THE EMERGING ROLE OF ENDOCANNABINOIDS IN SYNAPTIC PLASTICITY, REWARD, AND ADDICTION

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THE EMERGING ROLE OF ENDOCANNABINOIDS IN SYNAPTIC PLASTICITY, REWARD, AND ADDICTION

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EDITORIAL
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Editorial: The Emerging Role of Endocannabinoids in Synaptic Plasticity, Reward, and Addiction

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Keywords: endocannabinoid, CB1, plasticity, pain, ventral tegmental area, dopamine

Editorial on the Research Topic

The Emerging Role of Endocannabinoids in Synaptic Plasticity, Reward, and Addiction

INTRODUCTION

Endocannabinoids (eCBs) are lipid-signaling molecules that often work in a retrograde fashion. The most common eCBs are 2-arachidonoylglycerol (2-AG) and anandamide, which bind receptors such as cannabinoid receptor 1 (CB1) and CB2. Endocannabinoid signaling controls synaptic transmission throughout the central nervous system, and is important in modulating activity and behavior in the mesolimbic reward circuit, including the ventral tegmental area (VTA), nucleus accumbens (NAc), and lateral habenula (LHb). In these regions, the eCB system is essential for normal reward learning and for some maladaptive behaviors underlying drug abuse and addiction. Recently identified lipid-signaling eCB-like molecules are also now understood to shape mesolimbic system function and reward-related behaviors.

Further elucidating how the eCB system contributes to reward and addiction is especially pertinent given the recent legalization of medicinal or recreational marijuana throughout the world. The major psychoactive component in marijuana is Δ-9-tetrahydrocannabinol (THC), which binds CB1. Common effects of THC are short-term memory loss, appetite stimulation, and reward. There is still much to investigate concerning THC use, particularly the impact of adolescent use, with a focus on long-term alterations in eCB system function and behavioral changes. Further research is required to clarify the role of the endogenous eCB system, and the effect of exogenous CB1 or CB2-targeting drugs on mesolimbic function, including synaptic plasticity, to support reward behaviors and addiction.

This Research Topic focuses on endogenous eCB system function in the mesolimbic circuit with an emphasis on synaptic plasticity, reward behavior, novel eCB-like molecules, and pain.

COLLECTION PAPERS

Dopamine neurons in the VTA and their projections to NAc are essential to reward behavior, motivation, and addiction. The eCB system serves a key role in modulating synaptic transmission in the VTA and NAc to control dopamine signaling and behavior. Covey and Yocky review circuit mechanisms by which eCB signaling modulates dopamine release in the NAc and its implications in motivated behaviors. This review highlights the extraordinary complexity of NAc microcircuitry and discusses emerging techniques capable of dissecting how distinct cell types, their afferent projections, and local neuromodulators interact in the NAc to influence valence-based actions.

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Oleson et al. further discuss how dopamine-eCB interactions influence goal-directed behaviors. They review a variety of operant behavioral tasks used to dissect how eCBs shape dopamine release to affect distinct and overlapping aspects of motivation, reinforcement, attention, and habit formation.

In addition to canonical eCBs (2-AG and anandamide), eCB-like lipid-signaling molecules have emerged as important neuromodulators regulating dopamine transmission, reward, and addiction. Sagheddu et al. review recently discovered, naturally occurring *N*-acylethanolamines and *N-acyl* amino acids belonging to the lipid signaling system termed endocannabinoidome, and how these can be targeted to treat substance use disorders. Endogenous amides N-Oleoylglycine and N-Oleoylalanine, known to interfere with affective and somatic responses to acute naloxone-precipitated morphine withdrawal, are noted by Rock et al., as ineffective in the establishment of morphine tolerance elicited by morphine. Therefore, the effects of N-Oleoylglycine and N-Oleoylalanine on opiate dependence may be limited to naloxone-precipitated withdrawal effects.

Additional behavioral implications of the eCB system in VTA function include feeding behavior, where it is upregulated by orexin-A-induced enhancement of 2-AG tone and consequent disinhibition of dopaminergic neurons in obese mice, as highlighted by Tunisi et al. Also, Rodríguez-Manzo et al. describe eCB-mediated copulation satiety through changes in glutamate receptors and synaptic plasticity induction in the VTA.

Another important aspect of eCB studies in reward behavior is the long-term impact of adolescent cannabinoid exposure into adulthood, particularly regarding CB2 signaling. Initially, while CB2 receptors were thought to play functional roles only in the periphery, CB2 is expressed in the mesolimbic pathway and controls reward behavior. Ellner et al. demonstrate that CB2 blockade during adolescence has an impact on reward-learning behavior.

The LHb is associated with negative-reward behavior and provides input to the VTA to regulate aversion behavior. Shepard and Nugent provide a perspective on targeting eCB signaling as an intervention to treat mental illness following early life stress that leads to LHb dysfunction, causing disease states such as anxiety and substance use disorder.

Lastly, the eCB system has also been implicated in pro- and anti-nociceptive processes. Paulsen and Burrell highlight how eCBs can support a pro-nociceptive function through activity-dependent sensitization of non-nociceptive fibers through disinhibition of tonic GABA currents. The eCB system is also known to modulate synaptic plasticity to maintain homeostasis of excitatory and inhibitory networks. Pascual et al. used conditional CB1 knock-out in both glutamate and GABA neurons to examine compensatory transcriptomic changes that maintain homeostatic excitatory/inhibitory balance in the CA1 hippocampus. These data may have relevance to pathologies of homeostasis or stress-related disorders.

CONCLUDING REMARKS

Collectively, this Research Topic on eCBs highlights their important role in modulating synaptic transmission throughout the brain to control reward, motivation, pain, and addiction. Work reviewed in this issue discusses recent advancements that have the improved current understanding of the endogenous eCB system function in behavior, and will pave the way for novel therapeutics for psychiatric and neurological disorders.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Endocannabinoid-Like Lipid Neuromodulators in the Regulation of Dopamine Signaling: Relevance for Drug Addiction

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The family of lipid neuromodulators has been rapidly growing, as the use of different -omics techniques led to the discovery of a large number of naturally occurring *N*-acylethanolamines (NAEs) and *N*-acyl amino acids belonging to the complex lipid signaling system termed endocannabinoidome. These molecules exert a variety of biological activities in the central nervous system, as they modulate physiological processes in neurons and glial cells and are involved in the pathophysiology of neurological and psychiatric disorders. Their effects on dopamine cells have attracted attention, as dysfunctions of dopamine systems characterize a range of psychiatric disorders, i.e., schizophrenia and substance use disorders (SUD). While canonical endocannabinoids are known to regulate excitatory and inhibitory synaptic inputs impinging on dopamine cells and modulate several dopamine-mediated behaviors, such as reward and addiction, the effects of other lipid neuromodulators are far less clear. Here, we review the emerging role of endocannabinoid-like neuromodulators in dopamine signaling, with a focus on non-cannabinoid *N*-acylethanolamines and their receptors. Mounting evidence suggests that these neuromodulators contribute to modulate synaptic transmission in dopamine regions and might represent a target for novel medications in alcohol and nicotine use disorder.

Keywords: *N*-acylethanolamines, endocannabinoids, dopamine neurons, peroxisome proliferator-activated receptors-α, nicotine, alcohol

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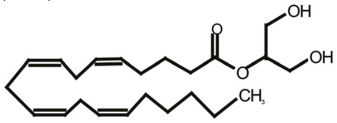
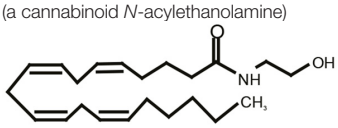
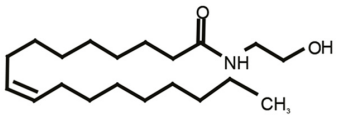
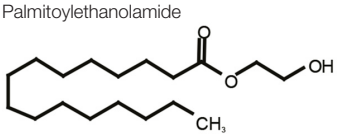
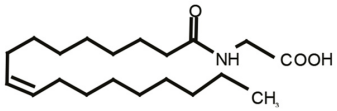
INTRODUCTION

One of the most fascinating fields in contemporary neuroscience is the emergence of lipids as signaling molecules, with a multitude of compounds recognized as mediators of communication within and between neurons (Piomelli et al., 2007). Among lipid neuromodulators, research in the last two decades has been focusing on synthesis, cellular effects, and catabolism of

TABLE 1 | Representative bioactive neural lipids, their cellular receptors, and their cellular effects on dopamine cells.

	Cellular receptors	Effects on dopamine cells
Endocannabinoids		
2-arachidonoylglycerol (2-AG) 	CB1 (Sugiura et al., 1995), CB2 (Mechoulam et al., 1995)	Short-term synaptic depression of glutamate and GABA inputs (Melis et al., 2004a)
Arachidonoylethanolamide (Anandamide) (a cannabinoid *N*-acylethanolamine) 	CB1 (Devane et al., 1992), CB2 (Mechoulam et al., 1995), TRPV1 (Zygmunt et al., 1999)	Facilitation of glutamate release and excitation (*via* TRPV1; Marinelli et al., 2003; Melis et al., 2008)
Non-cannabinoid *N*-acylethanolamines		
Oleoylethanolamide 	PPARα (Fu et al., 2003)	Phosphorylation and negative modulation of β*-nAChRs (Melis et al., 2008)
Palmitoylethanolamide 	PPARα (Lo Verme et al., 2005)	Phosphorylation and negative modulation of β*-nAChRs (Melis et al., 2008)
***N*-acyl amino acids**		
Oleoylglycine 	PPARα (Takao et al., 2015; Donvito et al., 2019)	Unknown

See text for abbreviations.

endogenous cannabinoids (eCBs) and their CB1 and CB2 cannabinoid receptors. The first characterized eCB, *N*-arachidonoylethanolamide (anandamide, AEA; Devane et al., 1992; **Table 1**), is one member of the *N*-acylethanolamines' (NAEs) family, also termed fatty acid ethanolamides. NAEs differ in the length and saturation of the hydrocarbon chain and their receptor affinity (Schmid et al., 1990; Hansen et al., 2000). Besides AEA, the saturated palmitoylethanolamide (PEA) and the monounsaturated oleoylethanolamide (OEA) have attracted attention due to their biological effects in the brain and periphery (**Table 1**). *N*-acyl amino acids are another related family of lipid signaling molecules in which an amino acid is covalently linked by an amide bond to the acyl moiety of a long-chain fatty acid. Among *N*-acyl amino acids, *N*-acyl glycines (particularly *N*-arachidonylglycine and *N*-oleoylglycine) are emerging as an intriguing class of neuromodulators, although largely uncharacterized so far (Bradshaw et al., 2009; Burstein, 2018; Battista et al., 2019; **Table 1**).

Among NAEs, the cannabinoid agonist AEA binds to CB1 and CB2 receptors and transient receptor potential vanilloid type 1 (TRPV1) at physiologically relevant concentrations, the others display an affinity for peroxisome proliferator-activated receptor-α (PPARα; Lo Verme et al., 2005; Hansen, 2010; Petrosino et al., 2010; Pistis and Muntoni, 2017), G protein-coupled receptors such as GPR55 (Baker et al., 2006) and GPR119, and TRPV1 (Piomelli, 2013). *N*-acyl amino acids are less characterized; however, evidence suggests a role for GPR18, GPR55, and GPR92, and PPARα in mediating some of the actions of *N*-oleoylglycine (Burstein, 2018; Donvito et al., 2019), which is one of the most studied among these molecules.

Although several of these molecules were known for decades, physiological activities of NAEs or *N*-acyl amino acids in the CNS and their role in neurological and psychiatric disorders, ranging from substance use disorder, neurodegenerative diseases, epilepsy, and mood disorders (Pistis and Melis, 2010; Melis and Pistis, 2014; Scherma et al., 2016; Pistis and Muntoni, 2017) has been characterized only relatively recently.

N-ACYLETHANOLAMINES AND *N*-ACYL AMINO ACIDS: SYNTHESIS AND CATABOLISM

Both AEA and other non-cannabinoid NAEs share both biosynthetic and catabolic pathways. Unlike typical neurotransmitters, their levels are regulated on-demand by enzymes responsible for their synthesis and degradation (Ueda et al., 2010a; Rahman et al., 2014) and not by vesicular release. They are synthesized from membrane-derived *N*-acylphosphatidylethanolamines (NAPEs; Hansen et al., 2000; Okamoto et al., 2004; Hansen, 2010; Ueda et al., 2010b; Rahman et al., 2014). The first step is the generation of the corresponding NAPE by a Ca^{2+}-dependent *N*-acyltransferase (NAT; Hansen et al., 2000; Hansen and Diep, 2009); NAPE is then hydrolyzed by NAPE-hydrolyzing phospholipase D (NAPE-PLD) with the generation of NAEs (Rahman et al., 2014; **Figure 1**).

Very little is known about the biosynthesis of *N*-acyl amino acids, except for *N*-acyl glycines, where it is hypothesized that glycine is directly condensed with the free fatty acid or CoenzymeA derivative acyl moiety by cytochrome C or glycine *NAT*-like 2 and 3 enzymes (Huang et al., 2001; McCue et al., 2008; Waluk et al., 2010; see Burstein, 2018; Battista et al., 2019; for comprehensive reviews of *N*-acyl amino acids).

When catabolized, NAEs and *N*-acyl amino acids are hydrolyzed to free fatty acids and ethanolamine or amino acids (Cravatt et al., 1996; Deutsch et al., 2002; Battista et al., 2019), respectively (**Figure 1**). This hydrolysis is catalyzed mainly by two major intracellular enzymes, although alternative pathways have been described (Bornheim et al., 1993; Hampson et al., 1995; Ueda et al., 1995; Yu et al., 1997; Kozak et al., 2002). The first enzyme to be characterized is the fatty acid amide hydrolase (FAAH; Cravatt et al., 1996). FAAH hydrolyzes all NAEs and several *N*-acyl amino acids with high efficiency, and it is expressed in many different tissues and cell types, including in the brain. The second major enzyme is the NAE-hydrolyzing acid amidase (NAAA; Tsuboi et al., 2005), which displays a significant preference for unsaturated NAEs such as PEA (Tsuboi et al., 2007). NAAA displays lower expression in the brain, yet significant CNS effects are obtained with selective inhibitors (Sagheddu et al., 2019), suggesting that despite low expression levels, this enzyme exerts physiologicallyrelevant actions by controlling brain levels of NAEs.

Stimuli triggering NAEs' synthesis vary between AEA and other NAEs. Endocannabinoids like AEA and 2-arachidonoylglycerol (2-AG) are synthesized following activation of metabotropic glutamate, muscarinic, or dopamine D2 receptors (Melis et al., 2004a,b; Kano et al., 2009). Besides the activation of metabotropic receptors, both AEA, 2-AG, and non-cannabinoid NAEs synthesis is initiated by a rise in intracellular Ca^{2+} (Luchicchi and Pistis, 2012; Melis et al., 2013b). The reason for this similarity is presumed to be the Ca^{2+}-dependency of the NAT enzyme. In experimental settings, the contribution of specific lipid neuromodulators can be disentangled with pharmacological tools, i.e., selective antagonists at their cellular receptors. Interestingly, both the canonical eCB signaling mediated by AEA/2-AG and the non-canonical OEA/PEA signaling have been characterized in dopamine neurons. However, it is still not clear whether these two separate signaling systems coexist in the same cell. As they are activated by different stimuli, they might fulfill different physiological functions. This scenario is consistent with the idea that NAE signaling displays exquisite anatomical and functional specificity. For example, activation of glutamate afferents in dopamine cells induces synthesis of 2-AG that, *via* CB1 receptors, dampens glutamate release (Melis et al., 2004a). On the other hand, acetylcholine release activates the OEA/PEA signaling (see below; Melis et al., 2010). Thus, both 2-AG and OEA/PEA signaling converge to reduce dopamine cell excitability, contributing to diminishing cellular overdrive induced by excitatory afferents. The functional relevance of these two distinct yet parallel systems needs to be fully understood.

The subcellular localization of NAPE-PLD in the brain is indicative of the possible functional significance of NAEs in the CNS (Cristino et al., 2008; Egertová et al., 2008; Nyilas et al., 2008; Suárez et al., 2008; Reguero et al., 2014; Merrill et al., 2015), as NAPE-PLD mRNA and immunoreactivity are detected both presynaptically and postsynaptically, but with a preferential distribution in postsynaptic dendrites (Reguero et al., 2014). The preferential postsynaptic localization of NAPE-PLD and NAEs affinity to nuclear receptors (see below) indicates that they may act as autocrine or paracrine signals at receptors expressed in the same or neighboring cells.

N-ACYLETHANOLAMINE RECEPTORS: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-α (PPARα)

PEA, OEA, and *N*-oleoylglycine targets have been identified as the PPAR, and specifically the α-isoform (PPARα). Very little is known about the functional relevance of *N*-acyl amino acids and their receptors such as GPR18, GPR55, or GPR92; this topic is discussed in Burstein (2018) and Battista et al. (2019).

In the brain, considerable evidence suggests that NAEs display activity through PPARα, receptors ubiquitously expressed in the CNS by neurons and glial cells (Braissant et al., 1996; Auboeuf et al., 1997; Mandard et al., 2004; Moreno et al., 2004; Galan-Rodriguez et al., 2009; Fidaleo et al., 2014).

PPARs belong to the large superfamily of transcription factors, composed of three isotypes: PPARα, PPARγ and PPARβ/δ (Germain et al., 2006). The large ligand-binding site of PPARs can accommodate a variety of diverse lipophilic endogenous ligands and synthetic agonists, including fibrates, clinically approved for decades for the treatment of hypertriglyceridemia. Hence, PPARα is a transcriptional regulator of genes involved in peroxisomal and mitochondrial β-oxidation, and fatty acid transport (Xu et al., 2002). PPARα is also engaged in the anti-inflammatory response, as it negatively regulates pro-inflammatory pathways and signals involved in the acute phase response in models of systemic inflammation

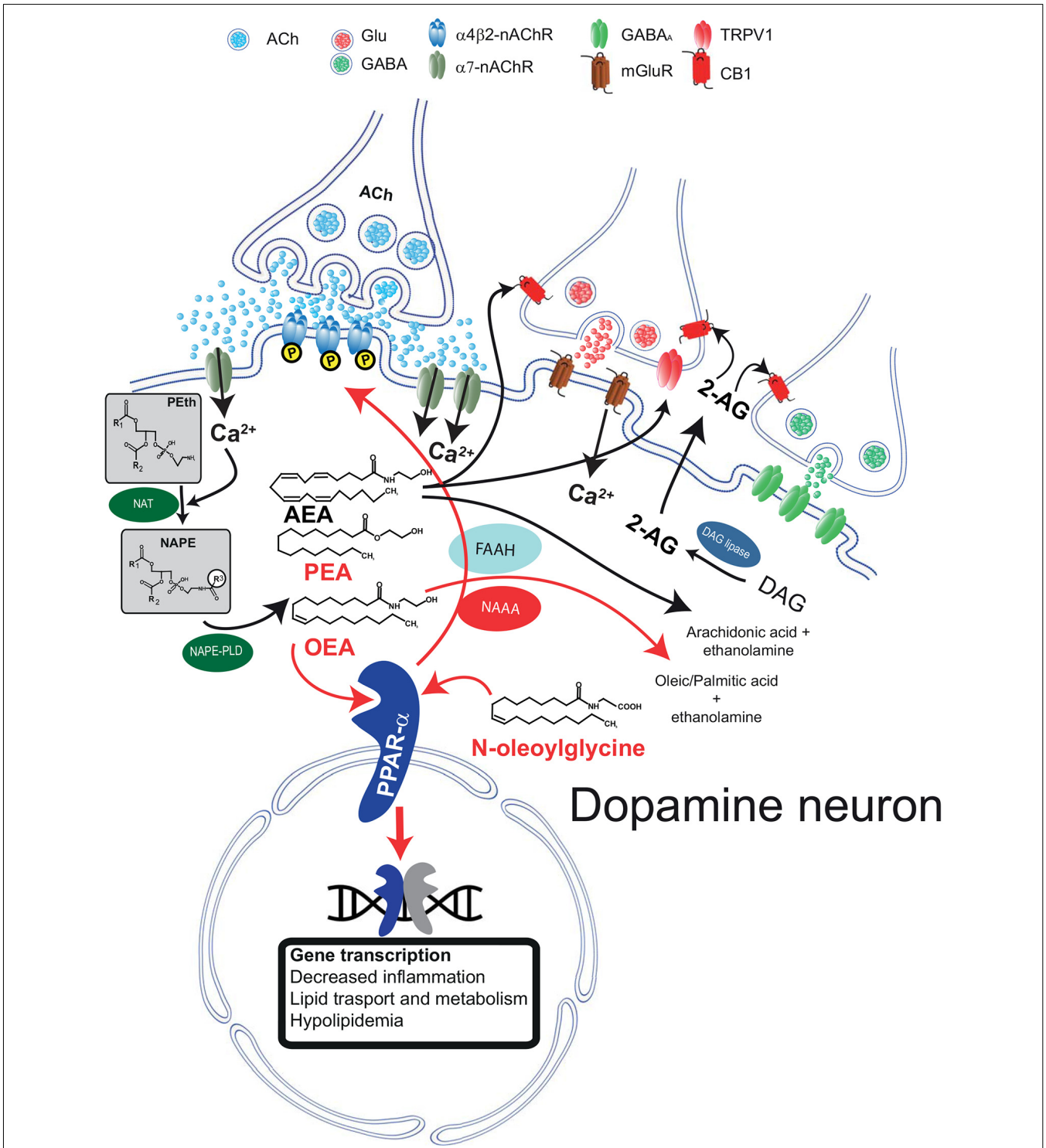


FIGURE 1 | Schematic diagram illustrating the biosynthetic and catabolic pathways for *N*-acylethanolamine (NAE) and canonical endocannabinoid formation and catabolism, and their cellular mechanisms of actions through their receptors. Phosphatidylethanolamine (Peth) is converted into *N*-acyl-phosphatidylethanolamine (NAPE) by *N*-acyltransferase (NAT). Ca^{2+} entry mediated by α7-nAChRs activates NAEs synthesis through the Ca^{2+} dependent NAT. The resulting NAPE is hydrolyzed by NAPE-PLD to the corresponding NAEs anandamide (AEA), oleoyl ethanolamide (OEA), and palmitoylethanolamide (PEA). Activation of PPARα by NAEs results in genomic effects (gene transcription) and in non-genomic actions, such as activation of a tyrosine kinase and phosphorylation of β2*nAChRs (i.e., α4β2). Fatty acid amide hydrolase (FAAH) and NAE-hydrolyzing acid amidase (NAAA) are the major inactivating enzymes for OEA, PEA, and AEA and convert them in ethanolamine and corresponding fatty acids (oleic, palmitic, and arachidonic acids, respectively). NAAA preferentially hydrolyzes PEA. *N*-oleoyl glycine is one

(*Continued*)

FIGURE 1 | Continued
member of the *N*-acyl amino acid family and is known to activate PPARα. The figure illustrates that AEA and 2-arachidonoylglycerol (2-AG) are produced on demand by NAPE-PLD and DAG lipase, respectively. Raises in intracellular Ca^{2+} can be induced, as in the example, by activation of metabotropic glutamate receptors (mGluR). 2-AG and AEA bind to presynaptic CB1 receptors expressed on GABA and glutamate terminals and depress neurotransmitter release. AEA also activates TRPV1 receptors located on presynaptic glutamatergic terminals. Abbreviations: NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase D; DAG, diacylglycerol; MAG, monoacylglycerol; FAAH, fatty acid amide hydrolase; Glu, glutamate; CB1, cannabinoid type-1 receptor; TRPV1, transient receptor potential vanilloid type-1; PPARα, peroxisome proliferator-activated receptor type-α; nAChRs, nicotinic acetylcholine receptors. This figure is adapted, with permission, from Melis and Pistis (2012) and Pistis and Muntoni (2017).

(Berger and Moller, 2002; Gervois et al., 2004; Moreno et al., 2004; Glass and Ogawa, 2006; Bensinger and Tontonoz, 2008).

PPARα AND DOPAMINE CELLS

The anatomical and functional segregation between cannabinoid and non-cannabinoid NAEs in their cellular effects is evident in dopamine cells, where these two signaling systems have been characterized. Dopamine cells synthesize and release eCBs following activation of metabotropic receptors, membrane depolarization, and Ca^{2+} entry (Melis et al., 2004a,b). Released eCBs bind to presynaptic CB1 receptors expressed by GABA and glutamate terminals (Melis et al., 2004b, 2013a, 2014; Pistis et al., 2004; **Figure 1**). The functional relevance of eCB signaling is reviewed elsewhere (Melis and Pistis, 2007, 2012; Melis et al., 2012). Here, it suffices to say that eCBs sculpt short- and long-term forms of synaptic plasticity and fine-tune firing activity of dopamine cells, specifically in tasks where these neurons are engaged, such as reward and motivation.

A different scenario is emerging when NAEs are concerned. In dopamine cells, NAE synthesis is triggered by enhanced nicotinic cholinergic transmission, and the switch was identified as the low-affinity extrasynaptic α7 nicotinic acetylcholine receptor (α7-nAChRs; Jones, 2004; Yang et al., 2009; **Figure 1**). The interpretation for this finding is that this receptor, expressed in sites distant from cholinergic axon terminals (Jones, 2004), is a sensor for an intense cholinergic drive, being activated by acetylcholine (Yang et al., 2009) spilled over from cholinergic synapses impinging onto dopamine cells. α7-nAChRs are permeable to Ca^{2+} ions, and their activation by acetylcholine or exogenous ligands (i.e., nicotine) evokes an increase in Ca^{2+} permeability and a rise in intracellular Ca^{2+}, which is necessary for the activity of the Ca^{2+}-dependent NAT isoform (Ogura et al., 2016; Hussain et al., 2018). The result is a rise in levels of PEA and OEA, which, differently from eCBs, bind to intracellular receptors within the dopamine cell, acting as autocrine-like signals (Melis et al., 2013b; **Figure 1**) in a fashion similar to other neuromodulators, such as neurotrophic factors (Herrmann and Broihier, 2018). Other laboratories have confirmed the interaction between α7-nAChRs and PPARα in different settings: Donvito et al. (2017) observed that the antinociceptive effects of α7-nAChR agonists were mediated by a rise in PEA and activation of PPARα and Jackson et al. (2017) confirmed that PPARα is involved in the effects mediated by α7-nAChR agonists in nicotine dependence.

These effects have the potential to regulate synaptic functions. Our studies show that PPARα activation in VTA dopamine cells triggers, *via* endogenous hydrogen peroxide and consequent activation of tyrosine kinase(s) (Melis et al., 2008, 2010), phosphorylation of the β2 subunits of the nAChRs (Melis et al., 2013b). Phosphorylation of nAChR subunits is an efficient way to regulate receptor functions by inducing a faster desensitization rate or a downregulation *via* internalization (Huganir and Greengard, 1990). Cholinergic inputs control firing rate and burst firing of midbrain dopamine cells *via* nAChRs (Mameli-Engvall et al., 2006), thus the functional regulation of β2 subunits, that together with α4 are the main nAChR subunits expressed by VTA DA neurons (Champtiaux et al., 2003), might prove useful in nicotine addiction (see below) dopamine-related neurological or psychiatric disorders.

While modulation of nAChRs by PPARα is one of the likely mechanisms by which these nuclear receptors acutely control dopaminergic transmission, we must take into account that genomic effects might also be highly relevant, e.g., anti-inflammatory effects. This is particularly important in psychiatric and neurological disorders when altered synaptic transmission and neuroinflammation interact to generate pathological phenotypes.

As non-cannabinoid NAEs are engaged by dopamine cells as an autocrine-like signal through PPARα to regulate afferent projections and their own pattern of activity, it is not surprising that these lipid neuromodulators might play a major role in substance use disorders (SUD).

An extensive literature substantiates the role of the dopamine system in addiction and SUD. Dopamine facilitates the development of long-lasting forms of synaptic adaptations that determine the effectiveness of reward and reward predictors to control subsequent seeking behavior (Wise and Robble, 2020). Among several aspects of dopamine function related to addiction, the phasic firing of dopamine neurons sculpts learning processes, particularly when learning is associated with rewarding stimuli or its opposite, aversion (Wise and Robble, 2020).

Evidence is accumulating that metabolic enzymes and receptors of these eCB-like signals might be a target for medications in SUD, and specifically alcoholism or nicotine dependence. In contrast, evidence linking them to psychostimulant or opioid use disorders is still very limited.

ROLE OF NAEs AND PPARα IN ALCOHOL USE DISORDER

It is well established that the eCB system in dopamine regions contributes to the motivation to consume alcohol. Evidence derives, among others, from the observation that the innate extent of susceptibility to alcohol use disorders (AUD) depends on increased eCB levels within mesolimbic dopamine regions

(Basavarajappa et al., 2006; Sagheddu and Melis, 2015), and that administration of CB1 receptor antagonists reduces alcohol drinking in animal models of alcoholism (Colombo et al., 1998). Consistently, alcohol self-administration is controlled by CB1 receptors in the VTA-NAc circuit of alcohol-preferring rats (Malinen and Hyytia, 2008), and Sardinian alcohol-preferring rats show enhanced eCB-mediated synaptic plasticity in the VTA when compared with Sardinian non preferring rats as controls (Melis et al., 2014).

Evidence is recently accumulating on non-cannabinoid NAEs' contribution to AUD (Orio et al., 2019). OEA has been shown to reduce behavioral expression of withdrawal, such as manifest signs of distress and alcohol-seeking (Bilbao et al., 2016). In rats, this is associated with the molecular effects of OEA, which counteracts alcohol-induced glial and neuronal alterations in brain regions processing drug reward (Rivera et al., 2019). Being antioxidant, anti-inflammatory, and neuroprotective, OEA, and PEA are considered molecules with therapeutic potential in comorbid disorders, including depression and anxiety in AUD (Pistis and Muntoni, 2017).

The role of NAEs in alcohol dependence has been extensively explored by studying the catabolic enzyme FAAH, both in rodents and humans. Several studies stress out the importance of FAAH genetic variants (Zhou et al., 2016; Sloan et al., 2018), or its enzymatic functionality as a factor contributing to the severity of the pathology. Recently, a PET scan study for a FAAH radiotracer was conducted in the brain of AUD patients during early abstinence. It showed transiently reduced FAAH levels, while its substrates AEA, OEA, and *N*-docosahexaenoyl ethanolamide (DEA) were elevated in the plasma (Best et al., 2020). Low FAAH levels are related to drinking behaviors and increased preference for alcohol, as demonstrated in several studies using animal models with genetic deletions (Basavarajappa et al., 2006; Blednov et al., 2006; Vinod et al., 2008; Pavón et al., 2018); or following pharmacological manipulation (Zhou et al., 2017).

PPARα is upstream of diverse genes that are modulated by ethanol or involved in ethanol-induced effects (Ferguson et al., 2014). Preclinical studies showed that modulation of PPARα by the synthetic agonist fenofibrate reduced motivational and reinforcing properties of ethanol, as measured by voluntary drinking in mice (Ferguson et al., 2014; Blednov et al., 2016) and rats (Karahanian et al., 2015); and corroborated by the self-administration paradigm in rats (Haile and Kosten, 2017). Considering that fibrates are approved for medical conditions, these studies suggest that regulation of PPARα deserves further clinical investigation in AUD, as recently detailed elsewhere (Karahanian et al., 2015; Matheson and Le Foll, 2020). Nonetheless, there are no pending clinical trials to date. An interesting pharmacological approach takes advantage of combining drugs acting at PPARα and other receptors. PPARα/γ dual agonists have proven to reduce alcohol consumption in both mice (Blednov et al., 2015) and rats (Alen et al., 2018). Dual CB1 antagonist/PPARα agonist reduced voluntary ethanol intake and self-administration in rat models of AUD (Alen et al., 2018), arising a promising step forward to the safe pharmacological manipulation of the eCB system.

ROLE OF NAEs AND PPARα IN ADDICTION: NICOTINE DEPENDENCE

Tobacco use is associated with high morbidity and mortality, it being the most preventable cause of death in the world (World Health Organization, 2019). Nicotine, the main psychoactive component in tobacco, is one of the most addictive substances (Picciotto and Mineur, 2014) and exerts its effects through nAChRs.

Both tobacco smoke and nicotine can affect the eCB system. Tobacco smoke alters FAAH, NAPE-PLD, and MAGL levels in the striatum (Torres et al., 2019), while nicotine modifies eCB signaling according to the administration protocol. For instance, nicotine self-administration decreased OEA and increased AEA and 2-AG levels, while nicotine infusion, as well as mecamylamine-induced nicotine withdrawal, only increased 2-AG levels (Buczynski et al., 2013; Saravia et al., 2017). A critical role for 2-AG was also demonstrated in nicotine reinforcement (Buczynski et al., 2016) and in nicotine-induced dopamine release (Cheer et al., 2007). The overlap of nAChRs and the receptors of the eCB system in brain areas critical to nicotine effects, such as the mesolimbic system, shows that the eCB system plays an important role in nicotine dependence (Gamaleddin et al., 2015).

The involvement of the eCB system in nicotine dependence was demonstrated by the effect of FAAH inhibitors. FAAH inhibitors suppress many reward-related effects of nicotine in rats and non-human primates, such as nicotine self-administration and reinstatement of nicotine seeking (Scherma et al., 2008; Forget et al., 2009; Gamaleddin et al., 2015; Justinova et al., 2015); nicotine-induced excitation of dopamine neurons in the VTA (Melis et al., 2008), and dopamine release (Scherma et al., 2008). Importantly, the involvement of both CB1 and PPARα receptors was reported.

Besides the effect of the major eCBs, there is increasing evidence of the involvement of PEA and OEA in nicotine addiction, as they have a crucial role as endogenous modulators of cholinergic transmission (Melis et al., 2013b). Moreover, they also inhibit nicotine addictive behaviors, changing dopamine cell excitability. All these actions are due to the activation of PPARα, rather than CB1 receptor (Melis et al., 2008), being dependent on PEA and OEA synthesis as a result of activation of the cholinergic system, *via* α7-nAChRs, and subsequent increase of intracellular Ca^{2+} (Melis et al., 2013b; **Figure 1**). Using *in vivo* and *in vitro* strategies, Melis et al. (2010) confirmed that the β2 subunit is crucial for PPARα effects, as re-expression of β2 receptors in VTA dopamine cells in β2 knockout mice was sufficient to rescue PPARα effects. Consistently, *N*-oleoyl glycine was shown by Donvito et al. (2019) to counteract several effects related to nicotine reward and dependence, including the withdrawal syndrome, with a PPARα-dependent mechanism. It is not known if this *N*-acyl amino acid is synthesized in dopamine cells and acts as an endogenous neuromodulator in a similar fashion of other NAEs with a dopamine moiety such as *N*-arachidonoyldopamine or *N*-oleoyldopamine (Ferreira et al., 2009; Sergeeva et al., 2017), so its role in the modulation in dopamine-mediated behaviors such as SUD are not clear yet.

Based on the mechanisms described, the suppression of nicotine-induced responses of dopamine neurons by PPARα agonists raised the interest on these ligands as a promising strategy to prevent nicotine relapse (Melis and Pistis, 2014; Matheson and Le Foll, 2020). To date, two clinical studies investigated the effects of PPARα agonists—gemfibrozil and fenofibrate—in smoking cessation, as a drug repositioning strategy. The authors did not observe beneficial effects of gemfibrozil or fenofibrate in treatment-seeking smokers (Perkins et al., 2016; Gendy et al., 2018). It must be pointed out that these disappointing results might be due to the low agonist potency and limited brain permeability in humans of clinically approved fibrates, which might result in low brain concentrations of these drugs, insufficient to achieve an optimal PPARα activation in the CNS. Indeed, doses of fenofibrate higher than those tested in the studies mentioned above were beneficial in a form of epilepsy induced by a gain of function of nAChRs, the sleep-related hyper motor epilepsy (SHE; formerly termed nocturnal frontal lobe epilepsy, NFLE; Puligheddu et al., 2017).

A way to circumvent the limited brain permeability of fibrates is to increase brain levels of endogenous PPARα agonists, such as PEA and OEA. The recent development of brain-permeant selective NAAA inhibitors offers the advantage to modulate levels of PEA and OEA selectively, and not AEA, therefore concurrently limiting psychiatric side effects due to eCB-CB1 alteration. Similar to direct PPARα agonists, also NAAA inhibitors display potential as anti-smoking medications, as they block nicotine-induced excitation of dopamine cells, dopamine elevations in the nucleus accumbens, and conditioned place preference in a PPARα-dependent manner (Sagheddu et al., 2019).

CONCLUDING REMARKS

The expanded eCB system, the "endocannabinoidome," is a hotbed for a large number of lipid signaling molecules, enzymes, and receptors and represents a Pandora's box for drug discovery (Cristino et al., 2020).

This review article summarizes evidence suggesting that NAE/PPARα signaling shows promise as a target in the treatment of SUD, particularly alcohol and nicotine use disorder. A parsimonious unifying hypothesis for this effect is NAE/PPARα's ability to modulate dopamine cell activity by specifically dampening stress-evoked excitatory drive from cholinergic afferents on VTA dopamine cells. Hence, a heightened cholinergic transmission has long been postulated to contribute to detrimental effects induced by stress, such as depression (Janowsky et al., 1972) and drug addiction (Morel et al., 2018; Shinohara et al., 2019). Consistently, selective inhibition of cholinergic neurons in the laterodorsal tegmentum, which provides a major cholinergic input to dopamine cells, prevents stress-induced cellular adaptations within VTA dopamine cells and the appearance of anhedonia and social withdrawal (Fernandez et al., 2018). Additionally, PPARα activation attenuates effects induced by stress (Scheggi et al., 2016; Ni et al., 2018; Song et al., 2018; Locci and Pinna, 2019), corroborating the idea that the interplay between stress, cholinergic inputs, dopamine neurons, and PPARα signaling might play a pivotal role in the potential favorable effects of NAEs in SUD.

SUD represents an unmet clinical need, with drugs currently in use that show limited efficacy or untoward side effects. Indeed, results reported for members of the NAE and *N*-acyl amino acid family suggest that analogs of these lipid neuromodulators could become potential drug candidates.

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frontiers in Synaptic Neuroscience

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Orexin-A/Hypocretin-1 Controls the VTA-NAc Mesolimbic Pathway via Endocannabinoid-Mediated Disinhibition of Dopaminergic Neurons in Obese Mice

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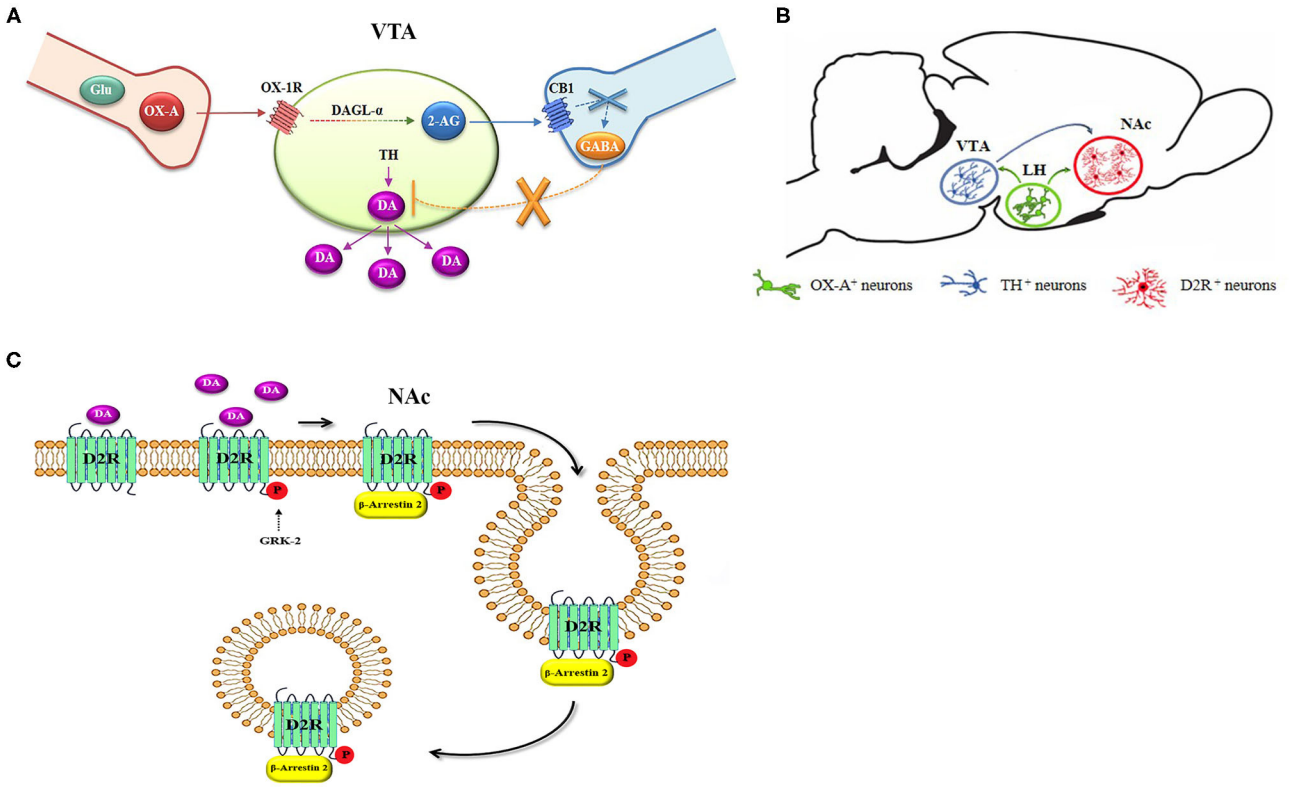
Disinhibition of orexin-A/hypocretin-1 (OX-A) release occurs to several output areas of the lateral hypothalamus (LH) in the brain of leptin knockout obese *ob/ob* mice. In this study, we have investigated whether a similar increase of OX-A release occurs to the ventral tegmental area (VTA), an orexinergic LH output area with functional effects on dopaminergic signaling at the mesolimbic circuit. By confocal and correlative light and electron microscopy (CLEM) morphological studies coupled to molecular, biochemical, and pharmacological approaches, we investigated OX-A-mediated dopaminergic signaling at the LH-VTA-nucleus accumbens (NAc) pathway in obese *ob/ob* mice compared to wild-type (wt) lean littermates. We found an elevation of OX-A trafficking and release to the VTA of *ob/ob* mice and consequent orexin receptor-1 (OX1R)-mediated over-activation of dopaminergic (DA) neurons via phospholipase C (PLC)/diacylglycerol lipase (DAGL-α)-induced biosynthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG). In fact, by retrograde signaling to cannabinoid receptor type 1 (CB1R) at inhibitory inputs to DA neurons, 2-AG inhibited GABA release thus inducing an increase in DA concentration in the VTA and NAc of *ob/ob* mice. This effect was prevented by the OX1R antagonist SB-334867 (30 mg/Kg, i.p.), or the CB1R antagonist AM251 (10 mg/Kg, i.p.) and mimicked by OX-A injection (40 μg/Kg, i.p.) in wt lean mice. Enhanced DA signaling to the NAc in *ob/ob* mice, or in OX-A-injected wt mice, was accompanied by β-arrestin2-mediated desensitization of dopamine D2 receptor (D2R) in a manner prevented by SB-334867 or the D2R antagonist L741 (1.5 mg/Kg, i.p.). These results further support the role of OX-A signaling in the control of neuroadaptive responses, such as compulsive reward-seeking behavior or binge-like consumption of high palatable food, and suggest that aberrant OX-A trafficking to the DA neurons in the VTA of *ob/ob* mice influences the D2R response at NAc, a main target area of the mesolimbic pathway, via 2-AG/CB1-mediated retrograde signaling.

Keywords: obesity, leptin, reward, orexin, cannabinoid receptor (CB1R), endocannabinoids



Graphical Abstract | Scheme of the anatomical and functional pathway regulating the reinforcement of DA signaling in obese mice. **(A)** A Functional cross-talk between OX-A and 2-AG occurs in the DA neurons of the VTA. **(B)** OX-A and DA interplay at LH-VTA-NAc neuronal circuitry. **(C)** Scheme of the DA-mediated D2R desensitization by phosphorylation of G protein receptor kinase-2 (GRK-2) and β-arrestin2 signaling in the NAc, culminating in the internalization of the D2R receptor in endosomes.

INTRODUCTION

Orexin-A/hypocretin-1 (hereafter referred to as OX-A) plays a prominent role in conditioned responses to stimuli associated with food and drug rewards by regulating the functional activity of DA neurons in the VTA (Harris et al., 2005; Tung et al., 2016). DA neurons are innervated by orexinergic fibers (Fadel and Deutch, 2002) and those projecting to the shell of NAc forming the mesolimbic pathway undergo an OX-mediated increase of firing rate (Baimel et al., 2017). Although both type 1 and 2 of orexin receptors, named OX1R and OX2R, are expressed in striatal regions, OX-A promotes consumption of highly palatable food mainly through OX1R activation (Harris et al., 2005; Borgland et al., 2006; Baimel and Borgland, 2015; Barson et al., 2015). Accordingly, i.p. or intracerebroventricular (i.c.v.) injection of OX-A increases food-intake in *ad-libitum* fed lean mice (Morello et al., 2016) whereas intra-VTA injection of OX-A increases intake of a diet with high contents of fat and sucrose solution in *ad-libitum* fed rats (Terrill et al., 2016), as well as a hedonic reaction to sucrose when injected in the rostromedial NAc (Castro et al., 2016). Moreover, OX-A injection in the paraventricular nucleus (PVN) of the thalamus increases the intake of a sucrose solution (Kay et al., 2014) and the release of DA in the NAc, thus promoting reward-related feeding in rats (Choi et al., 2012).

Acute consumption of an obesogenic high-fat diet (HFD) (Valdivia et al., 2014), or optogenetic stimulation of LH fibers projecting to DA neurons of the VTA (Nieh et al., 2015), promote a compulsive reward-seeking behavior inhibited by an OX1R antagonist (Piccoli et al., 2012). Accordingly, systemic injection of the OX1R antagonist SB-334867 reduces drinking of a sucrose solution (Terrill et al., 2016), acute intake of highly palatable food, and binge-like consumption of sucrose, together with reduced c-*Fos* activation in the VTA (White et al., 2005; Alcaraz-Iborra et al., 2014; Valdivia et al., 2014). Noteworthy, *Hcrtr1* knock-down rats undergo a reduction of both hedonic feeding and preference in the consumption of an HFD (Choi et al., 2012; Kay et al., 2014). Although both OX1R and OX2R are expressed in neurons of the mesolimbic regions, the neurobiological effects in reward-seeking behaviors are mainly due to OX-A binding to OX1R, whereas OX2R is more likely involved in sleep/wake cycle regulation and was suggested to participate in ethanol self-administration (Harris et al., 2005; Borgland et al., 2006; Barson et al., 2015; Baimel et al., 2017).

Endocannabinoids, especially 2-AG, have been implicated in food-seeking behaviors and obesity in a manner prevented

by CB1R antagonists (Silvestri and Di Marzo, 2013). The endocannabinoid 2-AG is biosynthesized on demand to produce retrograde inhibition of neurotransmitter release at presynaptic CB1 receptors. We and others demonstrated that 2-AG can be generated when OX1R is activated by OX-A (Ho et al., 2011; Cristino et al., 2013). Activation of Gq by OX1R can trigger PCL/DAGL-α-mediated production and release of 2-AG from its diacylglycerol biosynthetic precursors (Imperatore et al., 2016; Chen et al., 2018). Furthermore, we have previously shown that obesity-associated changes occur at hypothalamic circuits in *ob/ob* mice because of the switch from predominantly excitatory to inhibitory CB1R-expressing inputs to OX-A neurons. These changes are accompanied by DSI-mediated disinhibition of OX-A neurons with consequent elevation of OX-A trafficking and release to many brain target areas including the periaqueductal gray (PAG) and arcuate nucleus (ARC) (Cristino et al., 2013, 2016; Morello et al., 2016; Becker et al., 2017).

Based on this background, we exploited the model of leptin knockout obese *ob/ob* mice to investigate if an enhancement of OX-A trafficking and release also occurs from the LH to the VTA. Obese *ob/ob* mice represent a good model to study the effect of aberrant OX-A signaling on dopamine trafficking in the mesolimbic pathway. This is of special relevance to the pivotal role of orexinergic, dopaminergic, and endocannabinoid signaling in the control of neuroadaptive responses underlying compulsive reward-seeking behaviors, which can contribute to obesity.

MATERIALS AND METHODS

Animal and Drugs

The study has been performed according to the ARRIVE Guidelines to improve the reporting of bioscience research using laboratory animals. Experiments were performed in compliance with the European Union animal welfare guidelines [European Communities Council Directive of September 22, 2010 (2010/63/EU)] and the Italian Decree n.26/2014, authorization n. 152/2020-PR, Ministry of Health, Italy. Adult (16-wk-old) male C57BL/6j mice were purchased from Charles River Laboratories (Sulzfeld, Germany); male mice with spontaneous nonsense mutation of the *ob* gene for leptin (*ob/ob*, JAX mouse strain) B6.Cg-Lepob/J and WT *ob* gene expressing homozygous siblings of different ages were obtained from breeding *ob* gene heterozygotes and genotyped with PCR. Since orexin levels exhibit a diurnal fluctuation (concentrations increase during the dark period or active phase (i.e., ZT13- 24) and decrease during the light period or rest phase), the animals were maintained under a 12 h light:12 h dark cycle, light on at 8:00 PM, i.e., ZT0, for at least 4 weeks before euthanizing at ZT20-22. For the same reason, all the pharmacological treatments were performed at ZT20-22. Adult (16-wk-old) male B6.Cg-Tg(TH-GFP)21-31 (C57BL/6JJcl) mice were used for the electron microscopy analysis.

All mice were housed in controlled temperature and humidity conditions and fed *ad libitum* to exclude the effects of fasting on endocannabinoid-mediated plasticity in the VTA (Godfrey and Borgland, 2020). Wt and *ob/ob* mice were injected i.p. with several treatments as following: OX-A (Tocris, 40 μg/kg, 2 h), Leptin (Sigma Aldrich, 5 mg/kg, 2 h), SB-334867 (Tocris, 30 mg/kg for wt and 60 mg/kg for *ob/ob* mice, 3 h alone or 1 h before OX- A injection), AM251 (Tocris, 10 mg/kg, 3 h alone or 1 h before OX-A injection), O-7460 (Cayman, 12 mg/kg, 1 h alone or 30 min before OX-A injection), L741 (Tocris, 1.5 mg/kg, 30 min).

Immunohistochemical Procedures

Single Antigen Immunohistochemistry

Animals were euthanized under isoflurane anesthesia, perfused transcardially with 0.9% saline and 4% paraformaldehyde/0.1 M phosphate buffer (PB), pH7.4. Brains were cryoprotected with 30% sucrose in PB and cut with Leica CM3050S cryostat into 10 μm-thick through the coronal plane. The sections were collected in three alternate series and maintained frozen until being processed.

For immunoperoxidase-labeling, the sections were pretreated with 0.75% H_2O_2 in an aqueous solution and then incubated overnight with primary antibody goat anti-Orexin-A (Santa Cruz Biotechnology, Inc., Dallas, Texas—USA) or mouse anti-tyrosine hydroxylase (TH) (Millipore), diluted 1:200 in TBS-T (tris-buffered saline solution (TBS), pH 7.3). Primary antibodies were revealed with appropriate biotin-conjugate secondary antibodies (diluted 1:100 in TBS-T; Vector Laboratories, Burlingame, CA) and avidin-biotin complex by using 3,3'-diaminobenzidine-4 (DAB) (Sigma Fast, Sigma-Aldrich, Louis, MO—U.S.A.) as chromogen substrate. The immunodensity of OX-A or TH immunoreactivity in the VTA was evaluated by quantifying the optical density (O.D.) of immunoperoxidase signal from a region of interest (ROI) having a square-shaped area of 1 mm^2 selected, per each hemisphere, through the entire VTA area from Bregma −2.92 to −3.88 mm by analyzing $n \geq 3$ sections/mouse (n = 6 mice/genotype). Concerning the OX-A immunoreactivity, the O.D. value of the portion of tissue devoid of stained fibers in the ROI was measured and subtracted as background from the O.D. value of fibers of interest. Concerning the TH immunoreactivity, the O.D. was calculated from the single-immunoreactive cells included in the ROI and by selecting only those exhibiting the largest nucleus in the focal plane. All the O.D. quantifications were performed by using the LAS AF MetaMorph Imaging Software (Leica, Wetzlar, Germany) according to the formula: $OD = \log_{10}(255/I)$, where the "I" was the pixel intensity value given by the imaging software. All the analyzed sections were exposed to the same parameters of brightness, contrast and magnification.

Multiple Labeling With Immunofluorescence

Alternate brain slices (10 μm of thickness) containing dorsal or ventral striatum of mice were collected in three series of slides and then processed for multiple immunofluorescence by overnight incubation in a mixture of the following primary antibodies, each diluted 1:100 in PB-T: (1) goat anti-OX-A (Santa Cruz Biotechnology, Inc., Dallas, Texas–USA) and mouse anti-TH (Millipore); (2) mouse anti-TH (Millipore) and rabbit anti-Neuronal Marker (anti-NeuN) (Abcam); (3) goat anti-OX1R (Santa Cruz Biotechnology),

guinea pig anti-diacylglycerol lipase-α (anti-DAGL-α) (kindly provided by Prof. Ken Mackie) and mouse anti-TH (Millipore); (4) goat anti-OX-A (Santa Cruz Biotechnology), guinea pig anti-vesicular glutamate transporter (anti-VGluT1) (Synaptic Systems) and mouse anti-TH (Millipore); (5) rabbit anti-CB1R antibody (anti C terminus 461-472, Abcam), guinea pig anti-vesicular GABA transporter (anti-VGAT) (Synaptic Systems) and mouse anti-TH (Millipore); (6) rabbit anti-dopamine D2 Receptor (anti-D2R) (Millipore) and goat anti-βarrestin2 (Santa Cruz Biotechnology); (7) goat anti *c-Fos* (Santa Cruz Biotechnology) and rabbit anti-D2R (Millipore).

After incubation with primary antibodies, the sections were treated with appropriate Alexa-488, −546, or −350 donkey anti-IgGs (Invitrogen LifeTechnology) secondary antibodies before being counterstained with nuclear dye DAPI (4′,6-diamidino-2-phenylindole). Controls of specificity of immunolabeling in multiple fluorescence experiments were performed by omission of primary and secondary antibodies or by preabsorption of primary antibodies with the respective blocking peptides.

Immunofluorescence was analyzed by the confocal microscopy Nikon Eclipse Ti2 and images acquired with the digital camera DS-Qi2 (Nikon) and processed by Image analysis software NIS-Elements C (Nikon, Florence, Italy). $N = 6$–10 z stacks were collected through each analyzed section every 0.5 μm throughout the area of interest to be processed by the imaging deconvolution software. For each section, the optical density zero value was assigned to the background (i.e., a tissue portion devoid of stained cell bodies or fibers). Unbiased stereological counting method based on the MetaMorph Imaging Software (Leica, Wetzlar, Germany) was applied for quantification of the relative abundance of TH-immunoreactive neurons by counting the number of TH/NeuN double immunoreactive neurons vs. the total number of NeuN-immunoreactive neurons quantified in 1 mm^2 of a counting square-shaped frame selected, per each hemisphere, through the entire VTA area from Bregma −2.92 to −3.88 mm ($n \geq 3$ sections/mouse; $n = 6$ mice/genotype). Quantification of the relative abundance of c-Fos/D2R double immunoreactive neurons was performed in 1 mm^2 of a counting square-shaped frame selected, per each hemisphere, through the entire NAc area from Bregma +1.34 to +2.2 mm by analyzing $n \geq 3$ sections/mouse ($n = 6$ mice/genotype).

CLEM: OX1R and CB1R Immunoreactivity in Pre-embedding Electron Microscopy

Double pre-embedding immunogold labeling for observation at transmission electron microscopy (TEM) was performed in the VTA (50-μm-thick) of TH-eGFP fed *ad libitum* mice fixed with 3% paraformaldehyde/0.5–1% glutaraldehyde (vol/vol) in PB. The TH-eGFP neurons were selected by observation under appropriate epifluorescence microscopy by being easily recognizable at 488 nm excitation wavelength. The selected sections were incubated free-floating overnight at 4°C with the primary antibodies (rabbit anti-CB1R antibody, anti-C terminus 461–472, Abcam; and goat anti- OX1R, Santa Cruz), all diluted 1:100 in donkey serum blocking solution with 0.02% saponin. Subsequently, the sections were incubated in a mixture of 6 nm (for CB1R) and 10 nm (for OX1R) gold-conjugated secondary antibodies (Aurion), diluted 1:30 in donkey serum blocking solution with 0.02% saponin. Sections were treated with 0.5% OsO_4 in PB for 30 min at 4°C, dehydrated in an ascending series of ethanol and propylene oxide, and embedded in TAAB 812 resin (TAAB). Ultrathin (50 nm thickness) sections were cut by vibratome (Leica), collected on Formvar-coated, single- or multiple-slot (50-mesh) grids, and stained with 0.65% lead citrate. Electron micrographs were taken with the TEM microscope (FEI Tecnai G2 Spirit TWIN). The TEM observation was limited to series sectioned up to 0.6–0.8 μm depth from the external surface of pre-embedded immunolabeled tissue. Additional sections were processed in parallel as controls of reaction by omitting both or one of the primary antibodies from the mixture. No labeling was detected in the control material.

Lipid Extraction and 2-AG Measurement

VTA samples were dissected by micropunch of 1.5 mm diameter from each hemisphere and pooled for each mouse ($n = 8$ mice/genotype per group) and homogenized in 5 vol chloroform/methanol/Tris HCl 50 mM (2:1:1 by volume) containing 50 pmol of d5-2-arachidonoylglycerol (d5-2-AG) as internal standards. Homogenates were centrifuged at 13,000 × g for 16 min (4°C), the aqueous phase plus debris were collected and four times extracted with 1vol chloroform. The lipid-containing organic phases were dried and pre-purified by open-bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for 2-AG measurement were obtained by eluting the columns with 9:1 (by volume) chloroform/methanol and then analyzed by liquid chromatography atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS).

DA Measurement in VTA and NAc

DA levels were measured by using the DA enzyme-linked immune specific assay (ELISA) kit (Cusabio Biotech, Wuhan, China). According to the manufacturer's instructions, the tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS, and stored overnight at −20°C and, after two freeze-thaw cycles, the homogenates were centrifuged for 5 min at 5,000 × g in refrigerated condition (5°C). The absorbance was measured at 450 nm a wavelength by using the 96-well microplate spectrophotometer Multiskan GO (Thermo Scientific, Waltham, MA). DA quantification was performed in VTA or NAc samples dissected by micro punch of 1.5 mm diameter from each separate hemisphere of a mouse, $n = 9$ mice/genotype per group. Visceral tissue samples were used as controls to check the specificity of the assay and to avoid non-specific-binding and false-positive results.

Co-immunoprecipitation Assay and Western Blotting

Punched micro-dissected Nac samples from both hemispheres were pooled for each mice ($n = 8$ mice/genotype) and protein extracts were homogenized in an appropriate ice-cold lysis buffer and the concentration was determined using the Lowry protein assay (#55000111, Bio-Rad). The co-immunoprecipitation was

performed with the Dynabeads Protein G-Kit (Invitrogen Life Technology) according to the manufacturer's instructions. For western blot, the membranes were incubated overnight with rabbit anti-Dopamine D2 Receptor antibody (Millipore, catalog #AB5084P) or rabbit anti-β-Arrestin2 antibody (Cell Signaling Technology, catalog #3857) dissolved in TBS-BSA (4%) and then incubated for 1 h at room temperature with goat anti-rabbit IgG (H+L)-HRP conjugate antibody (Biorad, #1706515). The reactive bands of membranes were detected by chemiluminescence after a 5 min incubation without light with ECL (#170-5061, Bio-Rad) and visualized using Chemidoc MP Imaging System (#17001402, Bio-Rad). The images were analyzed and quantified using ImageJ software (imagej.nih.gov/ij/). The intensity of the bands of immunoblotted β-Arrestin2 protein was normalized with the D2R protein as the loading control.

Statistical Analyses

Data are expressed as mean ± SEM and were analyzed with GraphPad Prism 6 software, version 6.05 (GraphPad, Inc.). Data are presented as a histogram with mean ± sem. D'Agostino-Pearson's normality test was used to confirm the normal distribution of the data. Statistical differences among the two groups were determined by a two-tail *t*-test with Welch's correction. When more than two groups were analyzed statistics were computed by one-way ANOVA followed by Bonferroni test or two-way ANOVA followed by *post hoc* Tukey tests for comparison among means. A level of confidence of $P < 0.05$ was employed for statistical significance.

RESULTS

OX-A and TH Immunoreactivities Are Enhanced in the VTA of *ob/ob* Mice

Increased OX-A trafficking was found in the fibers projecting from the LH to the VTA (**Figure 1A**) of *ob/ob* in comparison to wt mice (**Figures 1B–E**), a feature confirmed by optical densitometry analysis of OX-A immunoreactivity (**Figure 1F**) and in line with our previous results (Cristino et al., 2013, 2016; Morello et al., 2016). DA neurons are unequivocally identified by the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis (Daubner et al., 2011). By confocal imaging OX-A/TH immunolabelling, we found a large distribution of OX-A-ir puncta as apposed to TH-ir neurons in the VTA of *ob/ob* compared to wt mice (**Figure 2**), in line with the enhancement of OX-A trafficking documented before (**Figure 1F**), and of TH-ir found in the vast majority of the VTA neurons by optical microscopy (**Figures 3A–D**). The densitometric measure of TH-ir in the VTA confirmed the enhanced expression of this rate-limiting enzyme of dopamine synthesis in *ob/ob* vs. wt mice (**Figure 3E**). The measure of the TH/NeuN ratio did not reveal a change in the percentage of the TH-positive neurons between *ob/ob* (92.89% ± 3.49) and wt (87.44% ± 4.23) mice through the entire volume of the VTA (**Figure 3F**). This result is in line with data showing that the vast majority of VTA neurons are dopaminergic (Bok et al., 2018).

Altogether, these data show that in the VTA of *ob/ob* mice an elevation of OX-A trafficking occurs concurrently with an increased expression of TH.

The OX-A-OX1R- DAGL-α Cascade Enhances 2-AG Levels in TH-ir Neurons of *ob/ob* Mice

We and others have previously demonstrated that 2-AG can be biosynthesized upon activation of postsynaptic OX1Rs by OX-A in the periaqueductal gray area (Ho et al., 2011; Cristino et al., 2016) and arcuate nucleus of mice (Morello et al., 2016). Furthermore, 2-AG synthesis can be triggered by OX-A in DA neurons via GqPCR(OX1R)-mediated activation of the PLC-DAGL-α pathway (Tung et al., 2016). Starting from this background, we sought to investigate: (i) if enhancement of 2-AG levels occurs in the VTA of *ob/ob* vs. wt mice as an effect of increased OX-A content in this area (**Figure 1F**); (ii) the anatomical network which underlies the OX-A-OX1R-DAGL-α cascade in the VTA.

2-AG Levels Are Increased in the VTA of Obese *ob/ob* Mice

By LC-APCI-mass spectrometric analysis of lipids extracted from the mouse VTA, we found an enhancement of 2-AG levels in *ob/ob* mice (15.04 ± 0.70 pmol/mg lipids, $n = 6$ mice per group) in comparison to wt mice (8.08 ± 0.41 pmol/mg lipids, $n = 6$ mice per group), as well as in the VTA of wt mice injected with OX-A (40 μg/kg, i.p., 2 h) (12.73 ± 0.46 pmol/mg lipids, $n = 6$ mice per group) in comparison to the wt vehicle-injected mice (8.08 ± 0.41 pmol/mg lipids). This enhancement was prevented by SB-334867 injection (30 mg/kg, i.p., 1h before OX-A treatment) (8.70 ± 0.39 pmol/mg lipids, $n = 6$ mice per group). In the VTA of SB-334867-treated *ob/ob* mice (60 mg/kg, i.p., 1 h) we found decreased levels of 2-AG (10.52 ± 0.35 pmol/mg lipids), similar to vehicle-injected wt mice (8.08 ± 0.41 pmol/mg lipids). These data demonstrate that the increase of endocannabinoid tone in the VTA is due specifically to increased OX-A signaling at OX1R. The enhancement of 2-AG levels in *ob/ob* mice and wt-OX-A injected mice was also counteracted by injection of O-7460 (12 mg/kg, i.p., 30 min), a selective inhibitor of DAGL-α (10.18 ± 0.38 pmol/mg lipids in *ob/ob* mice; 8.63 ± 0.42 pmol/mg lipids in wt-OX-A injected mice), which supports the putative activation of PLC-DAGL-α pathway downstream to the OX-A-mediated activation of OX1R (**Figure 3G**).

Anatomical and Molecular Substrates of Functional OX-A/2-AG Cross-Talk in the VTA

To study the molecular substrate of the functional cross-talk between OX-A and 2-AG at DA neurons, and its anatomical distribution in the VTA, multiple OX1R/DAGL-α/TH or OX-A/VGluT1/TH or CB1R/VGAT/TH immunofluorescence was performed in coronal sections of the VTA.

Confocal analysis of OX1R/DAGL-α/TH immunolabeling revealed expression of DAGL-α, the main biosynthesizing enzyme of 2-AG, close to OX1R immunoreactivity on the plasma membrane of TH-positive neurons (**Figure 4A**).

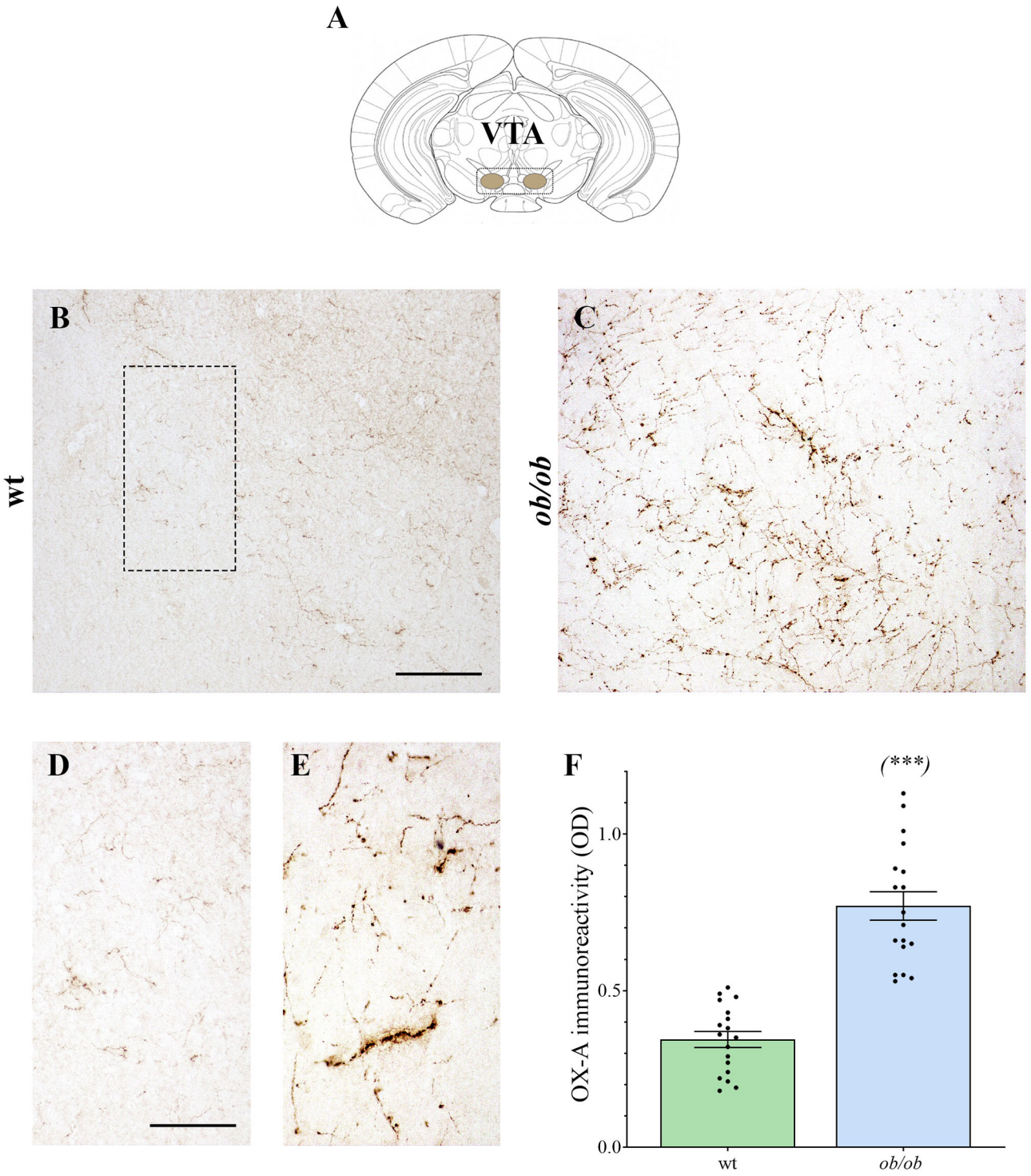


FIGURE 1 | (A) Adaptation from the (Franklin and Paxinos, 1997) Mouse Brain Atlas, illustrating the VTA. **(B–E)** Representative peroxidase-based OX-A-immunoreactive staining in the VTA of wt and *ob/ob* mice showing a dense plexus of orexinergic fibers intensely immunoreactive in obese mice. Higher magnification of fields is depicted in **(D)** (wt) and **(E)** (*ob/ob*). Scale bar: 150 μm **(B,C)**, 50 μm **(D,E)**. **(F)** Bar graph of OX-A peroxidase-based optical density (OD). Data are means ± SEM from n ≥3 VTA sections/mouse, $n = 6$ mice/genotype; ***$P < 0.001$; two-tail t-test with Welch's correction.

Since orexinergic fibers originating from the LH and projecting to the different target areas, including the VTA, are mainly excitatory (Rosin et al., 2003), multiple TH/OX-A immunofluorescence was performed associated with detection of immunoreactivity for VGluT1, i.e., the main vesicular transporter of glutamate, as a presynaptic marker of excitatory

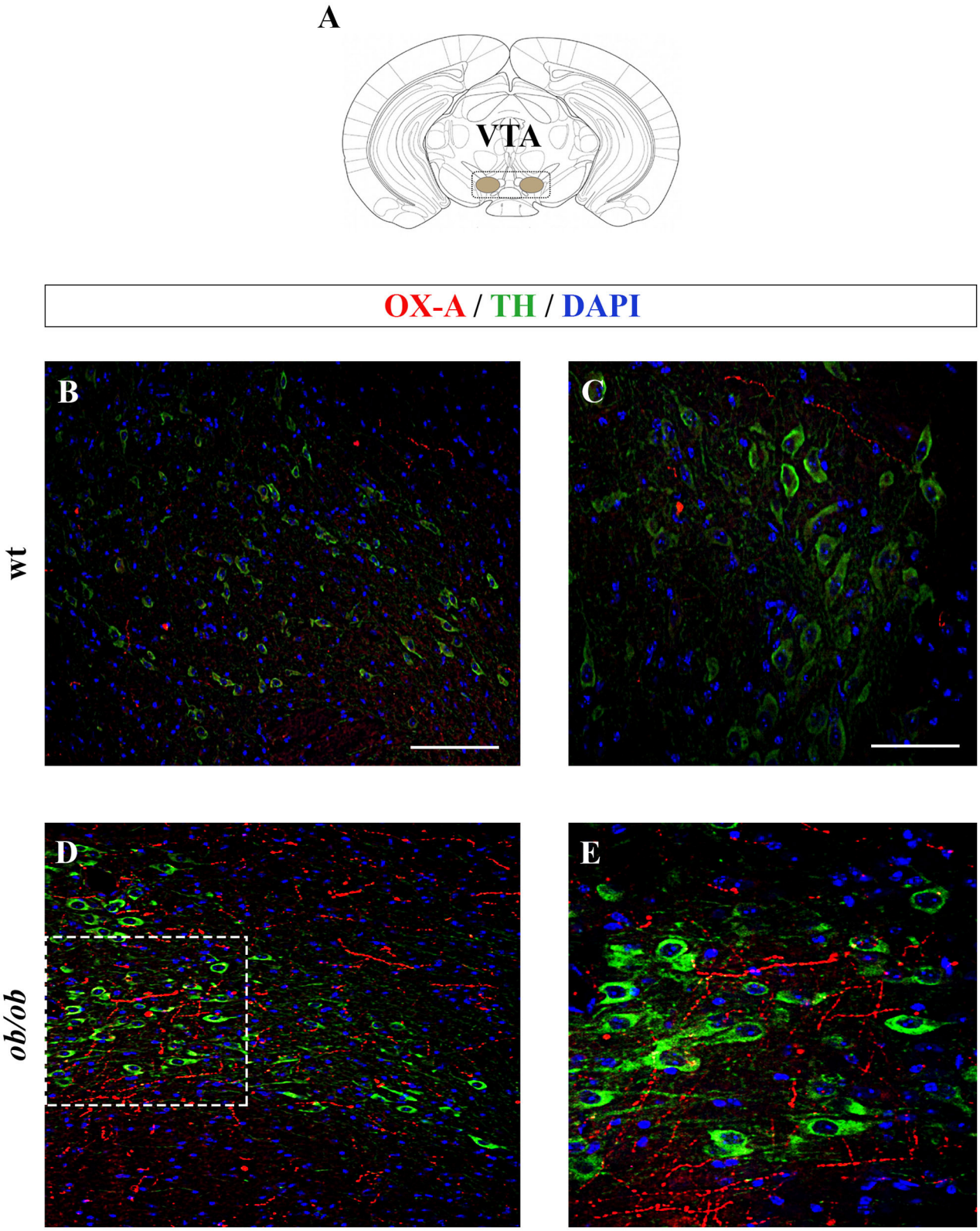


FIGURE 2 | (A) Schematic representation of a coronal section of the mouse brain highlighting the anatomical localization of the VTA (adapted from the mouse brain atlas, (Franklin and Paxinos, 1997). **(B–E)** Confocal microscopy images showing OX-A immunolabeled axon terminals apposed to TH-expressing neurons of adult wt and *ob/ob* mice. Immunofluorescent signals are red for OX-A, green for TH, and blue for DAPI. Note the enhancement of OX-A and TH immunoreactivities in the VTA of *ob/ob* mice **(D,E)** compared to the wt mice **(B,C)**. **(E)** is a higher magnification of the boxed area in **(D)**. [Scale bar: 100 μm **(B,D)**, 50 μm (**C,E)**].

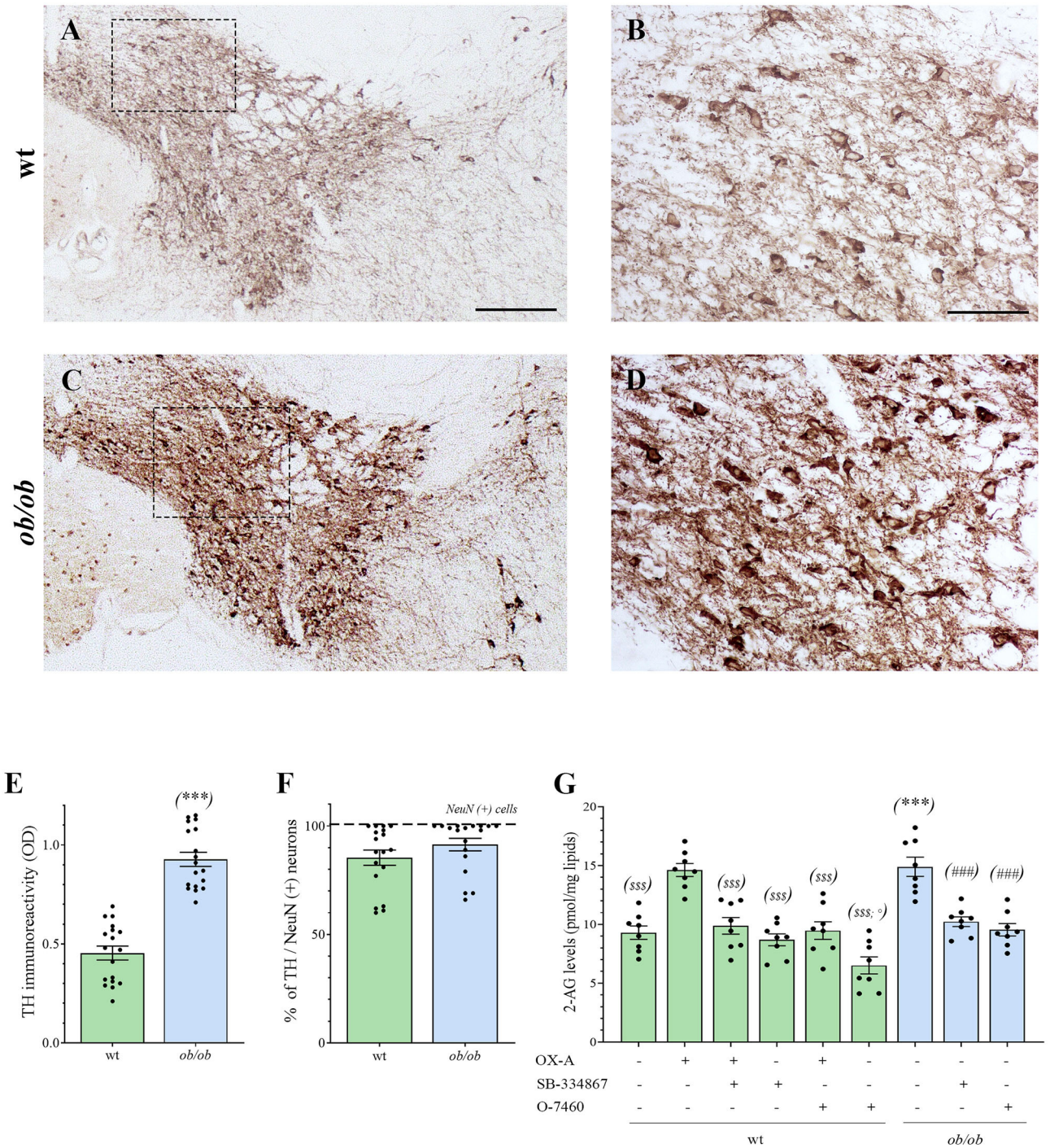


FIGURE 3 | (A–D) Representative peroxidase-based TH staining in the VTA of wt and *ob/ob* mice. **(B,D)** are higher magnification of the boxed areas in **A** and **C**, respectively [Scale bar: 300 μm **(A,C)**, 100 μm **(B,D)**]. Note the enhancement of TH immunoperoxidase labeling in *ob/ob* mice. **(E)** Bar graph of TH peroxidase-based optical density of immunoreactivity (O.D). Data are means ± SEM from $n \geq 3$ sections/mouse, $n = 6$ mice/genotype; $^{***}P < 0.001$ *ob/ob* vs. wt mice; two-tail *t*-test with Welch's correction. **(F)** Bar graph showing the mean percentage of TH–expressing neurons calculated as ratio vs. the number of the neuronal marker NeuN-expressing cells (TH/NeuN ratio). Data are means ± SEM from $n \geq 3$ sections/mouse, $n = 6$ mice/genotype; $P = 0.33$; two-tail *t*-test with Welch's correction. **(G)** Levels of 2-AG in the VTA of wt (green bars) and *ob/ob* mice (blue bars). Data are from $n = 8$ mice per group and are expressed as means ± SEM; $^{\$\$\$}P < 0.001$ wt+vehicle or wt treated vs. wt+OX-A mice; $^{\circ}P < 0.05$ wt+O-7460 vs. wt+vehicle mice; $^{***}P < 0.001$ *ob/ob* vs. wt mice; $^{\#\#\#}P < 0.001$ *ob/ob* treated vs. *ob/ob*+vehicle mice; one-way ANOVA followed by Bonferroni test.

orexinergic inputs to dopamine neurons in this area. A wide OX-A/VGluT1 co-localization was found in the fibers projecting to the vast majority of TH-positive neuronal somata (**Figure 4B**). Along with excitatory orexinergic inputs, DA neurons are regulated also by inhibitory inputs coming from the striatal areas (Kalivas, 1993). Therefore, the study of the anatomical pathway underlying the control of the OX-A/DA network was further extended to the inhibitory component of this circuitry by performing a multiple TH/VGAT/CB1R immunofluorescence study. In agreement with a previous study (Li et al., 2010), immunoreactivity for VGAT, i.e., the main vesicular transporter of GABA, was assumed as a presynaptic marker of inhibitory inputs to TH-expressing somata. We found inhibitory CB1R-immunolabeled GABAergic synaptic endings opposed to the somata of TH-positive neurons in the VTA (**Figure 4C**).

To further demonstrate the ultrastructural details of the anatomical substrate wherein orexin, endocannabinoids, and dopamine interact, we performed a CLEM study of OX1R and CB1R immunolabeling in the VTA of TH-eGFP mice. CLEM analysis revealed that TH-eGFP somata of DA neurons express OX1R labeling (10 nm immunogold particles) at postsynaptic sites of asymmetrical (i.e., putative excitatory) inputs and receive immunogold labeled CB1R-positive puncta (6 nm immunogold) at symmetrical (i.e., putative inhibitory) synapses (**Figure 4D**).

OX-A Enhances Dopamine Levels in the VTA and NAc

By considering the anatomical circuitry underlying OX-A/2-AG/DA interactions in the VTA, we moved forward to investigate the functional effect of aberrant OX-A and 2-AG signaling on DA neurons, either in wt or *ob/ob* mice. This part of the study was carried out both in the VTA and NAc, the main VTA target area in the mesolimbic pathway forming the master circuitry that regulates reward-seeking behaviors and addiction (Alonso-Alonso et al., 2015).

ELISA assays revealed the enhancement of DA concentrations in the VTA and NAc of *ob/ob* mice in comparison to wt (VTA: 895.89 ± 29.12 pg/mL in *ob/ob* vs. 506.44 ± 20.79 pg/mL in wt) (NAc: 249.11 ± 13.72 pg/mL in *ob/ob* vs. 109.44 ± 10.32 pg/mL in wt) (**Figures 5A,B**). Such elevation was dependent on OX-A signaling since it was mimicked by OX-A injection in wt mice (40 μg/kg, i.p., 2 h) (in VTA: 692 ± 29.67 pg/mL; in NAc: 172 ± 12.66 pg/mL) in a manner attenuated by the OX1R antagonist SB-334867 (30 mg/kg, i.p., 3 h) (in VTA: 521.33 ± 25.99 pg/mL; in NAc:133.89 ± 14.50 pg/mL) and by the CB1R antagonist AM251, in wt mice (10 mg/kg, i.p., 3 h) (in VTA: 514.67 ± 16.94 pg/mL; in NAc: 118.44 ± 12.50 pg/mL). In the VTA of OX-A-injected (40 μg/kg, i.p., 2 h) *ob/ob* mice the DA levels were similar to the control *ob/ob* mice (901.11 ± 36.32 pg/mL) and, in several samples, it resulted very close to the maximum limit of detection (1,000 pg / mL) reported for the used ELISA kit. In *ob/ob* mice the DA levels were reduced by SB-334867 (60 mg/kg, i.p., 3 h) (in VTA: 552.78 ± 34.10 pg/mL; in NAc: and 138.56 ±15.90 pg/mL) or AM251 (10 mg/kg, i.p., 3 h) (in VTA: 544.11 ± 37.94 pg/mL; in NAc: 144 ± 11.74 pg/mL) injection either *per se* or before OX-A treatment [SB-334867 (60 mg/kg, i.p., 1 h) (in VTA: 645.67 ± 32.46 pg/mL; in NAc: 179.56 ± 15.30 pg/mL)] [(AM251 (10 mg/kg, i.p., 1 h) (in VTA: 651.44 ± 34.16 pg/mL; in NAc: 190.89 ± 12.06 pg/mL)]. Furthermore, DA levels were lowered in the VTA and NAc of *ob/ob* mice injected with leptin (5 mg/kg, i.p., 2 h) (in VTA: 583.22 ± 36.48 pg/mL; in NAc: 118.11 ± 17.33 pg/mL), in agreement with our previous finding concerning the inhibitory effect by this adipokine on the disinhibition of OX-A release from the LH of obese mice (Cristino et al., 2013) (**Figures 5A,B**).

OX-A Signaling Drives β-Arrestin2-Mediated Desensitization of D2 Receptors in the NAc of Obese Mice

To further investigate if OX-A, via 2-AG-mediated disinhibition of DA neurons in the VTA, could affect mesolimbic pathway possibly by regulating sensitivity to DA receptors in the NAc target area, we studied the immunohistochemical expression of β-arrestin2 in the NAc. Since, among the D1-D5 subtypes of DA receptors, the D2 subtype is mainly involved in reward processing of drugs and natural stimuli including food (Zlomuzica et al., 2018), we analyzed β-arrestin2 expression in D2R-positive neurons by performing β-arrestin2/D2R co-immunostaining and co-immunoprecipitation assays in the NAc of wt and obese mice. By confocal microscopy analysis of D2R/β-arrestin2 immunosignal we found that, contrary to wt mice (**Figure 6A$_1$**), a wide colocalization was found in the soma of NAc neurons of *ob/ob* mice (**Figure 6A$_3$**) wherein merged yellow signals were more frequently observed (see in **Figure 6** the comparison between inset A_2 and A_4 as a high magnification of the cell in the dotted boxed area in A_1 and A_3, respectively). These data suggest a pivotal role of OX-A in the overactivation, and subsequent desensitization, of DA signaling at the mesolimbic circuitry, which could underlie subsequent overeating and food-addictive behaviors. In agreement with immunohistochemical data, the β-arrestin2/D2R co-immunoprecipitation assay showed a significant increase of β-arrestin2/D2R ratio in the co-immunoprecipitate complex from the NAc of *ob/ob* vs. wt mice. OX-A injection in wt lean mice (40 μg/kg, i.p., 2 h) enhanced the coupling of β-arrestin2 to D2R, similar to what observed in *ob/ob* mice (1.83 ± 0.15 for wt + OX-A vs. 2.40 ± 0.21 for *ob/ob* mice) (**Figures 6B,C**). On the contrary, SB-334867 injection prevented, or strongly reduced, the formation of the β-arrestin2/D2R complex in wt (SB-334867, 30 mg/kg, i.p., 3 h) (0.97 ± 0.11) and wt OX-A injected mice (SB-334867, 30 mg/kg, i.p, 1h, followed by OX-A injection: 40 μg/kg, i.p., 2 h) (1.22 ± 0.12), as well as in *ob/ob* mice (SB-334867: 60 mg/kg, i.p., 3 h) (0.86 ± 0.09), thus confirming the involvement of OX-A and OX1R in β-arrestin2-mediated D2R desensitization. In the same way, obese *ob/ob* mice injected with leptin (5 mg/kg, i.p., 2 h) or with the selective D2R antagonist L741 (1.5 mg/Kg, i.p. 30 min) showed a lower β-arrestin2/D2R ratio as compared to the basal condition of vehicle-injected *ob/ob* mice (0.85 ± 0.09 for *ob/ob* + leptin and 1.02 ± 0.07 for *ob/ob* + D2R antagonist vs. 2.40 ± 0.21 for *ob/ob* mice), and similar to leptin- or D2R antagonist-injected wt mice

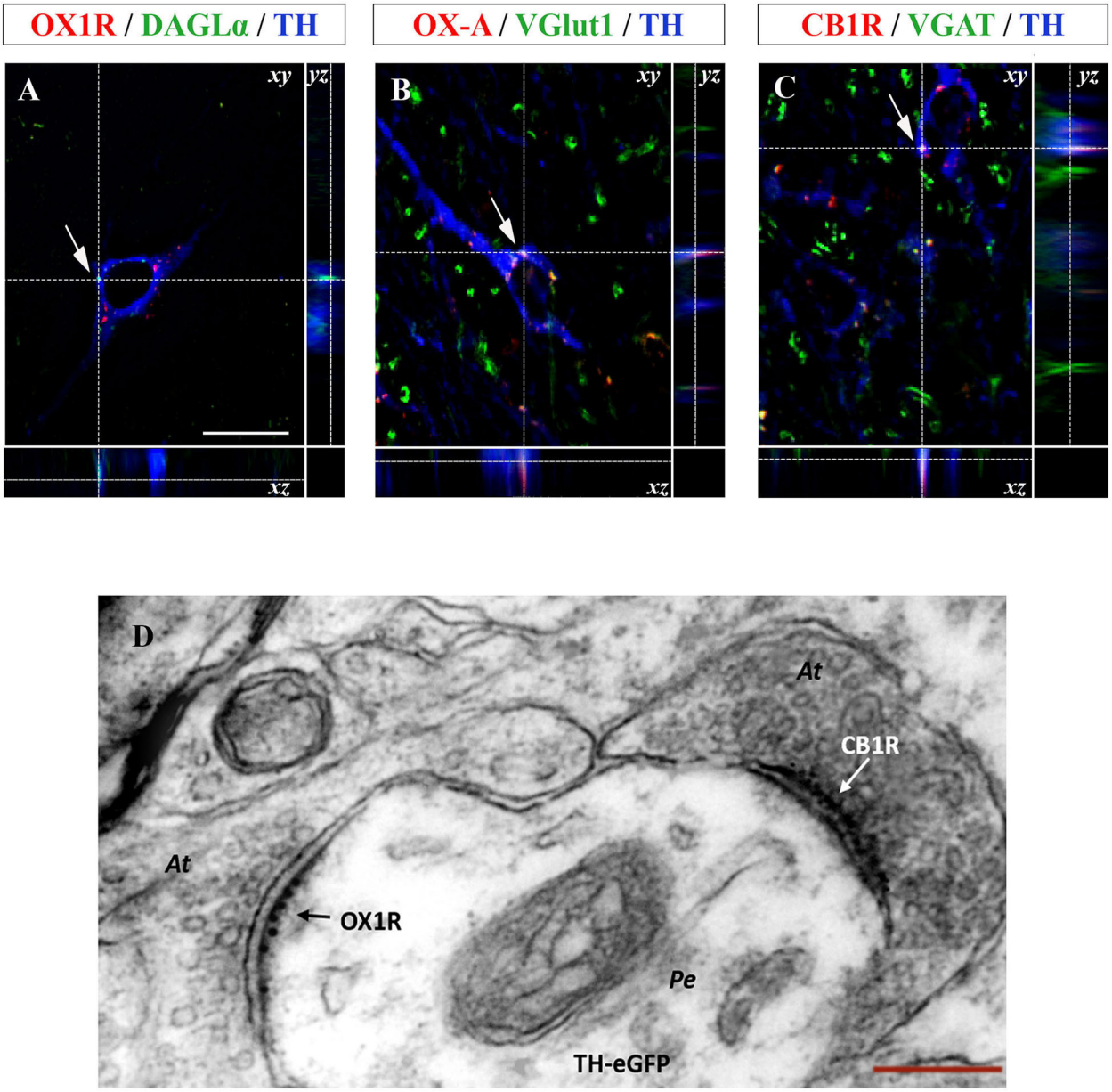


FIGURE 4 | (A–C) Confocal microscopy images (maximum intensity projections) of three multiple immunostained sections of the VTA. **(A)** Representative image of a dopaminergic neuron showing the co-expression of OX1R (red), DAGL-α (green), and TH (blue) immunoreactivities indicated by arrows. **(B)** Representative image of a dopaminergic neuron showing OX-A/VGluT1/TH co-expression (arrow). **(C)** Representative image of a TH positive neuron (blue) receiving CB1R immunolabeled puncta (red) colocalizing with VGAT immunoreactive inputs (green). Arrows indicate the merged immunolabeling at *xy* optical intersection, which is represented by high magnification in the lateral insets showing the *yz* and *xz* intersection of the dotted lines [scale bar: 30 μm]. **(D)** Representative electron micrograph showing double OX1R/CB1R immunogold reactivity in the VTA of TH-eGFP mice. Note asymmetrical, i.e., putative excitatory, axosomatic (At) input to the perikaryon (Pe) of a TH-eGFP neuron expressing OX1R-immunogold labeling and receiving symmetrical, i.e., putative inhibitory, CB1R immunogold labeled input at a different domain of the cell membrane (right) [Scale bar: 150 nm].

(1.14 ± 0.08 for wt + leptin mice and 0.93 ± 0.06 for wt + D2R antagonist-treated mice) (**Figures 6B,C**). In agreement with the abundant formation of the β-arrestin2/D2R complex in *ob/ob* mice, as revealed by the co-immunoprecipitation assay, a reduced percentage of c-*Fos*/D2R positive neurons was found in the NAc of *ob/ob* (29 ± 5.58%) in comparison to wt mice. This reduction was mimicked by OX-A injection in wt mice (40 μg/kg, i.p., 2 h) (40 ± 4.12%) and counteracted by SB-334867 injection in both wt (30 mg/kg, i.p., 3 h) (68.13 ± 5.21%) and *ob/ob* mice (60 mg/kg, i.p., 3 h) (55 ± 6.31%) (**Figure 6D**). Consistent with the immunohistochemical and molecular findings (**Figures 6A,B**) which identify the D2R as the main target of DA signaling in the NAc, the pharmacological blockade of D2R by the administration of selective D2R antagonist L741 (1.5 mg/kg,

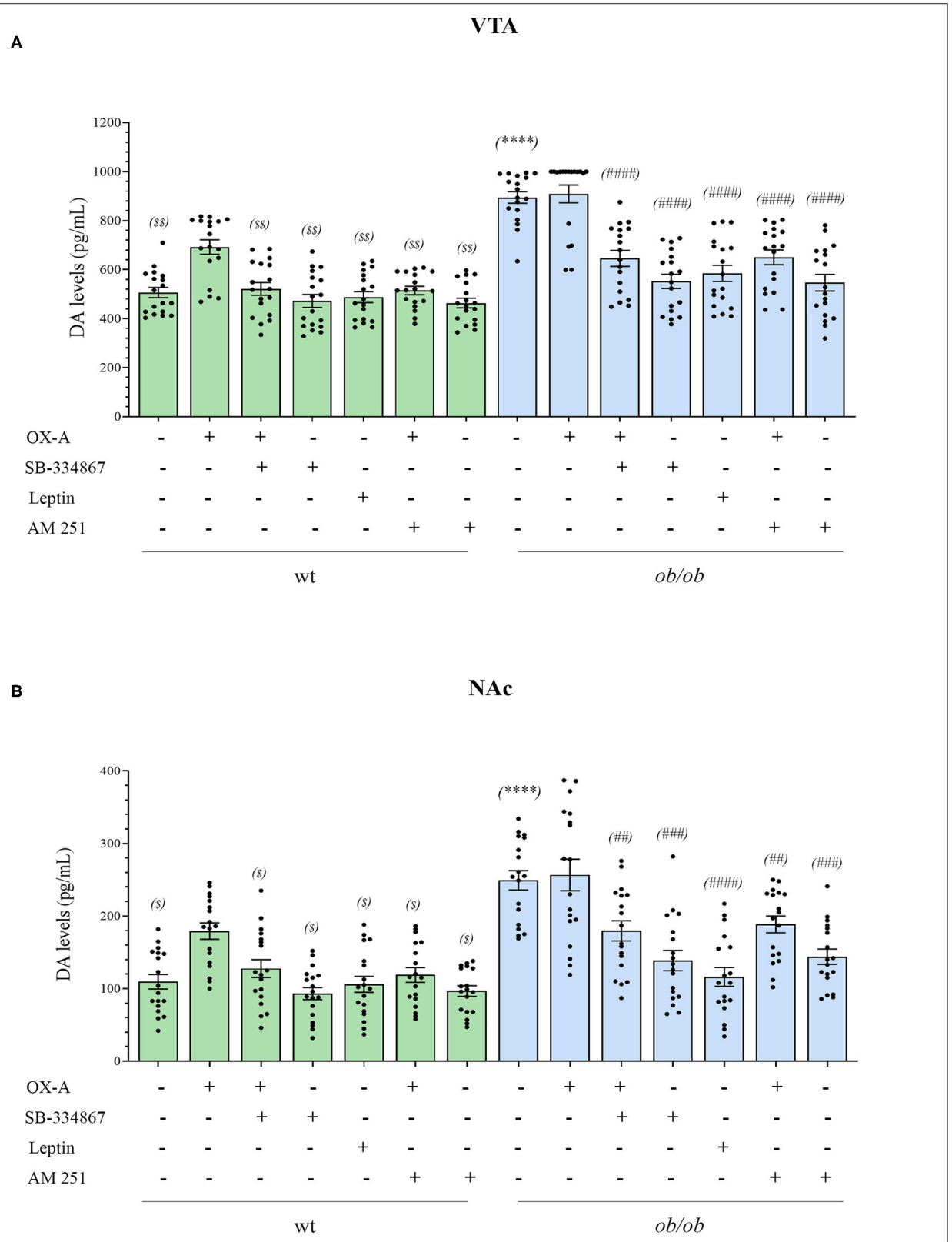


FIGURE 5 | Bar graph of dopamine levels in the VTA **(A)** and NAc **(B)** of wt lean (green bars) and *ob/ob* obese (blue bars) mice. Data are means ± SEM from $n \geq 18$ VTA or Nac sampled by punch microdissection (~1.5 mm diameter) from each brain hemisphere of a mouse, $n = 9$ mice/genotype per group; $^{\$}P < 0.05$, $^{\$\$}P < 0.005$ wt+vehicle or wt treated vs. wt+OX-A vs. mice; $^{****}P < 0.0001$ *ob/ob* vs. wt mice; $^{\#\#}P < 0.005$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ *ob/ob* treated vs. *ob/ob*+vehicle; two-way ANOVA followed by *post hoc* Tukey test.

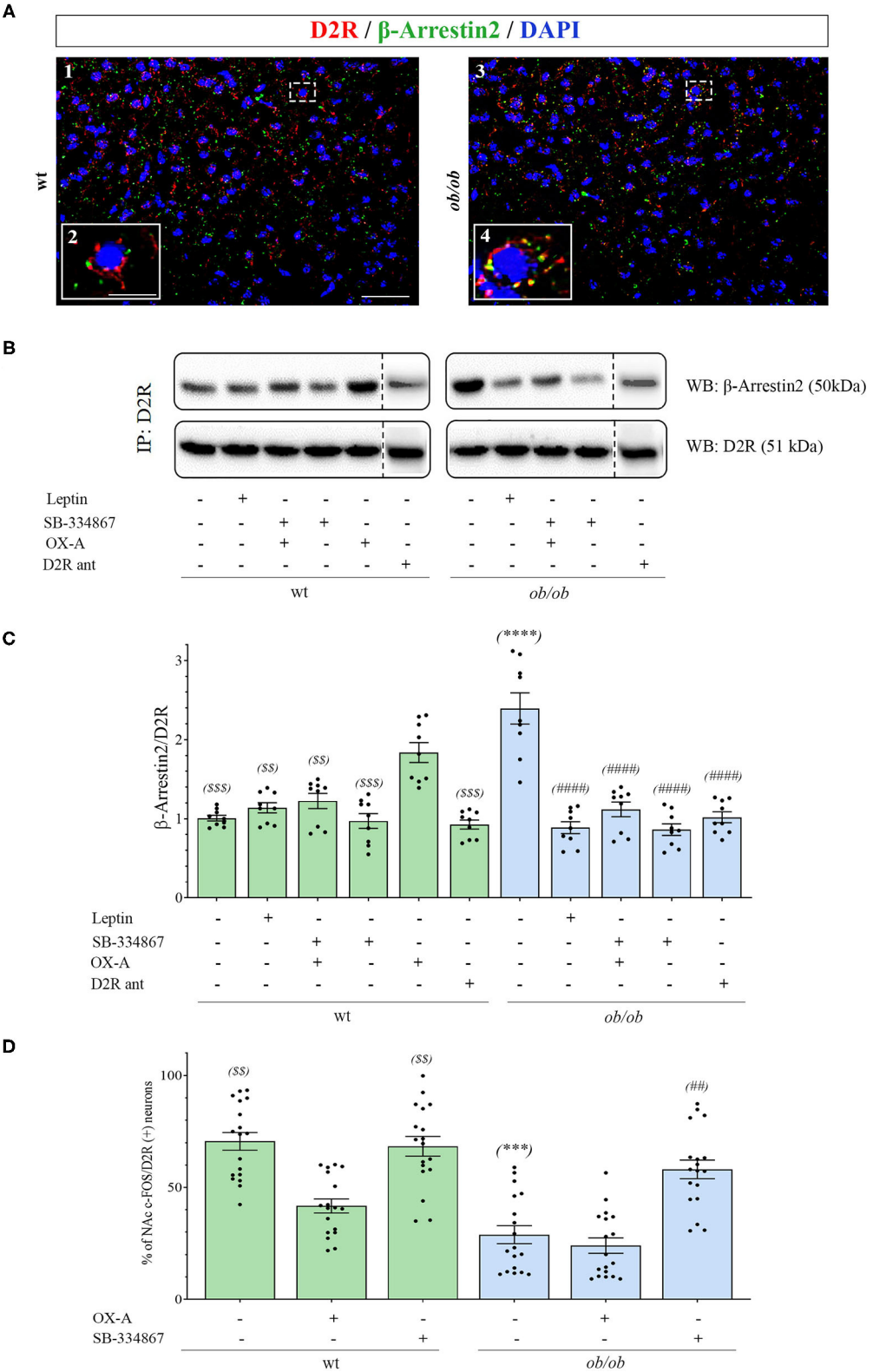


FIGURE 6 | (A) Confocal microscopy showing D2R (red) and β-arrestin2 (green) immunoreactivities in neurons of NAc in wt (A1) and *ob/ob* mice (A3). Note the high D2/β-arrestin2 colocalization (yellow) in *ob/ob* mice in comparison to wt mainly at the plasmatic membrane as revealed by the high magnification of a representative cell in the dotted boxed area in A1 and A3 and depicted in the respective inset as A2 and A4. Images are representative of $n \geq 6$ mice per group. [Scale bar: scale

(Continued)

FIGURE 6 | bar: 60 μm (A1 and A3), 30 μm (A2 and A4)]. **(B)** Representative immunoblots from D2R/β-arrestin2 co-immunoprecipitation assays from the NAc of wt and *ob/ob* mice. OX-A treatment (40 μg/kg, i.p., 2 h) in wt mice strongly increased the formation of the D2R/β-arrestin2 complex in comparison to vehicle-treated mice. In *ob/ob* mice the binding of D2R to β-arrestin2 was sensitive to treatment with leptin (5 mg/kg, i.p., 2 h) and SB-334867 (60 mg/kg, i.p., 3 h). **(C)** Densitometric analysis of the D2/β-arrestin2 complex. Data are means ± SEM from $n = 8$ mice/genotype per group; $^{\$\$}P < 0.005$, $^{\$\$\$}P < 0.001$ wt+vehicle or wt treated vs. wt+OX-A mice; $^{****}P < 0.0001$ *ob/ob* vs. wt mice; $^{\#\#\#\#}P < 0.0001$ *ob/ob* treated vs. *ob/ob*+vehicle mice; one-way ANOVA with Bonferroni *post hoc* **(D)** Percentage of c-*Fos***/**D2R positive neurons in the NAc of wt and *ob/ob* mice with or without SB-334867 injection (i.p., 3 h, 30 mg/kg, for wt and 60 mg/kg for *ob/ob* mice), or OX-A treatment (40 μg/kg, i.p., 2 h). Data are means ± SEM for n ≥3 sections/mouse, $n = 6$ mice/genotype per group; $^{\$\$}P < 0.005$ wt+vehicle or wt+SB-334867 vs. wt+OX-A mice; $^{***}P < 0.001$ *ob/ob* vs. wt mice; $^{\#\#}P < 0.005$ *ob/ob*+SB-334867 vs. *ob/ob*+vehicle mice; two-way ANOVA followed by *post hoc* Tukey test.

i.p. 30 min) prevented D2R desensitization in obese mice by reducing the β-arrestin2/D2R co-immunoprecipitation ratio vs. that observed in vehicle-injected *ob/ob* mice (**Figure 6C**).

In summary, in this part of the study we found that:

1. D2R represents the main target of DA signaling in the NAc. OX-A-mediated enhancement of DA signaling in obese mice eventually reduced D2R activity by promoting the formation of D2R/β-arrestin2 complex and D2R desensitization.
2. Almost half of the D2R-positive neurons in the NAc of *ob/ob* mice was constitutively insensitive to DA signaling as revealed by c-Fos immunoreactivity. This finding was reproduced by OX-A administration to wt mice and prevented by administration of the selective D2R antagonist L741, or by the administration of OX1R antagonist SB334867, *per se*, in obese mice, or before OX-A injection, in wt mice.

Both these findings suggest that the motivational aspects of reward processing mediated by D2R are altered in obese mice.

DISCUSSION

Because OX-A and 2-AG have been implicated in addiction-related behaviors (España, 2012; Oleson and Cheer, 2012; Baimel et al., 2015; Hernandez and Cheer, 2015), and OX-A triggers 2-AG biosynthesis via the PLC/DAGL-α pathway downstream to OX1R, we were interested in identifying the neural circuit underlying the OX-A/2-AG interplay in the VTA, and in testing if OX-A alters DA levels in the VTA by modulating local 2-AG synthesis and release in mice. With this aim we studied wt lean mice and leptin knockout obese *ob/ob* mice, this latter being characterized by an elevated basal tone of OX-A signaling due to the lack of leptin signaling (Cristino et al., 2013).

Despite the significant orexinergic innervation of the VTA, where both OX1R and OX2R receptors are found in neurons of the mesolimbic pathway (Marcus et al., 2001; Narita et al., 2006), OX-A/OX1R signaling represents the master player of dopaminergic neurons. Several studies have demonstrated that OX-A infusions into the VTA can increase DA concentration in the shell (Vittoz et al., 2008), core (España et al., 2011) or both, of the Nac (Narita et al., 2006). Moreover, an increase of local DA release in the NAc shell has been demonstrated following OX-A treatment of brain slices (Patyal et al., 2012). We report that 2 h following i.p. OX-A injection in mice can increase DA levels in the VTA and NAc in concomitance with the enhancement of 2-AG levels and via the potential CB1R-mediated disinhibition of GABAergic inputs to TH-ir neurons. OX-A-induced elevation of DA was prevented by the OX1R antagonist SB-334867 or by the CB1 antagonist AM251 injected i.p. 1 h before OX-A. In addition to glutamatergic signaling contributing to cocaine-induced sensitization (Borgland et al., 2006), GABAergic transmission is also an important modulator of VTA dopaminergic activity. OX-A application to VTA slices, in addition to increasing the firing of dopaminergic neurons, also directly decreased the firing of GABAergic neurons, suggesting the existence of another cellular mechanism related to OX-A modulation of GABAergic activity triggered by endocannabinoid signaling at the VTA and increasing VTA dopaminergic activity by retrograde disinhibition via CB1R inhibition of GABAergic inputs (Alger, 2002; Melis et al., 2004; Ho et al., 2011).

Electrophysiological studies further demonstrated that activation of OX1R in VTA dopaminergic neurons initiates a Gq/11-coupled PLC-DAGL pathway leading to the biosynthesis of 2-AG, which, retrogradely, activates CB1R at both inhibitory and excitatory inputs to DA neurons and regulating burst firing and DA release of VTA-DA neurons (Tung et al., 2016). In resting conditions, around 50% of DA neurons in this area are innervated by inhibitory GABAergic inputs (Grace and Bunney, 1984). Despite the fact CB1R was expressed at both GABAergic and glutamatergic inputs to VTA dopaminergic neurons (Mátyás et al., 2008), those innervated by OX-A afferences are mostly synaptically contacted by GABAergic CB1R expressing inputs (Tung et al., 2016). In line with this functional mechanism, we here provide analytical quantification for 2-AG levels in the VTA and show that OX-A/OX1R-mediated activation of the PLC-DAGLα pathway underlies enhanced 2-AG biosynthesis in the VTA of both OX-A-injected lean wt mice and obese *ob/ob* mice. In fact, this effect was prevented by SB334867 injection or by inhibition of DAGLα with 0-7460. Furthermore, confocal and electron microscopy analysis revealed the cytoarchitectonic distribution of the main enzymes, receptors, and neurotransmitters forming the OX-A-controlled DA network in the mouse VTA (Graphical abstract, A and B).

Consistent with the enhancement of OX-A trafficking from the LH to different target areas in the brain in *ob/ob* mice (Cristino et al., 2013; Morello et al., 2016), here we found intense OX-A immunoreactivity in the fibers projecting to the VTA and NAc. In both lean OX-A-injected and obese *ob/ob* mice, the elevation of OX-A signaling in the VTA was accompanied by enhancement of 2-AG and DA levels in the VTA and NAc. All these effects were prevented by OX1R antagonism and are in line with the reduced DA levels and release observed previously in *Hcrt*-KO mice (Shaw et al., 2017). From the neurobiological point

of view, the aberrant disinhibition of OX-A neurons described in *ob/ob* mice (Cristino et al., 2013; Becker et al., 2017) could explain the enhancement of orexinergic tone associated with the pathological hyperarousal typical of compulsive and addictive behaviors (Boutrel et al., 2005).

Several studies report that such behaviors, with either food or drugs of abuse, drive dopaminergic signaling via D2R, thereby inducing a paradoxical impairment in reward processes and to subsequent tolerance and addictive behavior (Wang et al., 2002; Koob and Le Moal, 2005; Borgland et al., 2006; Avena et al., 2008; Johnson and Kenny, 2010). Our results reveal that D2R undergoes β-arrestin2-mediated desensitization in the NAc upon longer-lasting activation induced by prolonged exposure to DA in obese mice (Graphical abstract, C). This condition is reflected by the reduction of c-*Fos* expression, a marker of neuronal functional activity, in D2R neurons. Both the D2R molecular internalization by the β-arrestin2 complex, and c-Fos reduction were prevented by injection of the selective D2R antagonist L741, as well as by SB334867, in agreement with similar results obtained in high fat fed obese mice (Valdivia et al., 2014).

Our immunohistochemical data reporting the enhancement of TH-ir in VTA neurons of *ob/ob* mice are, apparently, not in line with those referring to a reduction or no difference between *ob/ob and* wt mice (Fulton et al., 2006). This discrepancy could be possibly ascribed to a different method of TH quantification, which we based on single-cell optical imaging densitometry, hence avoiding to measure the tissue outside TH-ir perikarya. Additionally, the way and duration of the pharmacological treatments and the method for DA quantification were also different in our study, wherein leptin i.p. injection reduced DA levels in the VTA and NAc, in contrast to the DA elevation observed in leptin i.c.v.-injected *ob/ob* mice (Leinninger et al., 2009).

Several anatomical and functional interactions occur between the orexinergic and dopaminergic systems: (i) dendrites and somata of DA neurons receive OX-A-positive afferences, as demonstrated in this work and by others (Fadel and Deutch, 2002); (ii) OX-A increases the firing rate of TH-positive neurons in the VTA (Tung et al., 2016); (iii) OX-A increases the firing of VTA DA neurons projecting to the shell region of the NAc (Baimel et al., 2017); (iv) blocking D2R reduces hyperlocomotion and stereotypy induced by i.c.v. injection of OX-A (Nakamura et al., 2000). OX-A and drugs such as amphetamines share the ability to induce hyperlocomotion, grooming, and stereotypy in rats, typically considered behaviors of enhanced dopaminergic tone (Ida et al., 1999). These effects are inhibited by antagonism of D2R, thereby suggesting the involvement of the dopaminergic system in behavioral responses induced by OX-A (Nakamura et al., 2000). Accordingly, clinical observations have reported that human patients suffering from narcolepsy, due to almost complete loss of orexinergic neurons, despite long-term medical treatment with amphetamines and other wakefulness-stimulating substances, rarely develop an addiction to stimulant drugs of abuse (Kilduff and Peyron, 2000; Nishino et al., 2000; Barateau et al., 2016). In line with these clinical data in humans, orexin deficient mice (knock-out for *Hcrt*) show a lower consumption of sucrose when available *ad libitum* compared to littermate wt mice (Matsuo et al., 2011). Orexinergic neurons are activated by physiological homeostatic stimuli, including hypoglycemia and caloric restriction (Milbank and Lopez, 2019). Moreover, they are activated also in response to environmental cues like food, even during periods of relatively high energy abundance, thus indicating the ability of the orexinergic system to be activated in response to anticipation of rewards induced by high palatable food (Choi et al., 2010). This latter activates orexinergic neurons that project to the VTA by increasing motivational behavior that drives the craving for appetitive reinforcers (Valdivia et al., 2014). Likewise, the anticipation of preferred, palatable food and/or its consumption, with respect to a non-preferred food, also increases circulating 2-AG levels in human volunteers, and this response is accentuated in obese individuals and patients with binge eating disorder (Monteleone et al., 2016, 2018), in agreement with the enhanced OX-A/2-AG interaction that we have found here to occur in the VTA of *ob/ob* mice. Additionally, retrograde endocannabinoid signaling at CB1 receptors in the VTA is a well-established mechanism for the reinforcement of DA signaling in this area and of its behavioral consequences in food reward (Bacharach et al., 2018; Wenzel and Cheer, 2018). Altogether, our data suggest that aberrant OX-A signaling triggers a vicious OX1R/PLC-DAGL/2-AG/CB1 -mediated loop which promotes DA production and, possibly, release in the VTA and NAc. Excessive DA levels then act mainly via D2R in the mesolimbic area and cause D2R desensitization through the beta-arrestin2 pathway, with subsequent impairment of the rewarding process. These data put forward novel CB1R-mediated mechanisms for the regulatory action of OX-A in the mesolimbic dopaminergic system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by European Communities Council Directive of September 22, 2010 (2010/63/EU) and the Italian Decree n.26/2014, authorization n. 152/2020-PR.

AUTHOR CONTRIBUTIONS

LC conceived and designed the study. PG, VD, and LC contributed to the theoretical framework. VD and LC wrote the manuscript. LT prepared the figures. LT, LD'A, AF-R, and RI performed immunohistochemical studies. LT and RI performed electron microscopy study. AF-R and NF performed co-immunoprecipitation assay. LT, LD'A, and AF-R performed ELISA assay and FP performed LC-MS analysis. All the authors analyzed the data. All authors read and approved the final manuscript.

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N-Oleoylglycine and *N*-Oleoylalanine Do Not Modify Tolerance to Nociception, Hyperthermia, and Suppression of Activity Produced by Morphine

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The endogenous amide *N*-Oleoylglycine (OlGly) and its analog *N*-Oleoylalanine (OlAla), have been shown to interfere with the affective and somatic responses to acute naloxone-precipitated MWD in male rats. Here we evaluated the potential of a single dose (5 mg/kg, ip) which alleviates withdrawal of these endogenous fatty acid amides to modify tolerance to anti-nociception, hyperthermia, and suppression of locomotion produced by morphine in male Sprague-Dawley rats. Although rats did develop tolerance to the hypolocomotor and analgesic effects of morphine, they did not develop tolerance to the hyperthermic effects of this substance. Administration of neither OlGly nor OlAla interfered with the establishment of morphine tolerance, nor did they modify behavioral responses elicited by morphine on any trial. These results suggest that the effects of OlGly and OlAla on opiate dependence may be limited to naloxone-precipitated withdrawal effects.

Keywords: *N*-oleoylglycine, *N*-oleoylalanine, morphine, tolerance, analgesia

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INTRODUCTION

N-Oleoylglycine (OlGly) is an endogenous fatty acid amide that is structurally similar to *N*-aceylethanolamines, including the endocannabinoid, anandamide (AEA), as well as the endogenous peroxisome proliferator-activated receptor alpha (PPARα) agonists, *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA). Endogenous OlGly is released in the insular cortex of mice experiencing traumatic brain injury (Donvito et al., 2019). Since the insular cortex is a site critical for nicotine addiction (Naqvi et al., 2007, 2014; Forget et al., 2010), the laboratory of Raphael Mechoulam synthesized OlGly for evaluation of the potential of this fatty acid to mitigate nicotine addiction. Indeed, synthetic OlGly interfered with nicotine reward and dependence in mice. OlGly did not bind with cannabinoid 1 (CB_1) or cannabinoid 2 (CB_2) receptors *in vitro* and did not produce the typical CB_1 receptor tetrad (antinociception, hypothermia, catalepsy, and hypomobility) of behaviors in mice. OlGly was shown to bind with PPARα and to inhibit fatty

acid amide hydrolase (FAAH) *in vitro*. Indeed, the interference with nicotine reward by OlGly was reversed by a PPARα antagonist, a finding consistent with previous reports of PPARα agonists interfering with nicotine reward (Mascia et al., 2011; Justinova et al., 2015; Jackson et al., 2017). In contrast, a CB_1 receptor antagonist did not reverse OlGly interference with nicotine reward (Donvito et al., 2019).

Not only has OlGly been shown to interfere with nicotine dependence, but also with the aversive (Petrie et al., 2019) and somatic (Rock et al., 2020) withdrawal produced by acute naloxone-precipitated morphine withdrawal (MWD), at a dose of 1 or 5 mg/kg, intraperitoneal (ip), but not 20 mg/kg, ip, in rats. On the other hand, OlGly neither modified a morphine place preference (also reported in mice by Donvito et al., 2019), nor did it modify reinstatement of a previously extinguished morphine conditioned place preference (Petrie et al., 2019). At the effective dose of 5 mg/kg, OlGly had no effects on its own in the absence of MWD. Although interference with the affective properties of MWD by OlGly was prevented by pretreatment with a CB_1 receptor antagonist but not a PPARα antagonist (Petrie et al., 2019), interference with somatic MWD responses by OlGly was prevented by either pretreatment with a CB_1 receptor antagonist or a PPARα antagonist (which had no effects on their own). Since OlGly does not strongly bind to CB_1 receptors, the interference with the anti-withdrawal effects of OlGly on CB_1 receptors was most likely mediated by its action as a FAAH inhibitor, and subsequent elevation of endogenous AEA levels.

Because endogenous OlGly is rapidly deactivated by amidases, a more stable analog, *N*-oleoylalanine (OlAla), was synthesized by Mechoulam's group. OlAla, which is also an endogenous lipid (Bradshaw et al., 2009), also interfered with the affective symptoms of acute naloxone-precipitated MWD, but it was effective for a longer duration than OlGly through a CB_1 receptor and PPARα -dependent mechanism (Ayoub et al., 2020). Accordingly, OlAla was found to inhibit FAAH and activate PPARα *in vitro*.

If OlGly and OlAla are effective in the treatment of MWD, it is important to determine if they might also modify tolerance to morphine at the most effective therapeutic dose of 5 mg/kg, ip, when tested under similar housing conditions as our previous work. Indeed, recent evidence indicates that the FAAH inhibitor, URB597, prevents tolerance to morphine nociception in the tail immersion test (Fotio et al., 2020). Here we evaluated the potential of OlGly and OlAla to modify tolerance to nociception, hyperthermia, and suppression of activity produced by morphine under a similar morphine treatment regime as employed by Fotio et al. (2020).

MATERIALS AND METHODS

Animals

All animal procedures complied with the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee at the University of Guelph. Naïve male Sprague-Dawley rats (n = 64), weighing between 215 and 244 g on the day of habituation, obtained from Charles River Laboratories (St Constant, Quebec), were used. The rats were pair-housed in opaque plastic shoebox cages (48 × 26 × 20 cm), containing bed-o-cob bedding from Harlan Laboratories, Inc. (Mississauga, Ontario), a brown paper towel, and Crink-l′Nest™ (The Andersons, Inc., Maumee, Ohio). Additionally, the rats were provided with a soft white paper container that was 14 cm long and 12 cm in diameter. The colony room was kept at an ambient temperature of 21°C with a 12/12 h light-dark schedule (lights off at 7 am). The rats were tested in their dark cycle and were maintained on chow and water *ad libitum*.

Drugs

Morphine (MOR) was prepared in saline (SAL) at a concentration of 10 or 20 mg/ml and administered subcutaneously (sc) at 1 ml/kg (10 or 20 mg/kg, respectively). OlGly and OlAla were dissolved in a vehicle (VEH) mixture of ethanol, Tween 80, and physiological SAL in a 1:1:18 ratio. OlGly and OlAla were first dissolved in ethanol, Tween 80 was then added to the solution, and the ethanol was evaporated off with a nitrogen stream, after which, the SAL was added. The final VEH consisted of 1:9 (Tween80:saline). OlGly and OlAla were prepared at a concentration of 5 mg/ml and was administered intraperitoneally (ip; 1 ml/kg).

Apparatus

Locomotor Test

To assess locomotor activity, a locomotor chamber made of black Plexiglas (60 × 25 × 25 cm) was placed in a room illuminated by a red light. The locomotor activity of each rat was captured by a video camera placed above the chamber and sent to the Ethovision software program (Noldus, Inc., NL) to measure distance (cm) traveled.

Tail Flick Test of Nociception

For the tail flick test, a water bath was maintained at 50°C. The rats were lightly restrained, and the distal two-thirds of the tail was submerged in the water bath. The time (sec) the rat kept its tail in the water was recorded. Two withdrawal response latencies were taken, 1 min apart to obtain an average tail flick latency. To protect against tissue injury, the test was stopped after 10 s if the animal did not flick its tail.

Body Temperature

Body temperature measures were taken using an infrared digital ear thermometer (Model: KI-8170, Life Brand; Shoppers Drug Mart Inc., Canada). Rats were lightly restrained, and the probe tip of the thermometer was carefully inserted into the top of the ear canal and held in place until a reading was obtained. Three consecutive body temperature measures were taken to obtain an average body temperature measure.

Procedures

The experimental schedule is presented in **Table 1**. Following arrival in the facility, to establish baseline, all rats underwent the locomotor activity test, tail flick test and body temperature measures. On the baseline day and subsequent testing days, rats were first placed in the locomotor activity chambers for 15 min.

TABLE 1 | Experimental schedule.

Day	Morning Injection OlGly or OlAla Beginning at 7:50 AM MOR or SAL Beginning at 8 AM	Locomotion Test Beginning at 8:30 AM 15 min	Tail Flick Test Beginning at 8:50 AM	Temperature Beginning at 9 AM	Afternoon Injections OlGly or OlAla Beginning at 3:50 PM MOR or SAL Beginning at 4 PM	Morphine Dose (mg/kg, s)
Baseline		X	X	X		
Day 01	X	X	X	X	X	10
Day 02	X				X	20
Day 03	X		X	X	X	20
Day 04	X				X	20
Day 05	X	X	X	X	X	20
Day 06	X				X	20
Day 07	X		X	X	X	20
Day 08	X				X	20
Day 09	X	X	X	X	X	20
Day 10	X				X	20
Day 11	X		X	X	X	20
Day 12	X				X	20
Day 13	X	X	X	X	X	20

They were then tested in the tail flick test, followed by body temperature measures as depicted in **Table 1**. Rats were randomly assigned to receive an injection of OlGly (5 mg/kg, ip, Experiment 1), OlAla (5 mg/kg, ip, Experiment 2) or VEH 10 min prior to an injection of SAL or morphine (10 mg/kg on Day 01 and 20 mg/kg for all subsequent days, sc) injections twice daily (beginning at 8 AM and 4 PM) for 13 days. This resulted in the following groups for Experiment 1 (n = 8/group): VEH-SAL, VEH-MOR, OlGly-SAL, OlGly-MOR, and the following groups for Experiment 2 (n = 8/group): VEH-SAL, VEH-MOR, OlAla-SAL, OlAla-MOR.

Statistical Analysis

For Experiments 1 and 2, the distance (cm) traveled in the activity test of each rat were entered into a 2 × 2 × 5 mixed factors ANOVA with the factors of Chronic Treatment (SAL or MOR) × Pretreatment (VEH and OlGly or OlAla) × Day. The mean seconds to flick the tail from the two tests of nociception were entered into a 2 × 2 × 8 mixed factors ANOVA for Experiments 1 and 2. The mean body temperature of the three measures were entered into a 2 × 2 × 8 mixed factors ANOVA for Experiments 1 and 2.

RESULTS

Experiment 1

Ability of OlGly to modify tolerance to nociception, hyperthermia, and suppression of locomotor activity produced by morphine.

Rats developed tolerance to the hypolocomotor effects and to the analgesic, but not to the hyperthermic, effects of morphine. However, OlGly did not interfere with the establishment of tolerance. **Figure 1A** presents the mean (± sem) distance (cm) traveled in the locomotion test by the various groups across the 13 days of chronic morphine. The mixed factors ANOVA for activity revealed a significant effect of chronic treatment by day, $F(4, 112) = 25.4$; $p < 0.001$. On Days 01 and 05, rats administered morphine were less active than those administered saline (p's < 0.001), but not on Days 09, 13 or baseline. **Figure 1B** presents the mean (± sem) latency to tail flick from a hot water bath for the groups across the 13 days of chronic morphine. The mixed factors ANOVA for tail flick latency revealed a significant effect of chronic treatment by day, $F(7, 196) = 29.2$; $p < 0.001$. On Days 01–09, rats administered chronic morphine showed longer tail flick withdrawal latencies than those administered chronic saline (p's < 0.001), but they did not differ at baseline or Days 11–13. **Figure 1C** presents the mean (± sem) body temperature (°F) displayed by the various groups across the 13 days of morphine administration. The mixed factors ANOVA for body temperature revealed a significant chronic drug by day effect, $F(7, 196) = 14.0$; $p < 0.001$. Across all days (except for baseline), rats administered chronic morphine had higher body temperatures than rats administered saline (p's < 0.001). OlGly did not modify any behavioral effect of morphine across the 13 days of chronic morphine exposure.

Experiment 2

Ability of OlAla to modify tolerance to nociception, hyperthermia, and suppression of locomotor activity produced by morphine.

Much like in Experiment 1, rats developed tolerance to the hypolocomotor effects and to the analgesic, but not to the hyperthermic, effects of morphine. However, OlAla did not interfere with the establishment of tolerance. **Figure 2A** presents the mean (± sem) distance (cm) traveled in the locomotion test by the various groups across the 13 days of chronic morphine. The mixed factors ANOVA for activity revealed a significant effect of chronic treatment by day $F(4, 112) = 14.6$; $p < 0.001$. On Days 01, 05 (p's < 0.001), and 13 ($p < 0.05$), rats administered chronic morphine were less active than those administered chronic saline, but not at baseline. **Figure 2B** presents the mean

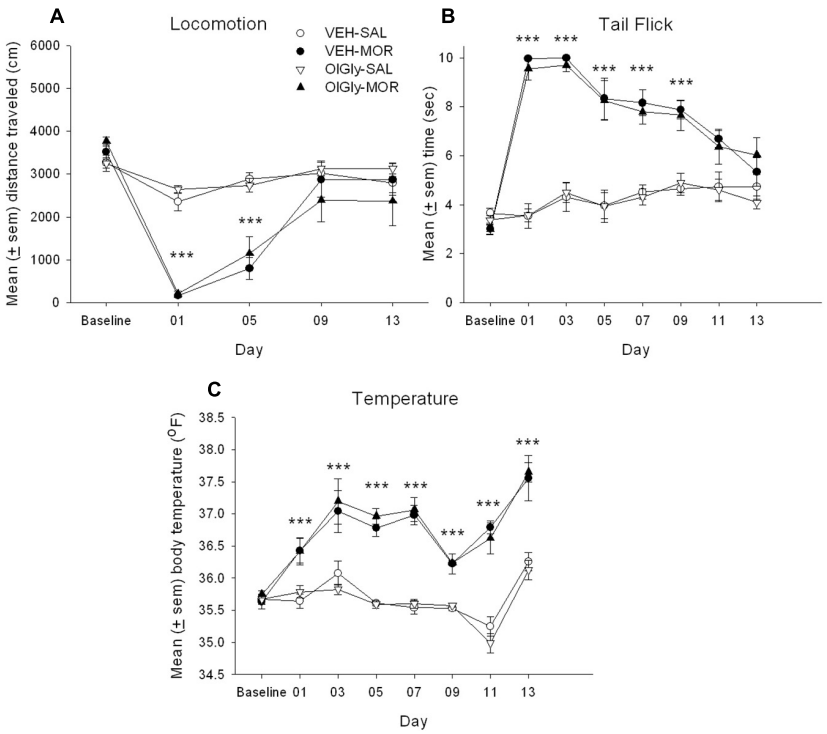

FIGURE 1 | OlGly **(A)** the mean (± sem) distance (cm) traveled in the locomotion test, **(B)** the mean (± sem) latency to tail flick from a hot water bath, **(C)** mean (± sem) body temperature (°F), displayed by the various groups across the 13 days of chronic morphine. Asterisks indicate a significant difference from SAL, $^{***}p < 0.001$.

(± sem) latency to tail flick for the groups across the 13 days of chronic morphine. The mixed factors ANOVA for tail flick revealed a significant effect of chronic treatment by day $F(7, 196) = 18.6$; $p < 0.001$. On Day 01–11 (p's < 0.001), and 13 ($p < 0.05$), rats administered chronic morphine showed longer tail flick withdrawal latencies than those administered chronic saline, but they did not differ at baseline. **Figure 2C** presents the mean (± sem) body temperature (°F) displayed by the various groups across the 13 days of morphine administration. The mixed factors ANOVA for body temperature revealed a significant chronic drug by day effect, $F(7, 196) = 41.9$; $p < 0.001$. Across all days (except for baseline), rats administered chronic morphine had higher body temperatures than rats administered saline (p's < 0.001). OlAla did not modify any behavioral effect of morphine across the 13 days of chronic morphine exposure.

DISCUSSION

Chronic dosing of morphine (twice daily 20 mg/kg, except day 1, for 13 days) resulted in tolerance to the hypolocomotor and the anti-nociceptive, but not to the hyperthermic, effects of morphine, as has been reported to the anti-nociceptive effects of morphine by Fotio et al. (2020). Here rats were injected with OlGly (5 mg/kg, ip, Experiment 1) or OlAla (5 mg/kg ip, Experiment 2) prior to each chronic treatment with morphine or saline to determine if these fatty acid amides that act *in vitro* as FAAH inhibitors and PPARα agonists (Donvito et al., 2019; Ayoub et al., 2020), would interfere with morphine tolerance as has been reported for the FAAH inhibitor, URB597 (Fotio et al., 2020). However, at least at a dose of 5 mg/kg, ip, neither OlGly nor OlAla administration interfered with the establishment of tolerance to the anti-nocicieptive and the hypolocomotor effects of morphine. These results suggest that, at the most effective dose for interference with acute naloxone-precipitated MWD responses (Petrie et al., 2019; Ayoub et al., 2020; Rock et al., 2020), neither OlGly nor OlAla are likely to prevent the establishment of tolerance to the chronic effects of morphine. However, it is possible that a higher dose of OlGly or OlAla would be required to chronically reduce tolerance than that required to acutely reduce opioid withdrawal. It is also possible that the doses of OlGly or OlAla employed here would interfere with tolerance to a lower dose of chronically administered morphine. As well, as this work has been conducted only with male rats, it is conceivable that female rats may respond differently to the effects of these fatty acid amides on tolerance to morphine or on naloxone-precipitated MWD effects.

Unlike OlGly and OlAla, the FAAH inhibitor URB597, has recently been shown to prevent tolerance to morphine anti-nociception in the tail flick test using a similar procedure and dosing schedule as was used here (Fotio et al., 2020). The effect of URB597 on morphine tolerance was reversed by pretreatment with a cannabinoid 2 (CB_2) receptor antagonist (AM630) and partially suppressed by pretreatment with a

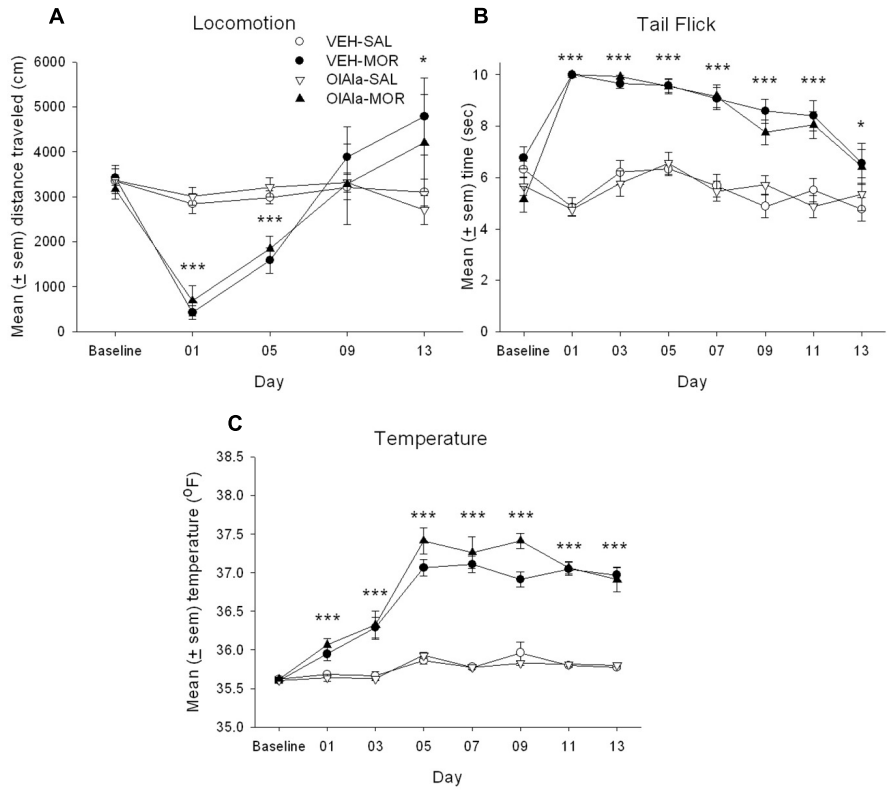

FIGURE 2 | OlAla **(A)** the mean (± sem) distance (cm) traveled in the locomotion test, **(B)** the mean (± sem) latency to tail flick from a hot water bath, **(C)** mean (± sem) body temperature (°F), displayed by the various groups across the 13 days of chronic morphine. Asterisks indicate a significant difference from SAL, ***$p < 0.001$, *$p < 0.05$.

CB_1 receptor antagonist (AM251) or the PPARα antagonist (GW6471). Additionally, AEA mobilization and the mRNA levels of the PPARα receptor were elevated in the spinal cord of morphine tolerant mice (Fotio et al., 2020). Since OlGly and OlAla interfere with naloxone-precipitated MWD by both CB_1 and PPARα antagonism (Ayoub et al., 2020; Rock et al., 2020), we predicted that OlGly and OlAla would also interfere with tolerance to morphine as does URB597 (Fotio et al., 2020). The lack of an effect on tolerance to morphine may have been a function of the relatively weak efficacy of OlGly to inhibit FAAH (IC_{50} = 8.65 μM; Donvito et al., 2019) relative to the more potent FAAH inhibitor, URB597 (4.6 nM; Mor et al., 2004). Similar to OlGly, OlAla weakly inhibited FAAH by about 40% at 10 μM (Ayoub et al., 2020). Unfortunately, neither FAAH activity nor AEA levels were measured in the tissue of these animals. Thus, it is possible that, at the dose used, neither compound elevated the brain concentrations of AEA and other *N*-acylethanolamines to an extent sufficient to activate CB1 or PPARa, and that a higher dose of OlGly and/or OlAla may have been more effective in inhibiting FAAH and may therefore have modified tolerance to morphine.

We have previously demonstrated that at a dose of 5 mg/kg, OlGly and OlAla interfere with naloxone-precipitated withdrawal from acutely administered morphine, without modifying morphine reward (Donvito et al., 2019; Petrie et al., 2019; Ayoub et al., 2020; Rock et al., 2020). Here we show that this effective dose of OlGly and OlAla did not affect the hypolocomotor effect, anti-nociceptive effect or the hyperthermic effect of morphine on any occasion across 13 treatment days and did not modify the development of tolerance to any of these effects. It is conceivable that either higher doses of the fatty acid amides or a different treatment regime of morphine may have revealed an effect of OlGly or OlAla on tolerance to one or more effects of morphine. As the work to date with OlGly and OlAla has been limited to acute naloxone precipitated MWD, future studies will determine the potential of OlGly to interfere with withdrawal reactions (spontaneous and naloxone-precipitated) from chronic treatment with opiates. However, on the basis of the acute MWD effects, it is unlikely that treatment with OlGly or OlAla to reduce MWD will reduce the potential of morphine to produce analgesia in clinical populations. These results suggest that these fatty acid amides may be used effectively in the future to combat MWD without compromising the beneficial therapeutic effects of opiates.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Guelph Animal Care Committee.

AUTHOR CONTRIBUTIONS

ER, CL, and MS performed the behavioral testing with assistance in daily injections from MD. LP, and ER wrote the manuscript, with suggestions and revisions from CL. LP, CL, and ER conceived and coordinated the work. All authors read, contributed to, and approved the final version of the manuscript.

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frontiers in Synaptic Neuroscience

ORIGINAL RESEARCH
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Dichotomic Hippocampal Transcriptome After Glutamatergic vs. GABAergic Deletion of the Cannabinoid CB1 Receptor

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Brain homeostasis is the dynamic equilibrium whereby physiological parameters are kept actively within a specific range. The homeostatic range is not fixed and may change throughout the individual's lifespan, or may be transiently modified in the presence of severe perturbations. The endocannabinoid system has emerged as a safeguard of homeostasis, e.g., it modulates neurotransmission and protects neurons from prolonged or excessively strong activation. We used genetically engineered mouse lines that lack the cannabinoid type-1 receptor (CB1) either in dorsal telencephalic glutamatergic or in forebrain GABAergic neurons to create new allostatic states, resulting from alterations in the excitatory/inhibitory (E/I) balance. Previous studies with these two mouse lines have shown dichotomic results in the context of behavior, neuronal morphology, and electrophysiology. Thus, we aimed at analyzing the transcriptomic profile of the hippocampal CA region from these mice in the basal condition and after a mild behavioral stimulation (open field). Our results provide insights into the gene networks that compensate chronic E/I imbalances. Among these, there are differentially expressed genes involved in neuronal and synaptic functions, synaptic plasticity, and the regulation of behavior. Interestingly, some of these genes, e.g., *Rab3b*, *Crhbp*, and *Kcnn2*, and related pathways showed a dichotomic expression, i.e., they are up-regulated in one mutant line and down-regulated in the other one. Subsequent interrogation on the source of the alterations at transcript level were applied using exon-intron split analysis. However, no strong directions toward transcriptional or post-transcriptional regulation comparing both mouse lines were observed. Altogether, the dichotomic gene expression observed and their involved signaling pathways are of interest because they may act as "switches" to modulate the directionality of neural homeostasis, which then is relevant for pathologies, such as stress-related disorders and epilepsy.

Keywords: CB1 receptor, homeostasis, glutamate transmission, GABA transmission, transcriptome analysis

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INTRODUCTION

Physiological homeostasis is the internal state of a dynamic equilibrium in which an organism functions most optimally. This process includes many parameters being kept within pre-set limits, homeostatic ranges, and the internal mechanisms and feedback loops to respond to internal or external insults (Cooper, 2008). Furthermore, the homeostatic range itself can change under the influence of environmental factors in a process called allostasis, which allows the organism to adapt to the new conditions. Although most of the allostatic changes are transient (e.g., adequate stress response), sometimes different factors establish an entirely new allostatic state, i.e., homeostasis significantly different from most of its conspecifics. The central nervous system (CNS) especially requires tight homeostatic regulation to ensure the correct functioning of neuronal circuits (Woods and Wilson, 2013).

The endocannabinoid system (ECS) acts retrogradely on presynaptic terminals to suppress neurotransmitter release, particularly on-demand after the postsynaptic site's depolarization. Thus, the ECS acts as a feedback loop to maintain homeostasis at the cellular and brain circuit levels. The neuromodulatory action of the ECS is mediated via the activation of the presynaptic cannabinoid receptor-type 1 (CB1), a Gi/o protein-coupled receptor, which activates different intracellular pathways to exert its function. CB1 is expressed in the two major neurotransmitter systems, i.e., in glutamatergic and GABAergic neurons, and many cell types in the brain, including glial cells (Busquets-Garcia et al., 2018; Lutz, 2020).

These observations have fueled numerous studies on the roles of CB1 specifically in different neural populations by using conditional CB1 knock-out (KO) mouse models, where CB1 is inactivated only in distinct neurons (Monory et al., 2006; Lutz et al., 2015; Busquets-Garcia et al., 2016) or glial cells (Han et al., 2012). We have focused mainly on mouse lines that lack CB1 on dorsal telencephalic glutamatergic neurons (Glu-CB1-KO) or forebrain GABAergic neurons (GABA-CB1-KO), which cause an excess of excitation or inhibition, respectively, and forces the brain to adapt to the increased neurotransmission. The use of these mutant mouse lines has shown a differential role for CB1 on glutamatergic and GABAergic neurons, respectively, at different levels, such as THC-induced tetrad effects (Monory et al., 2007; De Giacomo et al., 2020), THC-induced food intake (Bellocchio et al., 2010), THC-induced anxiety-like behavior (Rey et al., 2012), G-protein coupling (Steindel et al., 2013; Mattheus et al., 2016), and long-term hippocampal potentiation (Monory et al., 2015). Interestingly, these studies have also revealed a dichotomic phenotype between Glu-CB1-KO and GABA-CB1-KO animals, which means that each conditional CB1-KO reacts opposingly to the other upon the same stimulus. In some of these studies, it has been observed that CB1-WT and conditional CB1-KO mice showed indistinguishable behavior without prior stimulation (Jacob et al., 2009; Bellocchio et al., 2010; Rey et al., 2012), but only the stimulus revealed the opposing phenotypes. These observations have led us to reason that brain functioning of Glu-CB1-KO and GABA-CB1-KO individuals represent different allostatic states, i.e., homeostasis that is significantly and permanently different from CB1-WT animals. Considering previous research with these mouse lines, we wondered whether the hippocampal transcriptome would show such dichotomic features. To this end, we performed RNA-sequencing of hippocampal Cornus Ammonis (CA) samples from Glu-CB1-KO, GABA-CB1-KO, and corresponding CB1-WT mice. We then conducted a differential expression analysis (DEA) between conditional CB1-KO and CB1-WT mice in the basal state and after a mild behavioral activation. Differentially expressed genes (DEGs) that showed a dichotomic expression when comparing the two mouse lines would be especially relevant, as they could act as "switches" to modulate the directionality of the change in homeostasis, i.e., toward an increased excitatory or inhibitory state, respectively. The discovery of such genes would prove very useful for mental disorders caused due to excessive excitatory or inhibitory drive either in the whole brain or in specific brain regions.

MATERIALS AND METHODS

Mouse Lines

Conditional CB1-KO mice were generated via crossing CB1f/f (JAX Laboratory Stock No. 036107) mice with animals expressing Cre recombinase under the control of the regulatory elements of the genes *dlx5/6* (for deletion in GABAergic terminals; MGI Cat# 3758334, RRID:MGI: 3758334) and *nex1* (for deletion in glutamatergic terminals; MGI Cat# 3758333, RRID:MGI:3758333). This use of the Cre/LoxP system allowed for high specificity in the genetic deletion as shown previously (Monory et al., 2006). Each conditional CB1-KO group was compared to corresponding CB1-WT littermates to minimize possible unwanted variations caused by breeding, handling, age differences, etc. Genotyping was performed as previously described (Massa et al., 2010). Male mice had food and water *ad libitum* during their 12-h light-dark cycle (07:00–19:00) and were 8–10 weeks old when they were sacrificed.

Behavioral Hippocampal Activation

In order to study how the hippocampus of conditional CB1-KO mice react upon stimulation, we induced neuronal activity during the first half of the light cycle by exposing the animals to an open field arena (40 × 40 × 30 cm white box), a new environment that the mouse could explore undisturbed for 5 min (light intensity: 20–30 lux). This sufficient yet straightforward stimulus-induced neuronal activity throughout the hippocampus during arena exploration induced gene expression changes to be investigated.

Hippocampal Microdissection and Validation

In order to microdissect only the Cornus Ammonis from the hippocampus, we first removed the dentate gyrus (DG) from the rest of the hippocampus according to the protocol of Hagihara et al. (2009). The remaining hippocampal tissue was then dissected out in one-piece and snap-frozen on dry ice. We selected the CA1/CA2/CA3 region because this is the hippocampal subregion where we reported existing differences

in neuronal morphology and electrophysiological properties in these two mutant mouse lines (Monory et al., 2015). Moreover, we attempted to reduce the intrinsic heterogeneity observed within the hippocampus by excluding the DG due to distinct functionalities, e.g., in neurogenesis and as a gate input from the entorhinal cortex.

RNA Extraction and Reverse Transcription

In order to sustain the amount and quality of the hippocampal RNA for sequencing, the extraction and purification of RNA was performed as previously described (Lerner et al., 2018). To summarize, we followed the instructions from the RNeasy Mini kit (Qiagen) with a slight modification during the homogenization step. Frozen tissue was homogenized in 600 μL RLT buffer with 1% β-mercaptoethanol (according to the manufacturer's protocol), though with the addition of 200 μL chloroform. Apart from this step, there were no other changes made. Obtained RNA samples were treated with DNase I to degrade any possible contamination by genomic DNA. Samples were then eluted in 30 μL RNase-free water. The working bench and the tools used were cleaned before and during the extraction with RNase away plus (MßP, San Diego, CA, USA) to avoid any RNA degradation.

To perform qPCR, 1 μg of RNA was retrotranscribed into complementary DNA (cDNA) with the high-capacity cDNA reverse transcription kit (Life Technologies, Germany). This kit uses random primer hexamers for the reverse-transcription step. The resulting cDNA was diluted 1:10 in RNase-free water and stored at −80°C.

Hippocampal CA1/CA2/CA3 RNA-Sequencing

The hippocampal transcriptome sequencing occurred at the Core Facility Genomics (Institute of Molecular Biology gGmbH, IMB; Mainz). Next generation sequencing (NGS) library preparation was performed in two steps. First, cDNA was generated using NuGEN's Ovation RNA-seq system v2, from an input amount of 10 ng of total RNA, following the kit's instructions from the year 2012 (NuGEN, The Netherlands). Samples were amplified using the single primer isothermal amplification (SPIA) method from NuGEN. The resulting purified cDNA was quantified using the Qubit dsDNA HS assay kit in a Qubit 2.0 Fluorometer (Life Technologies, Germany). Afterward, the cDNA was profiled on a high sensitivity DNA chip using a 2100 Bioanalyzer (Agilent Technologies, Germany) as quality control. From the total cDNA, 1.5 μg were fragmented using a Covaris S2 focused-ultrasonicator (Covaris, UK), with the following parameters: (1) Duty cycle = 10%; (2) Intensity = 5; (3) Cycles/Burst = 200; (4) Time = 160 s; and (5) Water level = 15. After the fragmentation, the resulting material was once again quantified and profiled using a Qubit 2.0 and a 2100 Bioanalyzer, respectively, as described above. This process was repeated as an extra quality control point to ensure an optimal fragmentation.

Secondly, NGS libraries were generated from 100 ng of fragmented cDNA using NuGEN's Ovation ultralow system v2, following the kit's manual from the year 2014 (NuGEN, The Netherlands). Libraries were amplified in 7 PCR cycles and purified using beads. These purified libraries were quantified as described above, and profiled on a DNA 1000 Chip using a 2100 Bioanalyzer as the last quality control. Libraries representing all the experimental groups were pooled into individual pools containing 12 libraries, all of them in equimolar ratio. Each pool was loaded into four lanes of an Illumina's HiSeq flowcell and ran on a HiSeq 2500 in High-output mode (Illumina, USA), generating single-reads 50 base pairs long with an average yield of 52 million reads per library.

Quantitative Polymerase Chain Reaction (qPCR)

For the qPCR procedure, cDNA was amplified using the commercial TaqMan assays (Applied Biosystems) with an ABI7300 real-time PCR cycler (Applied Biosystems). Reactions were performed in duplicates, and *β-actin* was used as a reference gene. Analysis of the resulting data was performed using the 7300 system SDS software (Applied Biosystems).

Genes and their respective primers were selected according to the necessities of the experiment. *Arc* was used to measure hippocampal activation, whereas *TDO2* and *Lphn2* were used to prove the tissue purity resulting from the hippocampal microdissection. The rest of the genes (except for the reference gene) were chosen because they appeared as differentially expressed genes in the RNA-seq analysis (**Table 1**). We used an independent batch of mice (n = 7–9 animals per group) to validate genes of interest from our RNA-seq data.

Statistical Analysis of Behavioral and qPCR Data

Continuous variables were graphically represented as individual values and mean ± standard error of the mean (SEM). First, normality was checked for each parameter analyzed in this study. Next, conditional CB1-KO groups were statistically compared

TABLE 1 | List of TaqMan primers used for qPCR.

Gene symbol	Gene name	TaqMan primer code
β-actin	β-Actin	Mm00607939_s1
Arc	Activity-regulated cytoskeleton-associated protein	Mm01204954_g1
TDO2	Tryptophan 2,3-dioxygenase	Mm00451266_m1
Lphn2	Latrophilin 2	Mm01320597_m1
Npy	Neuropeptide Y	Mm00445771_m1
CRHBP	Corticotropin-releasing hormone binding protein	Mm01283832_m1
Cnr1	Cannabinoid receptor type-1	Mm01212171_s1
FosB	FBK osteosarcoma oncogene B	Mm00500401
Bdnf (exon V)	Brain-derived neurotrophic factor	Mm04230607
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	Mm00443060_m1
Grin2B	Glutamate receptor ionotropic NMDA-type subunit 2B	Mm00433820_m1

For each gene, the pair of primers was selected according to the manufacturer's suggestion and their gene coverage.

to their respective CB1-WT littermates with an unpaired *t*-Test (including Welch's correction when variances were significantly different) or a non-parametric Mann-Whitney test, depending on whether samples were normally distributed or not, respectively. Statistical analysis was performed with GraphPad Prism v5.0 (RRID:SCR_002798) and InVivoStat v4.1. *P*-values were two-tailed, and differences were considered statistically significant if the *p*-value was below 0.05.

Bioinformatic Analysis

An initial quality check of the raw sequencing data was done via FastQC version (v0.11.8) (RRID:SCR_014583). Then, bbduk.sh from the BBMap (BBmap, RRID:SCR_016965) suite of tools (version 38.06) was employed to perform adapter trimming and quality filtering of the raw sequence reads (q30 cutoff) (Zerbino et al., 2018). Afterward, the trimmed and cleaned sequences were mapped against the mouse reference genome mm10 from UCSC (downloaded via Illumina iGenome: http://support.illumina.com/sequencing/sequencing_software/igenome.html, download date 06/06/18), using the STAR aligner (version 2.6.0a) (RRID:SCR_015899) with default options (Dobin et al., 2013). Summarized quality results were created by MultiQC (version 1.8.dev0) (Ewels et al., 2016). Counting of reads per gene was done via featureCounts (version 1.6.2), with the -s 2 option, using the annotation file for the mm10 mouse genome from iGenomes (Liao et al., 2014).

The R package DESeq2 (version 1.30.0) was used for all subsequent differential gene expression (DGE) (Love et al., 2014). *P*-values were adjusted using the the Benhamini-Hochberg method for multiple testing correction (Benjamini and Hochberg, 1995), and the threshold for significantly differentially expressed genes (DEGs) was defined as the adjusted *p*-value (p-adjusted) < 5 %.

The subsequent Gene Ontology (GO) term analysis was executed by the R package clusterProfiler (version 3.18.0) using the standard over-representation test (Yu et al., 2012). All GO terms with an adjusted $p < 5$ % were considered significantly overrepresented for the respective gene subset.

In order to check the cell specificity of the DEGs resulting from the Glu-CB1 and GABA-CB1 comparisons, we downloaded the transcriptomic information of different brain cell types of *Mus musculus*, derived from single-cell RNA-seq analyses and published by the Allen Institute for Brain Sciences (https://portal.brain-map.org/atlases-and-data/rnaseq). The cell type specific expression patterns of the respective genes were extracted and visualized using the R package pheatmap (version 1.0.12) (**Supplementary Figures 1–4**).

EISA (exon-intron split analysis) by Gaidatzis et al. (2015) was performed using the EisaR package with standard settings, as per the vignette (Gaidatzis et al., 2015). The same significance threshold for post-transcriptionally regulated genes was used as previously described for the DGE analysis. Finally, the Pearson correlation between the $\log_2$ Fold changes derived from exons and introns was calculated per gene.

All plots were realized with the R packages ggplot2 (version 3.3.2) or venn (version 1.9) (Wickham, 2016; Dusa, 2020).

RESULTS

Open Field Paradigm to Induce Neuronal Activity and Sample Selection for RNA-Sequencing

For the transcriptomic analysis, we used 8–10 week-old male mice at basal (home cage) conditions with the following genotype and group size: Glu-CB1-WT ($n = 5$), Glu-CB1-KO ($n = 7$), GABA-CB1-WT ($n = 6$), GABA-CB1-KO ($n = 6$). For the open field stimulated state, mice were behaviorally characterized (**Figures 1A–C**). The group size of the open field groups were: Glu-CB1-WT ($n = 5$), Glu-CB1-KO ($n = 7$), GABA-CB1-WT ($n = 5$), and GABA-CB1-KO ($n = 7$). No significant genotype and treatment differences were observed in the 5-min open field exposure, neither in general locomotion (**Figure 1A**) nor in anxiety-like behavior (**Figures 1B,C**). Isolated hippocampi of all mice used for transcriptomic analysis were checked for the purity of the CA1/CA2/CA3 region by using RT-qPCR for *latrophilin 2* (*Lphn2*), a marker for the CA region. In contrast, *tryptophan 2,3-deoxygenase* (*TDO2*), a marker for dentate gyrus, was not detected (**Figure 1D**). Furthermore, the open field stimulus was monitored by observing significant increases in *Arc* mRNA levels (**Figure 1E**). CB1 deletion in mutants was substantiated by observing decreased *cnr1 (CB1)* mRNA levels (**Figure 1F**).

Dichotomies in the Transcriptomic Profiles of Glu-CB1-KO and GABA-CB1-KO Hippocampi in the Basal State

First, we analyzed the transcriptome differences between the allostatic states, represented by Glu-CB1-KO and GABA-CB1-KO hippocampi, and their respective homeostatic states, as represented by CB1-WT littermates at basal, i.e., unstimulated home cage conditions.

The differential expression analysis (DEA) revealed several differentially expressed genes (DEGs) when comparing the conditional CB1-KO mice with their respective CB1-WT littermates. The relatively low level of differences in expression, as evident by the number of differentially regulated genes above the significance threshold and the PCA plots of the test conditions, is in accordance with previous observations that conditional CB1-KO mice are very similar to wild-type animals in the absence of a stimulus (Jacob et al., 2009; Rey et al., 2012). Interestingly, we observed a strong difference in the number of DEGs between the mutant lines. While, we found 8 DEGs in the Glu-CB1 mouse line (**Figure 2A**), we observed 107 DEGs in the GABA-CB1 comparison (**Figure 2B**). Thus, we found only one overlapping dichotomous gene between the different analysis groups, namely the gene *B-cell linker* (*Blnk*) (**Figures 2C,D**). We further analyzed genes related to synaptic function and plasticity via RT-qPCR. Here, *Nr4a2*, a critical transcription factor for neuronal function, and *Grin2B*, a subunit of the glutamate NMDA receptor, were investigated as DEGs in an independent set of RNA samples (**Figures 2E,F**). Interestingly, no genes related to the ECS were found to be differentially

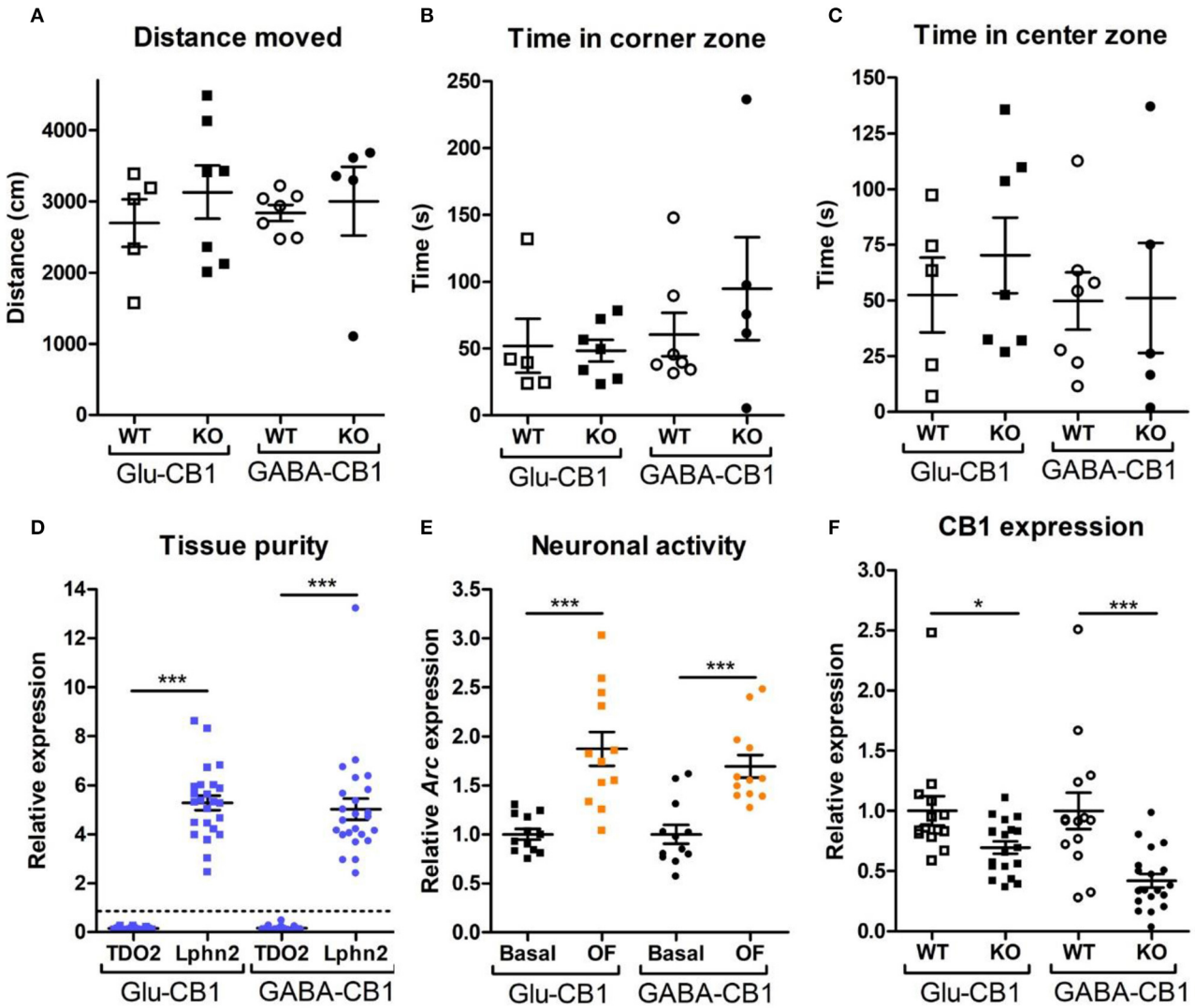

FIGURE 1 | Behavioral and qPCR data used for the selection of the final samples. Samples for RNA-sequencing were selected depending on the purity of the dissected tissue, as well as the anxiety-like behavior and the neuronal activity induced by the exposure to the open field. All animals that are shown were selected for the transcriptomic analysis. **(A)** Locomotion activity in the open field arena (statistical power = 9.06%; calculated by one-way ANOVA power analysis). **(B)** Time spent in the corner zones (anxiety-like behavior) of the open field arena (statistical power = 7.92%; calculated by one-way ANOVA power analysis). **(C)** Time spent in the center zone (anxiolytic-like behavior) of the open field arena (statistical power = 9.81%; calculated by one-way ANOVA power analysis). **(D)** Gene expression levels for the gene activity-regulated cytoskeleton-associated protein (*Arc*) showed increased neuronal activity upon brief exposure to a new environment (open field arena). CB1-WT and their respective conditional CB1-KO samples were pooled together in each of the groups shown. **(E)** The purity of the tissue resulting from the microdissection of the hippocampus was checked by measuring the expression levels of *tryptophan 2,3-deoxygenase* (*TDO2*) and *latrophilin 2* (*Lphn2*), which are specific for the DG and the CA. The graph shows only CA tissue samples that were normalized and compared to the respective DG samples (dashed line). **(F)** Gene expression levels of *CB1* (*cnr1*) in the CA regions of CB1-WT mice and the respective conditional CB1-KO animals. Samples from basal state and open field group were pooled together. Bars represent mean ± S.E.M. Unpaired *t*-Test with Welch's correction when variances between groups were different. $^{*}p < 0.01$; $^{***}p < 0.001$.

expressed when comparing each conditional CB1-KO group to their respective CB1-WT controls.

To find out whether the DEGs from Glu-CB1 and GABA-CB1 comparisons are exclusively expressed within certain cell-types, we extracted the expression patterns of the respective genes from the transcriptomic information of mouse brain-cell types published by the Allen Institute for Brain Sciences (https://portal.brain-map.org/atlases-and-data/rnaseq). We could not observe common patterns of gene expression in the DEGs resulting from the Glu-CB1 comparison (**Supplementary Figure 1**), most likely due to the low number of genes. However, the *in-silico* analysis revealed that most of the DEGs found in the GABA-CB1 comparison are expressed in both glutamatergic and GABAergic neurons. However, a small proportion of DEGs from this comparison are expressed mainly in glutamatergic populations (**Supplementary Figure 2**). We next performed

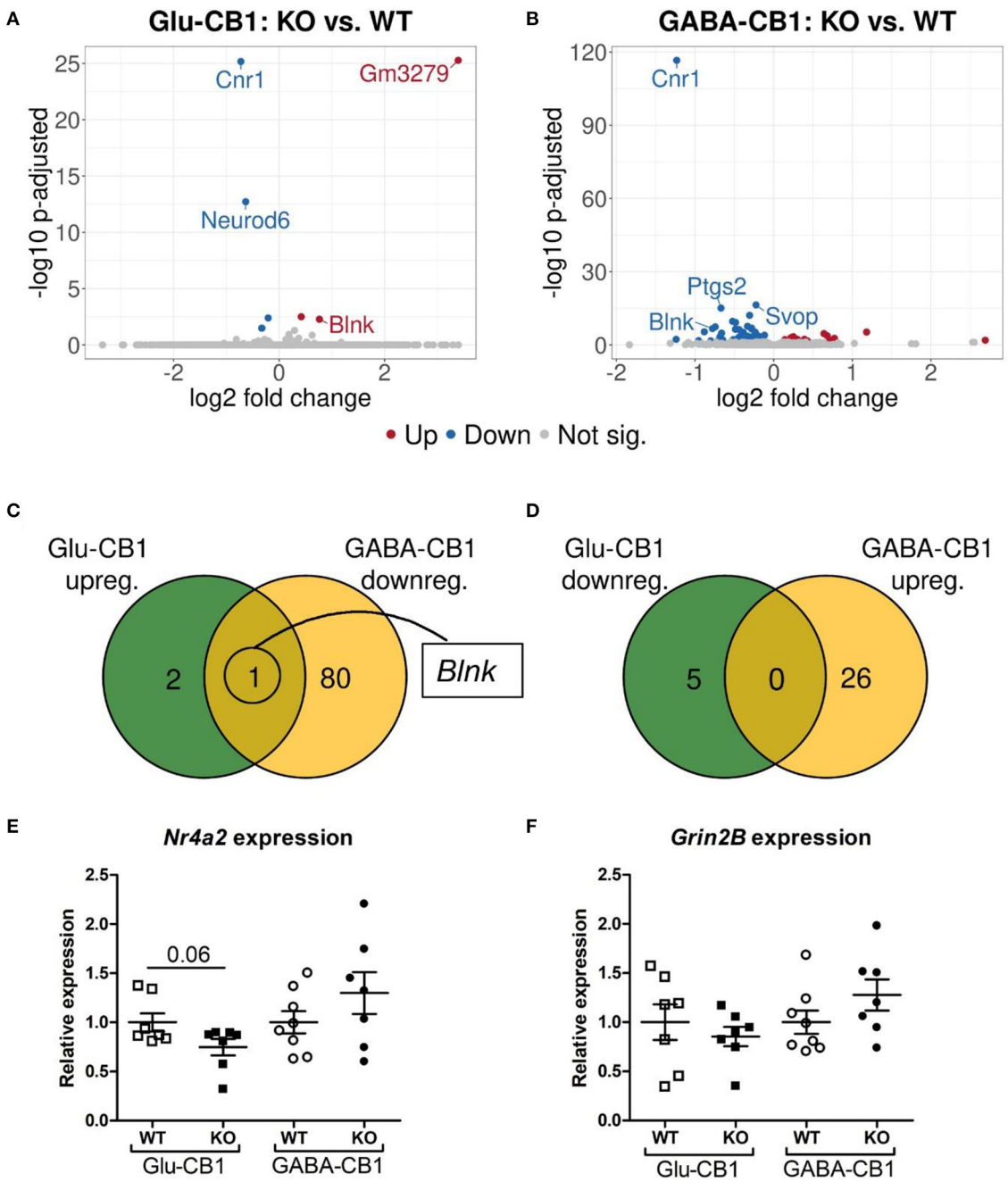

FIGURE 2 | Transcriptomic profiles from Glu-CB1-KO and GABA-CB1-KO mice in the basal state. **(A,B)** Volcano plots showing differentially expressed genes (red and blue dots) found when comparing Glu-CB1-KO **(A)** and GABA-CB1-KO **(B)** mice to their respective CB1-WT in their basal state. Red dots represent upregulated genes resulting from this comparison, whereas blue dots indicate downregulated genes. Labels of the top3, dichotomic, and qPCR-validated DEGs are shown. Non-significant genes are colored in gray. **(C,D)** Venn diagrams to visualize the number of dichotomic genes found between Glu-CB1 and GABA-CB1 DEGs in the basal state, i.e., those genes that appear up-regulated in one of the conditional CB1-KO lines and down-regulated in the other, or vice versa. **(E)** Expression levels derived from qPCR of the transcription factor *Nr4a2* for each of the genotypes analyzed in the basal state. **(F)** Expression levels derived from qPCR of the gene *Grin2B* for each of the genotypes analyzed in the basal state. RT-qPCR data normalized to *β-actin*. Bars represent mean ± SEM. Unpaired t-Test with Welch's correction when variances between groups were different.

a gene ontology (GO) enrichment analysis with the DEGs found. This analysis provided an insight into which biological processes, cell compartments, and molecular functions are altered, possibly also in compensation of the loss of CB1 in these two neuronal populations by increasing either excitatory or inhibitory neurotransmission, present in Glu-CB1-KO and

TABLE 2 | GO terms of the DEGs from GABA-CB1-KO mice in the basal state.

	GABA-CB1-KO in the basal state	Enrichment	*P*-value (adjusted)
Biological process	Modulation of chemical synaptic transmission	Down-regulated	0.04375221
	Regulation of trans-synaptic signaling	Down-regulated	0.04375221
	Learning or memory	Down-regulated	0.04375221
	Memory	Down-regulated	0.04375221
Cell compartment	Secretory vesicle	Down-regulated	0.02787115
	Axon terminus	Down-regulated	0.02787115
	Axon part	Down-regulated	0.02787115
	Neuron projection terminus	Down-regulated	0.02787115
	Distal axon	Down-regulated	0.02787115

Table showing the list of GO terms resulting after analyzing the DEGs in the comparison GABA-CB1-KO vs. CB1-WT in the basal state. GO terms marked as downregulated indicate that these terms are enriched for downregulated DEGs, and the opposite holds true for terms marked as upregulated.

GABA-CB1-KO hippocampi, respectively. Due to the low number of DEGs found when comparing Glu-CB1-KO and CB1-WT hippocampi in the basal state (**Figure 2A**), we could not allocate any overrepresented processes in this dataset. However, we found overrepresented GO terms of downregulated genes in GABA-CB1-KO mice compared with respective CB1-WT controls (**Table 2**). These processes comprised the terms of synaptic transmission, as well as learning and memory. Moreover, GO terms related to neuronal structures, such as neuronal projections, axonal structures, and secretory vesicles, were also found to be significantly enriched for downregulated genes in GABA-CB1-KO as compared to CB1-WT. In conclusion, our results showed significant differences between GABA-CB1-KO and Glu-CB1-KO animals in their basal state, presumably in adapting to increased inhibitory and excitatory neurotransmission, respectively.

Exposure to Open Field Induces Different Transcriptional Profiles in Glu-CB1-KO and GABA-CB1-KO Mice

A brief exposure to a new environment (e.g., open field arena) triggers neuronal activity in the hippocampus (Cohen et al., 2017). In order to study how each of the allostatic and homeostatic states, respectively, react upon such a stimulation, we exposed both Glu-CB1 and GABA-CB1 mice for 5 min to an open field arena (**Figure 3**) and isolated the tissue 60 min afterwards for transcriptomic analysis. The subsequent DEA revealed more DEGs in this stimulated condition than in the basal state. Thus, the open field exposure is sufficient to induce gene expression changes, although transcriptional differences between conditional CB1-KO and CB1-WT samples were relatively low. Interestingly, we found more genes that show dichotomic expression after open field exposure (**Figures 3C,D**) than in the basal state (**Figures 2C,D**), in particular for the Glu-CB1-KO mice. This observation suggests activity-induced neuronal pathways being modulated in the hippocampi of both Glu-CB1-KO and GABA-CB1-KO mice, although in opposing directions. Among the dichotomically regulated genes, we found transcription factors related to neuronal developmental processes (e.g., *Ldb2*), potassium channels involved in Ca^{2+} homeostasis (e.g., *Kcnn2*), neuropeptides (e.g., *Sst*), and proteins involved in the biogenesis of neuronal vesicles (e.g., *Rab3b*), the regulation of the cytoskeleton (e.g., *Sptb*) and the regulation of the stress response (e.g., C*rhbp*). In addition, we investigated via RT-qPCR (**Figures 4A–D**) the dichotomic expression levels of additional genes after behavioral activation, such as *FosB* (**Figure 4A**), *Crhbp* (**Figure 4B**), *BDNF* (**Figure 4C**), and *Npy* (**Figure 4D**).

The cell-type specificity analysis revealed that the DEGs resulting from the Glu-CB1 comparison were expressed in different neuronal populations. Interestingly, some of these DEGs had very high levels of expression in GABAergic neurons, suggesting that the lack of CB1 in Glu-CB1-KO mice caused some form of dysregulation on GABAergic populations (**Supplementary Figure 3**). In the case of the GABA-CB1 comparison, almost all DEGs were expressed in both glutamatergic and GABAergic populations (**Supplementary Figure 4**). Next, we analyzed GO term overrepresentations within those DEGs found after exposure to the open field arena. Contrary to their basal state, Glu-CB1-KO mice provided interesting GO terms after behavioral activation of the hippocampus (**Table 3**). The upregulated genes from the Glu-CB1 comparison were revealed to be significantly involved in the activity of adrenergic and G-protein coupled receptors, as well as neuropeptide hormones. The signaling pathways of the glutamate receptor and neuropeptides were also enriched for those upregulated genes, as well as different neuronal structures such as the dendritic shaft, dopaminergic synapses, the axon, and secretory granules. Interestingly, those genes that revealed to be downregulated comparing Glu-CB1-KO and CB1-WT mice after open field exposure were involved in voltage-gated potassium channel complexes. Lastly, biological processes such as the fear response, axon guidance or the regulation of neurotransmitter transport were enriched for upregulated genes observed in the comparison of Glu-CB1-KO with CB1-WT hippocampi after a mild stimulus.

In contrary, for DEGs in GABA-CB1-KO mice, only downregulated genes yielded enriched terms (**Table 4**). These terms included processes for a proper neuronal function, such as GABAergic and dopaminergic synaptic transmission, as well as the regulation of trans-synaptic signaling, the membrane potential, and the action potential. Specific neuronal structures (e.g., synapses and axons) were also found to be overrepresented for the downregulated genes in our analysis.

Interrogation on the Source of the Transcriptional Alterations Applying Exon-Intron Split Analysis (EISA)

EISA was conducted to determine whether the gene expression changes are due to transcriptional or post-transcriptional mechanisms via the analysis of intronic and exonic read changes in each comparison (**Figure 5**) (Gaidatzis et al.,

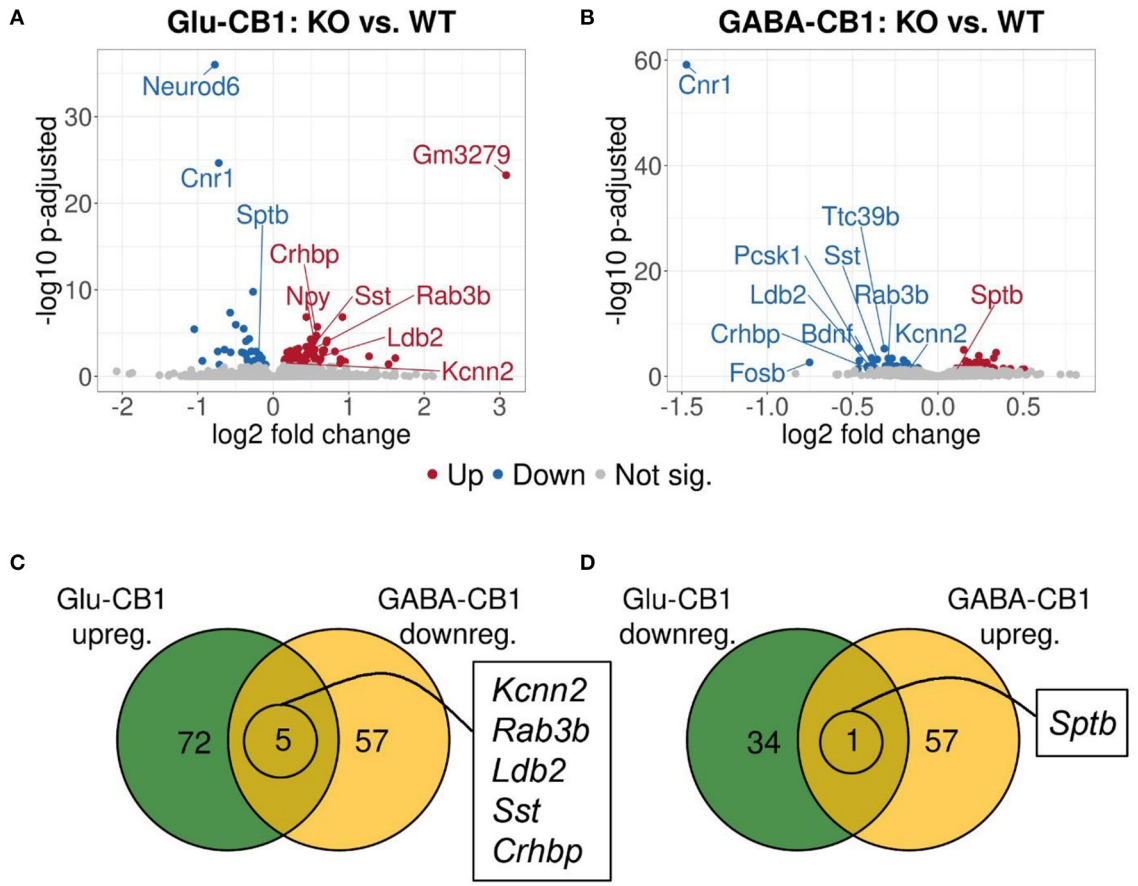

FIGURE 3 | Transcriptomic profiles from Glu-CB1-KO and GABA-CB1-KO mice after open field exposure. **(A,B)** Volcano plots showing differentially expressed genes (red and blue dots) found when comparing Glu-CB1-KO **(A)** and GABA-CB1-KO **(B)** mice to their respective CB1-WT after exposure to the open field arena. Red dots represent upregulated genes resulting from these comparisons, whereas blue dots indicate downregulated genes. Labels of the top3, dichotomic, and qPCR-validated DEGs are shown. Non-significant genes are colored in gray. **(C,D)** Venn diagrams to visualize the number of dichotomic genes found between Glu-CB1-KO and GABA-CB1-KO data after open field exposure, i.e., those genes that appear up-regulated in one of the conditional CB1-KO lines and down-regulated in the other, or vice versa.

2015). The Pearson correlation between intronic and exonic changes indicates whether the global gene expression differences originated at the transcriptional or posttranscriptional level, as represented by the correlation coefficient (R). A normal cell relies on a mixture of transcriptional or posttranscriptional mechanisms to regulate its gene expression. We performed EISA on each conditional CB1-KO mouse line compared to their WT littermates and found that only a few genes were found to be significantly post-transcriptionally regulated (**Figure 5**). Thus, we cannot pinpoint the overall origin of the transcriptomic changes observed either to dominantly transcriptional or post-transcriptional control. However, it is interesting to note that the Glu-CB1 line (**Figures 5A,B**) had slightly lower R-values than the GABA-CB1 line. This observation could indicate increased post-transcriptional regulation in Glu-CB1-KO mice to counterbalance the excess of excitatory neurotransmission. Nevertheless, more experiments are required to confirm this point.

DISCUSSION

Brain homeostasis and the modulation of neurotransmission is tightly connected to the ECS (Busquets-Garcia et al., 2018; Lutz, 2020). This function of the ECS, mediated by the CB1 receptor, is fundamental both at the neuronal and the circuit level, in order to avoid neuronal overexcitation and maladaptive behaviors (Lau and Tymianski, 2010; Olloquequi et al., 2018). Consequently, the ECS is used as a therapeutic target for many pathologies, and thus, to explore the mechanisms by which the ECS acts. However, the main receptor of the ECS (CB1) has a ubiquitous expression in the mammalian brain, which complicates the study of cell-specific mechanisms underlying CB1 action. Conditional CB1-KO mouse lines, in which CB1 is deleted in a specific neuronal or glial population, have boosted our knowledge of the ECS in specific cell populations. Thus, we used these mouse lines to study brain homeostasis and potential gene expression changes whereby the brain of these mice tries to counteract the

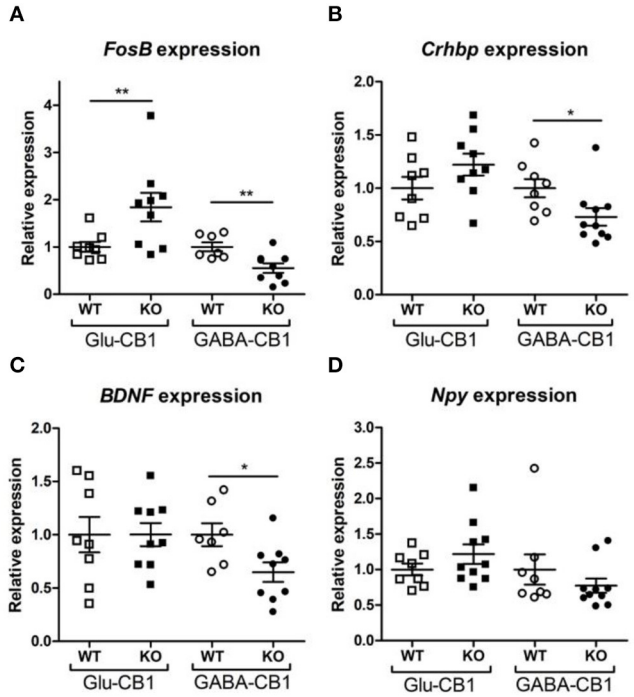

FIGURE 4 | Relative expression of genes of interest after open field exposure measured via RT-qPCR. The measurements were performed with independent samples ($n = 7$–9 replicates per group) to validate the results from our RNA-seq experiment. Genes that showed a potential dichotomy between the comparisons of both mouse lines were selected for further validation. **(A)** Expression levels of *FosB* proto-oncogene, AP-1 transcription factor subunit. **(B)** Expression levels of *corticotropin-releasing hormone binding protein* (Crhbp). **(C)** Expression levels of *brain-derived neurotrophic factor* (*BDNF*). **(D)** *Expression levels of neuropeptide Y* (*Npy*). The expression levels were normalized using *β-actin* as housekeeping gene. Bars represent mean ± S.E.M. Unpaired *t*-Test with Welch's correction when variances between groups were different. $^{*}p < 0.05$; $^{**}p < 0.01$.

TABLE 3 | GO terms of the DEGs from Glu-CB1-KO mice after open field.

	Glu-CB1-KO after open field	Enrichment	*P*-value (adjusted)
Molecular function	Receptor regulator activity	Up-regulated	0.0028834
	Receptor ligand activity	Up-regulated	0.00453663
	Neuropeptide hormone activity	Up-regulated	0.00453663
	G protein-coupled amine receptor activity	Up-regulated	0.01502056
	Adrenergic receptor activity	Up-regulated	0.03844831
Biological process	Glutamate receptor signaling pathway	Up-regulated	0.0285555
	Multicellular organismal response to stress	Up-regulated	0.0285555
	Axon guidance	Up-regulated	0.0285555
	Fear response	Up-regulated	0.03192236
	Regulation of neurotransmitter transport	Up-regulated	0.03192236
	Neuropeptide signaling pathway	Up-regulated	0.03192236
Cell compartment	Perikaryon	Up-regulated	0.00049563
	Axon terminus	Up-regulated	0.00841979
	Dendritic shaft	Up-regulated	0.00841979
	Axon part	Up-regulated	0.02405893
	Secretory granule	Up-regulated	0.03819382
	Dopaminergic synapse	Up-regulated	0.04162235
	Voltage-gated potassium channel complex	Down-regulated	0.04554603
	Potassium channel complex	Down-regulated	0.04554603

Table showing the list of GO terms resulting after analyzing the DEGs in the comparison Glu-CB1-KO vs. CB1-WT after open field exposure. GO terms marked as downregulated indicate that these terms are enriched for downregulated DEGs, and the opposite holds true for terms marked as upregulated.

imbalances of excitatory and inhibitory neurotransmission, as represented by Glu-CB1-KO and GABA-CB1-KO mice, respectively.

Interestingly, at basal state (home cage condition), we could observe over a hundred DEGs in GABA-CB1-KO hippocampi when comparing them to their respective CB1-WT animals, whereas we found only eight DEGs in Glu-CB1-KO samples. This observation suggests important differences in the adaptive mechanisms used by GABA-CB1-KO, rather directed toward promoting neuronal and network excitability, and by Glu-CB1-KO animals, rather directed toward compensating increased excitability and excitotoxicity. Thus, the differences in the number of DEGs indicate different compensatory strategies, some of which depend more than others on transcriptional processes. Moreover, the mere number of DEGs may not reflect the functional importance of the actual DEGs in the homeostatic response. Furthermore, we found that some genes also had a dichotomic expression when comparing Glu-CB1-KO and GABA-CB1-KO data, such as *Nr4a2*, an important transcription factor in neurons, and *Grin2B*, a subunit of the NMDA glutamate receptor. *Nr4a2* and *Grin2B* are especially relevant, as they are heavily involved in synaptic transmission and signaling (Endele et al., 2010; Rajan et al., 2020). *Grin2B* has a central role on long-term potentiation and synaptic plasticity (Keith et al., 2019), network excitability (Marquardt et al., 2019), and can promote neuronal health or regulate excitotoxicity (Hardingham and Bading, 2010). GO terms related to synaptic transmission and signaling, and the neuronal structures relevant for cell-cell communication were enriched for downregulated genes in GABA-CB1-KO samples compared to their CB1-WT counterparts. However, even at basal state, the transcriptome differences between mutants and wild-types were very mild, though, at the morphological level, e.g., dendritic branching, are strongly different (Monory et al., 2015), indicating that the accumulation of life events and environmental challenges are involved in these morphological alterations. The lack of expression changes in ECS-related

TABLE 4 | GO terms of the DEGs from GABA-CB1-KO mice after open field.

	GABA-CB1-KO after open field	Enrichment	*P*-value (adjusted)
Biological process	Synaptic transmission, GABAergic	Down-regulated	0.00588666
	Regulation of synaptic transmission, GABAergic	Down-regulated	0.0133165
	Regulation of action potential	Down-regulated	0.02260484
	Modulation of chemical synaptic transmission	Down-regulated	0.02260484
	Regulation of trans-synaptic signaling	Down-regulated	0.02260484
	Synaptic transmission, dopaminergic	Down-regulated	0.02260484
	Action potential	Down-regulated	0.02260484
	Regulation of membrane potential	Down-regulated	0.03951345
	Behavior	Down-regulated	0.0467256
Cell compartment	Presynapse	Down-regulated	0.00600701
	Distal axon	Down-regulated	0.01692391
	Secretory granule	Down-regulated	0.01719666
	Integral component of synaptic membrane	Down-regulated	0.01719666
	Secretory vesicle	Down-regulated	0.01719666
	Neuron projection terminus	Down-regulated	0.02740205
	GABA-ergic synapse	Down-regulated	0.02756174
	Axon part	Down-regulated	0.01719666

Table showing the list of GO terms resulting after analyzing the DEGs in the comparison GABA-CB1-KO vs. CB1-WT after open field exposure. GO terms marked as downregulated indicate that these terms are enriched for downregulated DEGs, and the opposite holds true for terms marked as upregulated.

genes could result from adaptive mechanisms during mouse development in order to secure the functioning of the ECS in the basal state, as they also lack CB1 during this life period. Moreover, our behavioral stimulus was brief and mild. It could be that more intense or long-lasting stimuli could induce wider changes in the transcriptomic profile that would ultimately affect the expression levels of ECS-related genes.

Along this line, we aimed at studying how these conditional CB1-KO mice react upon a mild challenge, represented by a brief exposure to an open field arena. This new environment was sufficient to induce changes in the neuronal gene expression patterns in Glu-CB1-KO and GABA-CB1-KO mice, as neuronal activity triggered an excess of excitatory or inhibitory neurotransmission, respectively. We found over a hundred DEGs in both comparisons and, surprisingly, several more genes showed a dichotomic expression between mouse lines compared to the basal state. We did not find any commonly upregulated or downregulated genes between comparisons, except for CB1. This suggests that no common mechanism was used by Glu-CB1-KO and GABA-CB1-KO mice to try restoring homeostasis. Among the dichotomic genes, we found genes involved in neuropeptide signaling (*Crhbp, Npy, Sst*), transcription factors (*Ldb2, FosB*), the cytoskeleton (*Sptb*), synaptic function (*Rab3b, Kcnn2*), or synaptic plasticity (*Bdnf*). *Sst*, e.g., is used as a neurotransmitter by inhibitory interneurons and is essential for the correct maturation of inhibitory synapses (Su et al., 2020) and for behavioral control (Anaclet et al., 2018). The neuropeptide Y (*Npy*) is also present in inhibitory interneurons throughout the brain and regulates behavioral responses, especially those related to anxiety and fear (Desai et al., 2014; Bartsch et al., 2020). Both *Sst* and *Npy* modulate the activity of neural networks through their modulation of GABAergic interneurons. *Rab3b* is also an important regulator of excitability, as it is known to prime synaptic vesicles release (Schlüter et al., 2006) and is required for endocannabinoid-dependent long-term depression at inhibitory synapses (Tsetsenis et al., 2011). Lastly, the potassium channel *Kcnn2* is critical for neuronal Ca^{2+} dynamics and homeostasis (Richter et al., 2016), thus modulating brain and neuronal processes such as synaptic plasticity (Sun et al., 2020). The relevance of these genes for neuronal cells means that these genes, or others along their respective pathways, could serve to modulate brain homeostasis towards an increased excitatory or inhibitory state or back to homeostasis.

Our *in-silico* cell-type specificity analysis showed that most of these DEGs were similarly expressed across all brain cell types. However, a proportion of DEGs found in the GABA-CB1 comparison in the basal state were only expressed in glutamatergic neurons. Interestingly, we observed a similar feature when analyzing the DEGs from the Glu-CB1 comparison after open field, as some of these DEGs had very high levels of expression in GABAergic populations. These results suggest that the lack of CB1 on a specific neuronal population not only alters the transcriptomic profile of said population, but also has an important effect on the transcriptomic landscape of other neuronal cell types. Another interesting observation is that the GO enrichment analysis performed on the DEGs found after open field exposure yielded very different results for each comparison. On the one hand, those GO terms among the upregulated genes in the Glu-CB1 suggest an increase in the activity and signaling of various receptors and neuropeptides, and the regulation of neurotransmitter transport. The upregulation of these genes could be a consequence of neuronal activity in an excessively excitatory environment. Voltage-gated potassium channel complex was the only GO term to be enriched for downregulated genes in this comparison. On the other hand, those genes that were downregulated in the GABA-CB1 comparison were significantly enriched for processes related to synaptic transmission and action potential, as well as the regulation of trans-synaptic signaling and the membrane potential. The downregulation of these genes could be the direct result of increased inhibitory drive induced by neuronal activity and the absence of CB1 on GABAergic neurons.

In summary, our results showed differences between the transcriptomic profiles of Glu-CB1-KO and GABA-CB1-KO mice in their basal state and after exposure to a mild stimulus.

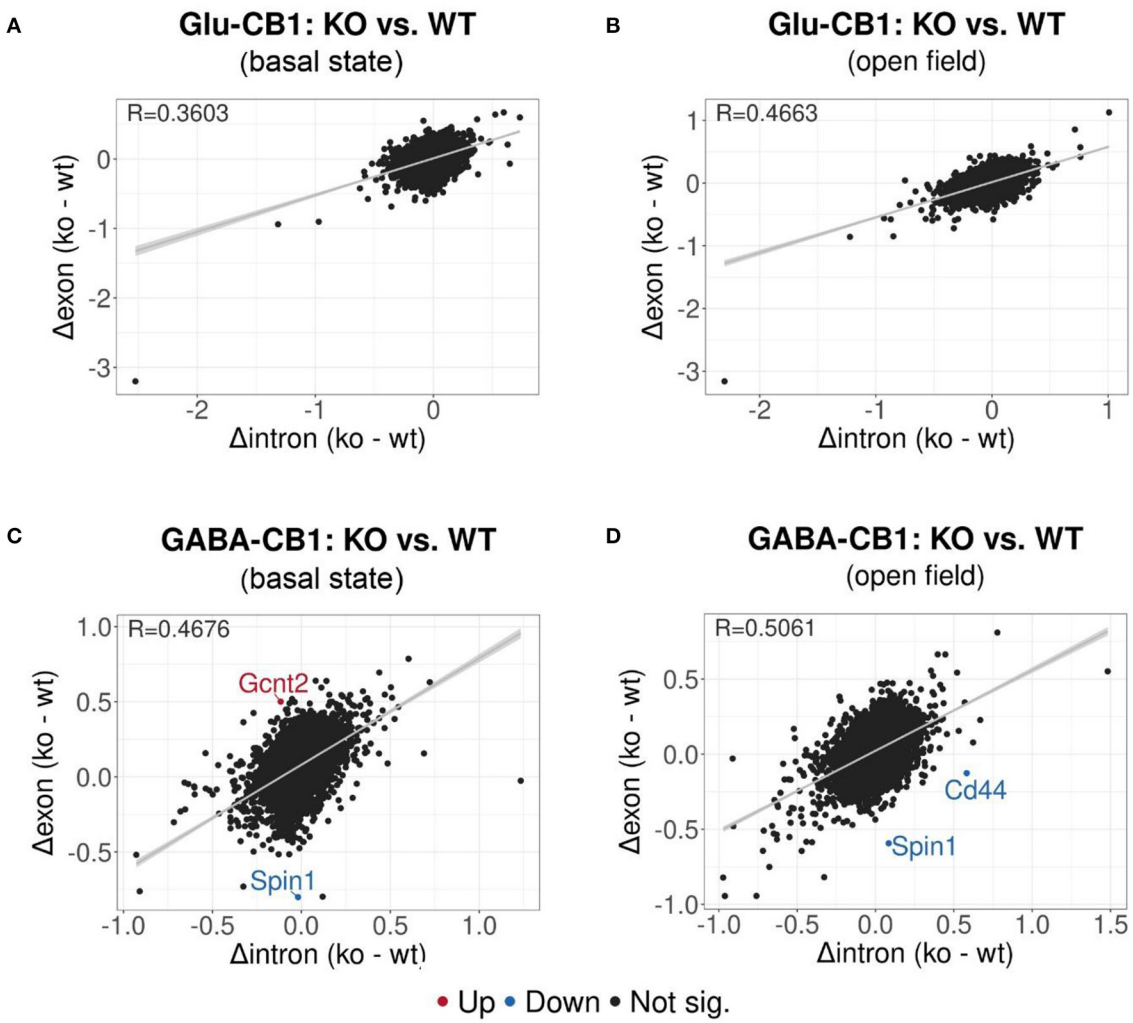

FIGURE 5 | EISA analysis of the Glu-CB1 and GABA-CB1 comparisons in the basal state and after open field exposure. Dot plots representing the change in exonic and intronic counts for each gene (dots). The value R at the top left corner of each graph indicates the slope of the regression curve, as well as the tendency on whether the overall changes in gene expression are due to transcriptional (R = 1) or posttranscriptional (R = 0) changes. **(A)** EISA data resulting from comparing Glu-CB1-KO vs. CB1-WT mice in the basal state. **(B)** EISA data resulting from comparing Glu-CB1-KO vs. CB1-WT mice after open field exposure. **(C)** EISA data resulting from comparing GABA-CB1-KO vs. CB1-WT mice in the basal state. **(D)** EISA data resulting from comparing GABA-CB1-KO and CB1-WT mice after open field exposure. Significant post-transcriptionally regulated genes comparing conditional CB1-KO and CB1-WT mice are labeled and colored in red for upregulation and blue for downregulation.

Furthermore, we found several genes with dichotomic expression levels when comparing both mouse lines (i.e., they are increased in one mouse line and decreased in the other). Understanding how these genes or their respective pathways are regulated and their interactions could shed new light into how the brain maintains its homeostasis, as well as into neuronal mechanisms protecting against E/I imbalances.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found in NCBI under the GEO accession number GSE168873 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168873).

ETHICS STATEMENT

The animal study was reviewed and approved by Landesuntersuchungsamt Rheinland-Pfalz.

AUTHOR CONTRIBUTIONS

DP performed all behavioral and molecular experiments, except the sequencing of RNA. AW and CH performed all bioinformatic

analysis of the sequencing data. DP wrote the manuscript with the support of AW and BL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsyn.2021.660718/full#supplementary-material

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PERSPECTIVE
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Targeting Endocannabinoid Signaling in the Lateral Habenula as an Intervention to Prevent Mental Illnesses Following Early Life Stress: A Perspective

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Adverse events and childhood trauma increase the susceptibility towards developing psychiatric disorders (substance use disorder, anxiety, depression, etc.) in adulthood. Although there are treatment strategies that have utility in combating these psychiatric disorders, little attention is placed on how to therapeutically intervene in children exposed to early life stress (ELS) to prevent the development of later psychopathology. The lateral habenula (LHb) has been a topic of extensive investigation in mental health disorders due to its prominent role in emotion and mood regulation through modulation of brain reward and motivational neural circuits. Importantly, rodent models of ELS have been shown to promote LHb dysfunction. Moreover, one of the potential mechanisms contributing to LHb neuronal and synaptic dysfunction involves endocannabinoid (eCB) signaling, which has been observed to critically regulate emotion/mood and motivation. Many pre-clinical studies targeting eCB signaling suggest that this neuromodulatory system could be exploited as an intervention therapy to halt maladaptive processes that promote dysfunction in reward and motivational neural circuits involving the LHb. In this perspective article, we report what is currently known about the role of eCB signaling in LHb function and discuss our opinions on new research directions to determine whether the eCB system is a potentially attractive therapeutic intervention for the prevention and/or treatment of ELS-associated psychiatric illnesses.

Keywords: CB1R, early life stress, endocannabinoids, FAAH, lateral habenula, LHb, MAGL

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INTRODUCTION

Early life stress (ELS) and childhood trauma have been recognized as critical risk factors that increase the probability of developing psychiatric disorders in both clinical observations and animal models (Heim and Nemeroff, 2002; Taylor, 2010; Targum and Nemeroff, 2019). Given our incomplete understanding of the neurobiological underpinnings and mental outcomes following ELS, as well as the wide array of maladaptive alterations in neural circuits induced by ELS, the development of efficacious interventional therapy has been hampered. At the time of writing this perspective, the coronavirus (COVID-19) pandemic

has fundamentally changed societal norms with detrimental economic impact leading to increased psychological and physical stress resulting from a multitude of factors such as unemployment, social isolation and homelessness (Salari et al., 2020). Importantly, one of the deleterious effects of the COVID-19 pandemic has been the closure of schools resulting in social isolation of children from their peers and disproportional access to educational resources, which are especially lacking in minority communities and children with behavioral/cognitive deficits (Wong et al., 2020). In addition, there have been concerns of possible increased child maltreatment and neglect, as well as food and housing insecurity due to pandemic shutdowns across the globe (Humphreys et al., 2020). As such, growing epidemiological evidence suggests that adolescents living through the pandemic are potentially at a greater risk for depression and anxiety (Gotlib et al., 2020; Guessoum et al., 2020). Therefore, the issue of the consequences of ELS on the future well-being of children, now more than ever, are at the forefront of public health and further necessitates a complete understanding of the etiology of behavioral disorders associated with ELS and the identification of potential novel interventional therapeutic targets.

In conjunction with efforts aimed at increasing our knowledge of the molecular and cellular alterations that perturb the central nervous system (CNS) function in mental health disorders, identifying critical brain regions and neural circuits has remained of high importance. Stress-induced dysfunction in a number of brain regions following ELS has been linked to stress-related disorders including the amygdala (Malter Cohen et al., 2013), prefrontal cortex (Herzberg and Gunnar, 2020), and ventral tegmental area (VTA; Shepard and Nugent, 2020). Although these structures have been extensively studied, evidence now suggests that the lateral habenula (LHb), an evolutionarily conserved epithalamic structure, is a critical brain region that is involved in depression, substance use disorder, anxiety, and sleep disorders (Hu et al., 2020). Earlier studies from our lab and others have demonstrated that rodent models of ELS promote LHb dysfunction through increased LHb neuronal excitability and altered synaptic transmission (Tchenio et al., 2017; Authement et al., 2018; Langlois et al., 2020; Simmons et al., 2020). Moreover, our longitudinal studies have demonstrated that these ELS-induced alterations are potentially long–lasting and span across multiple stages of development (Shepard et al., 2018b; Langlois et al., 2020).

MATERIALS AND METHODS

All experiments were conducted with approval from the Uniformed Services University (USU) Institutional Animal Care and Use Committee (IACUC) and guidelines from the National Institutes of Health (NIH) for the care and use of laboratory animals.

Maternal Deprivation and LHb Microdissection

Maternal deprivation (MD) was carried out as described previously (Authement et al., 2018; Shepard et al., 2018a,b, 2020; Simmons et al., 2020). At P9, male pups (Charles River) were isolated from the dam as a group in a separate room to avoid localization from other animals in the main housing area. MD pups were kept together and isolated from the dam for a total of 24 h before being returned to the home cage with the dam and remaining pups serving as the control group (non-maternally deprived, non-MD rats). Rats were kept with the dam until P28 where they were weaned into dyads based on stress conditions (non-MD vs. MD). Between postnatal days 70–80, adult male rats were anesthetized with isoflurane and decapitated by guillotine. Brains were quickly dissected in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.00 $MgSO_4$, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O_2–5% CO_2. Sagittal brain sections were taken at 300 μm and the LHb was grossly dissected from each as done previously (Authement et al., 2018). LHb was visualized by the slice containing both the stria medullaris and the fasciculus retroflexus.

Western Blot

WBs on LHb tissues were conducted as described previously (Authement et al., 2018). Twenty microgram of LHb protein lysates were loaded into precast polyacrylamide gels and separated by electrophoresis. Following transfer to nitrocellulose membranes, antibodies targeted against MAGL (1:500, Millipore ABN1000), FAAH (1:500, Abcam ab54615), GAPDH (1:1,000, Abcam ab9485) and vinculin (1:1,000, Abcam ab129002). HRP-linked secondary antibodies targeting either rabbit (1:2,000, Cell Signaling) or mouse (1:2,000, Cell Signaling) were used. Data was analyzed using ImageJ. MAGL and FAAH expression levels were normalized to the levels of either GAPDH or vinculin. Summary data is reported as a fold change and is normalized to the non-MD group.

Statistical Analysis

Values for WB experiments are presented as means ± SEM. Statistical significance was determined using unpaired Student's *t*-test and was set at a level of $p < 0.05$. Due to a smaller sample size, the Shapiro-Wilk test was used to assess normality. Statistical analyses were performed using GraphPad Prism.

The Lateral Habenula: a Lynchpin for the Development of Psychiatric Disorders?

The habenula (both medial and lateral divisions) together with the stria medularis and pineal gland (Roman et al., 2020) make up the epithalamus which is conserved from humans down to teleosts indicating a critical evolutionary role (Sutherland, 1982). Specifically, the LHb functions as an "anti-reward" structure which is activated in response to aversion and negative reward (Hikosaka, 2010). Serving as a critical hub for incoming afferents projecting from forebrain and limbic regions (Hikosaka et al., 2008; Hu et al., 2020; Roman et al., 2020), the primarily glutamatergic pathways from the LHb suppress the release of monoamines such as dopamine (DA) and serotonin from the VTA (Ji and Shepard, 2007; Matsumoto and Hikosaka, 2007; Hong et al., 2011) and dorsal raphe nucleus (DRN; Pasquier et al., 1976; Varga et al., 2003), respectively, through their

direct and indirect (GABAergic neurons of the rostromedial tegmental area, RMTg) projections. However, recent evidence suggests the existence of possible GABAergic interneurons in the LHb that could also provide local inhibitory signaling (Zhang et al., 2018; Flanigan et al., 2020; Webster et al., 2020; Li et al., 2021). It is important to note that LHb glutamatergic neurons are not homogenous and differ with respect to their physiological firing patterns, the expression of a variety of neuropeptidergic receptors, their downstream projections, and the inputs that they receive (Wallace et al., 2020). Studies have suggested that dysregulation of LHb function is involved in the pathophysiology of a variety of mental disorders (Lecca et al., 2014; Hu et al., 2020). Most notably, LHb hyperactivity has been consistently found in both clinical and animal models of depression (Proulx et al., 2014; Browne et al., 2018; Nuno-Perez et al., 2018; Yang et al., 2018; Cerniauskas et al., 2019; Gold and Kadriu, 2019). While LHb hyperactivity associated with depressive phenotypes have been found to occur uniformly across all LHb neuronal subpopulations (Li et al., 2013; Authement et al., 2018; Shepard et al., 2018b; Yang et al., 2018; Simmons et al., 2020), some studies suggest projection- and input-specific LHb subcircuit dysfunction in depression (Li et al., 2011; Cerniauskas et al., 2019). For example, the enhanced release of glutamate at glutamatergic synapses onto VTA-projecting LHb neurons correlates with learned helplessness behaviors in rats (Li et al., 2011) suggesting that depression-related hyperactivity in specific LHb subpopulations may arise from synaptic changes at distinct synaptic inputs to LHb subpopulations. Additionally, the entopeduncular (EP) nucleus (rodent homolog to the internal segment globus pallidus) co-releases glutamate/GABA to mediate aversion and "anti-reward" signaling. Antidepressants can alter changes in presynaptic glutamate/GABA release and thereby modulate LHb neuronal excitability (Shabel et al., 2012, 2014; Cerniauskas et al., 2019; Wallace et al., 2020). Consistently, chronic stress in mice increases the activity of VTA- but not DRN-projecting LHb neurons through enhanced presynaptic glutamate release from the EP, which underlies stress-induced increases in passive coping and reduced motivation, but not anxiety or anhedonia (Cerniauskas et al., 2019). Given that DA dysfunction is associated with ELS (Pruessner et al., 2004), our lab first focused on the effects of ELS on VTA DA function using an ELS model of MD in rats (Ellenbroek et al., 2005) in which rat pups are subjected to a single 24-h maternal separation (MS) from the dam. We demonstrated that MD induced VTA DA dysfunction through induction of GABAergic metaplasticity involving A-kinase anchoring scaffolding protein (AKAP150, also referred to AKAP79—the human equivalent to rodent AKAP150) signaling and histone deacetylases (HDACs). We showed that MD in juvenile rats selectively induces long-term depression (LTD) and shifts spike-timing-dependent plasticity (STDP) toward LTD at GABAergic synapses onto VTA DA neurons. This MD-induced metaplasticity involved epigenetic modifications to AKAP150 signaling that included an increase in HDAC2 and decreased histone acetylation which was reversible with HDAC inhibition (Authement et al., 2015; Shepard et al., 2018a, 2020; Shepard and Nugent, 2020). Taken together, this body of work demonstrates that MD alters GABAergic synaptic strength onto VTA DA neurons which potentially contributes to DA dysfunction in psychiatric disorders stemming from ELS.

Considering that the LHb regulates VTA DA signaling, we extended our studies to determine whether MD also perturbs LHb function. Studies from our lab using MD and others using maternal separation (MS; Tchenio et al., 2017) or the limited bedding and nesting models of ELS (Bolton et al., 2018) demonstrated that ELS can promote LHb hyperactivity (Authement et al., 2018; Shepard et al., 2018b; Simmons et al., 2020). Mechanistically, we have shown that LHb neurons in adolescent rats that underwent MD stress are hyperexcitable partly due to a shift in synaptic excitation and inhibition (E/I) balance towards excitation, as well as downregulation of small conductance (SK2) potassium channels and increased protein kinase (PKA) activity, resulting in induction of an intrinsic plasticity in LHb neurons. On the other hand, MS stress in mice decreases postsynaptic $GABA_BR$ signaling arising from entopeduncular nucleus GABAergic inputs which then contributes to MS-induced LHb hyperexcitability in adult mice (Tchenio et al., 2017). Given the epidemiological evidence for the increased predisposition towards developing psychiatric conditions of children subjected to early trauma, we also aimed to determine whether MD-induced LHb hyperactivity persists throughout adolescence into adulthood. Indeed, we have demonstrated persistent maladaptive alterations in LHb neuronal and synaptic function by MD from early adolescence (Authement et al., 2018; Shepard et al., 2018b; Simmons et al., 2020) into adulthood (Langlois et al., 2020). We also found that one of the possible mechanisms contributing to synaptic dysfunction in the LHb during early adolescence may be related to the potential impairment of eCB signaling (Authement et al., 2018) which we hypothesize partly contributes to the long-lasting ELS-induced LHb hyperexcitability.

eCB Signaling: a Key Player in Synaptic Regulation

Unlike neurotransmitters and neuropeptides, eCBs are lipids that are synthesized on-demand in an activity-dependent manner. The two major constituent eCB signaling messengers are N-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) which primarily act on cannabinoid receptors 1 and 2 (CB1R and CB2R) as retrograde signals, although they can also act on transient receptor potential vanilloid receptor type 1 (TRPV1) as non-retrograde signals (Castillo et al., 2012). There is a great deal of evidence to support that both AEA and 2-AG are synthesized by enzymes such as *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively, that are localized to postsynaptic neurons (Katona et al., 2006; Yoshida et al., 2006; Yoshino et al., 2011). Interestingly, eCB degradation enzymes are segregated spatially in that fatty acid amide hydrolase (FAAH, the main degradative enzyme for AEA) is principally found postsynaptically and monoacylglycerol lipase (MAGL, the enzyme responsible for 2-AG degradation) is found presynaptically. Given these differences in spatial organization, it is reasonable to assume that these enzymatic

synthesis/degradation pathways can be distinctly modulated with cell type-and brain region-specificity to regulate neuronal activity, synaptic strength (balance and/or coordination of excitatory to inhibitory inputs), and plasticity. Although there are a wide variety of receptors that eCBs can target independently of CB1Rs/CB2Rs, such as TRPV channels and other orphan receptors (Cristino et al., 2020), we will be discussing our perspective on eCB signaling within the context of CB1R engagement due to its high neuronal expression (Qureshi et al., 1998) and robust role in synaptic plasticity (Gerdeman and Lovinger, 2003; Castillo et al., 2012). We recommend the following in-depth reviews for the role of eCB signaling in synaptic function and plasticity (Castillo et al., 2012; Augustin and Lovinger, 2018; Zou and Kumar, 2018; Cristino et al., 2020).

CB1Rs are $G_{i/o}$-protein-coupled receptors (GPCRs) which once activated decrease the release of neurotransmitter presynaptically through a variety of mechanisms including decreased cyclic adenosine monophosphate (cAMP), membrane hyperpolarization through activation of potassium channels and decreased vesicular release of neurotransmitter *via* inhibition of voltage-gated calcium channels (VGCCs; Cristino et al., 2020). Although CB1Rs are primarily considered to be presynaptically localized, studies have also identified that CB1Rs can also function as autoreceptors when postsynaptically localized (Bacci et al., 2004). Lastly, others have identified that CB1Rs can also localize to glial cells, such as astrocytes (Navarrete and Araque, 2008). Moreover, depending on the localization of CB1Rs at distinct presynaptic inputs, eCBs are poised to differentially affect the strength of synaptic transmission in a cell-type and circuit-dependent manner. Importantly, eCBs are developmentally regulated and their levels fluctuate during critical developmental windows (Meyer et al., 2018). Not surprisingly, disease-based alterations in the neural function and behaviors involving eCB signaling have been implicated, making the eCB system a potential therapeutic target. Given the complexity of the eCB system that also includes several eCB-related mediators, their enzymes, and their molecular targets, the classical eCB signaling has been expanded to an "-ome"; the endocannabidiome (Cristino et al., 2020).

ELS Potentially Impairs eCB Signaling in the LHb

Studies on LHb physiology have demonstrated that eCB signaling profoundly controls LHb synaptic plasticity, neuronal activity, and associated behaviors (Valentinova and Mameli, 2016; Park et al., 2017; Authement et al., 2018; Berger et al., 2018). Potential alterations in eCB-mediated regulation of LHb neurons could contribute to LHb dysfunction associated with anhedonia, as well as motivational and social deficits in depression and other stress-related disorders.

In fact, CB1Rs are shown to be highly expressed in the LHb, specifically in presynaptic axon terminals, postsynaptic dendrites and glia (Berger et al., 2018). Intra-LHb manipulations of CB1Rs, has been shown to bi-directionally affect stress coping strategies where upon LHb CB1R blockade, rats display a more proactive behavioral style over avoidance (i.e., rats are more likely to explore and interact with a novel conspecific) whereas LHb CB1R activation increases avoidant behaviors (Berger et al., 2018). These data suggest that LHb eCB signaling has profound effects on motivational and social behaviors which could be mediated by eCB-mediated synaptic modifications in the LHb. The two forms of synaptic plasticity (long-term potentiation, LTP, and LTD) are both subject to eCB modulation (Castillo et al., 2012), although eCBs mostly depress synaptic transmission (through a decrease in presynaptic neurotransmitter release), inhibit LTP and promote the induction of a presynaptic LTD at both glutamatergic and GABAergic synapses (Castillo et al., 2012). In response to a traditional LTD protocol (low frequency stimulation), or upon activation of group I metabotropic glutamate receptor (mGluR I), LHb neurons can express an eCB-mediated presynaptic LTD at glutamatergic synapses (Valentinova and Mameli, 2016; Park et al., 2017). Importantly, a strong stressor (delivering unpredictable tail shocks while rats are restrained) is sufficient to block eCB-mediated glutamatergic LTD in LHb neurons, potentially enhancing the excitatory drive onto LHb neurons following this acute stress. The stress-induced impairment of eCB-mediated LTD is recovered by inhibition of CamKIIa (an important LHb molecular substrate in behavioral depression; Park et al., 2017). Interestingly, mGluR activation also triggers LTD at GABAergic synapses through PKC-dependent reduction of β2-containing $GABA_A$Rs in LHb neurons suggesting that LHb mGluR signaling bidirectionally regulates LHb neuronal outputs through induction of mGluR-dependent synaptic plasticity that could mediate opposing motivational behaviors (Valentinova and Mameli, 2016).

We also found that activation and inhibition of CB1Rs decreases and increases the probability of presynaptic GABA release in the LHb, respectively, suggesting a possible presence of a tonic eCB signaling and persistent CB1 receptor activation in the regulation of GABAergic transmission in the LHb (Authement et al., 2018). Importantly, we have shown that activation of corticotropin-releasing factor (CRF)-CRFR1-PKA signaling increases LHb excitability through selective suppression of presynaptic GABA release onto LHb neurons through retrograde eCB-CB1R signaling, as well as increases in intrinsic excitability of LHb neurons through PKA-dependent reduction of SK2 channels. More importantly, we observed blunted responses of LHb neurons to the excitatory effects of acute CRF signaling in LHb neurons due to MD-induced increases in PKA activity, possibly downregulating SK2 channels in LHb neurons (Authement et al., 2018).

Our longitudinal studies on the effects of MD on LHb function demonstrated the persistence of MD-induced LHb hyperexcitability during development (Shepard et al., 2018b; Langlois et al., 2020). Interestingly, we have also observed potentially long–lasting elevations in eCB-2AG levels in the LHb as we found decreased MAGL (2-AG degrading enzyme) protein levels, as well as decreases in CB1R expression in the adult rat LHb (**Figure 1**). This is in line with the studies from knock-out (KO) mice where either deletion of MAGL or chronic antagonism of MAGL can decrease CB1R expression in the CNS (Chanda et al., 2010; Schlosburg et al., 2014). Consistently, chronic unpredictable stress has been shown to alter eCB degradation and is associated with less CB1R binding

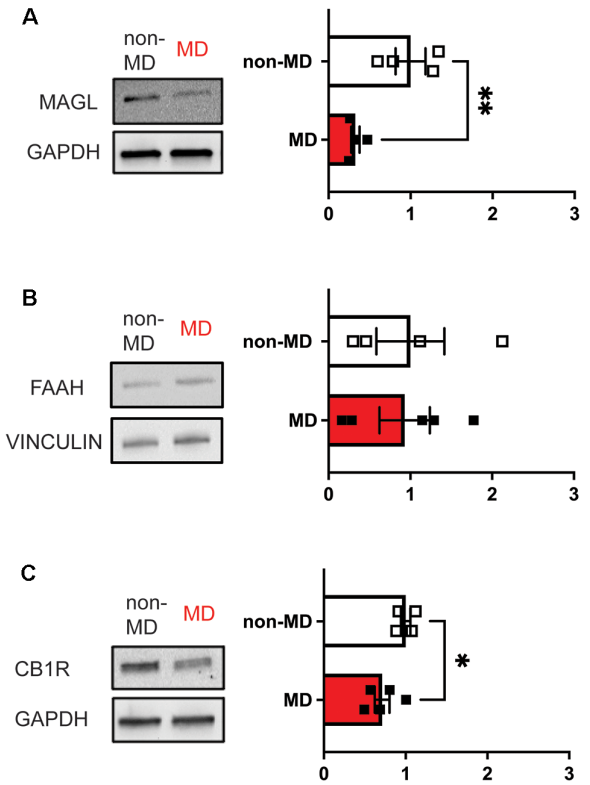

FIGURE 1 | Maternal deprivation (MD) decreases the abundance of both MAGL and CB1R in LHb whole protein lysates, but not FAAH in adulthood (Postnatal days 70–80) in response to ELS at Postnatal day 9. Bar graphs represent fold changes in the abundance of protein with respect to non-MD rats. **(A)** MD decreases total MAGL abundance (non-MD, $n = 4$; MD, $n = 4$; $t_{(6)} = 3.543$). **(B)** MD does not significantly affect FAAH abundance (non-MD, $n = 4$; MD, $n = 5$; $t_{(7)} = 0.1360$). **(C)** MD decreases CB1R abundance (non-MD, $n = 4$; MD, $n = 5$; $t_{(7)} = 2.522$). $^{*}p < 0.05$, $^{**}p < 0.01$ by unpaired t-test.

(Berger et al., 2018). Similarly, a recent study demonstrated that exposure to alcohol in rats decreases CB1R expression, but in contrast, increases MAGL with no change in FAAH. Interestingly, this study also found that activation of eCB signaling within the LHb is an analgesic and could reduce ethanol intake (Fu et al., 2021). Additionally, KO of MAGL can promote anxiety- and pro-depressive behaviors (Imperatore et al., 2015) providing additional evidence for a critical role of eCB modulation of neural circuits that regulate stress-related behaviors. Given that adult MD rats show an imbalance of excitation and inhibition towards excitation with LHb hyperactivity (Shepard et al., 2018b; Langlois et al., 2020), we assume that this could also contribute to an elevation in eCB 2-AG production. Although we show the interaction between CRF neuromodulatory systems with eCB signaling within the adolescent rat LHb following MD, whether MD-induced dysregulation of CRF signaling persists into adulthood and affects eCB-mediated regulation of LHb activity is unclear. Given that MD-induced CRF dysregulation seems to promote intrinsic LHb excitability and that eCB-CB1R signaling decreases both presynaptic glutamate and GABA release and mediates glutamatergic LTD in LHb, we predict that MD may decrease 2-AG degradation by MD-induced decreases in presynaptic MAGL specific to presynaptic GABAergic inputs to the LHb while selective MD-induced downregulation of CB1Rs in presynaptic glutamatergic terminals within the LHb could decrease eCB-mediated suppression of glutamate release as well as inhibit eCB-mediated LTD in LHb neurons. Interestingly, a recent study demonstrated that most cannabinoids suppress the reinforcing effects of optogenetic VTA DA neuron self-stimulation in mice, suggesting that cannabinoid receptor activation, in general, attenuates VTA DA reward or could exert aversive effects. This study also shows that VTA GABA and glutamate neurons express CB1Rs while VTA DA neurons express CB2Rs (Humburg et al., 2021). Given that VTA GABA neurons also provide inhibitory GABAergic input to LHb, it is possible that ELS-induced decreases in MAGL expression and persistent increases in eCB 2AG-CB1R-mediated suppression of VTA GABAergic input to the LHb promotes ELS-associated LHb hyperactivity. However, a technical limitation of our molecular data is the lack of cell-type and synapse-specific localizations of the enzyme and receptor which poses the question whether MD-induced downregulation of CB1R and MAGL occurs globally at all excitatory and inhibitory synaptic inputs and LHb neurons projecting to different downstream targets. Our studies show that MD increases the overall activity of LHb neurons and induces behavioral changes including increased immobility in the forced swim test, reduced sucrose preference, and decreased motivation for morphine self-administration (Shepard et al., 2018b; Langlois et al., 2020). Given that distinct LHb subcircuits may mediate specific behavioral phenotypes as shown following chronic stress model of depression in mice (Cerniauskas et al., 2019), future ELS research is necessary to investigate the precise contribution of ELS-induced hyperactivity of VTA-, RMTg- and DRN-projecting LHb neurons as well as eCB-mediated synaptic plasticity at specific synaptic inputs to the LHb in different behavioral deficits following ELS. Moreover, it is worthwhile to examine how different ELS models (MD, MS or LBN) affect stress neuromodulatory systems such as CRF (Authement et al., 2018) and kappa opioid receptor signaling (Simmons et al., 2020) within the LHb with their possible interaction with the eCB system in mediating motivational and anhedonic states associated with these ELS models as cell type and circuit-specific manner.

DISCUSSION

New advances in understanding neural circuits and the connectome have shaped our understanding of how discrete brain regions and circuits become dysregulated in psychiatric and neurological disorders. Given that eCB signaling plays a critical role in neuronal regulation across the CNS, greater emphasis needs to be placed on the role of eCB signaling with respect to specific inputs and brain structures. Advancements in mouse genetics and Cre-dependent manipulations of neuronal activity (optogenetics and DREADDs) and gene expression allow us to gain greater spatial and temporal control for cell type- and circuit-specific eCB neuromodulation and eCB-mediated

behaviors. In addition to using preclinical animal models to study the role of eCB signaling, it is also important to understand whether pharmacological manipulation of eCB signaling in a clinical setting is a viable treatment method. Until recently, one problem with imaging the habenula was due to its small size which makes functional imaging a challenge (Lawson et al., 2013). Recently, using magnetic resonance imaging (MRI) with better resolution has made it possible to functionally image the human habenula (Strotmann et al., 2013, 2014). However, it is still not possible to reliably distinguish between the medial and lateral divisions in humans.

Recent legislative actions have now allowed the use of medical marijuana for the treatment of a variety of conditions which vary across the United States and countries around the world. However, one concern of the use of marijuana and other derivatives as treatment is their potential for abuse, as well as the potential for inducing schizophrenia and other psychiatric disorders (Bostwick, 2012; Chadwick et al., 2013). Therefore, a large number of clinical trials have both been conducted and are currently underway to determine whether targeting CB1R directly or affecting the degradation rate of eCBs through FAAH and MAGL inhibitors can be used in a wide variety of disorders ranging from anxiety to epilepsy (van Egmond et al., 2021); however, there is still a lack of clinical data to determine whether these compounds will be efficacious across the general population. In addition, there is greater concern over whether treatment in pediatrics and adolescents would be safe (Fontanella et al., 2021) considering the critical role of the eCB system in development (Meyer et al., 2018). Therefore, future drug development could potentially focus more so on targeting downstream eCB signaling from CB1Rs.

Advances in proteomic screenings have started to allow the identification of protein-protein interactions of receptor complexes. In fact, many receptors can exist in nature as receptor complexes where accessory proteins or auxiliary subunits can impact receptor trafficking, kinetics, and pharmacology (Maher et al., 2017). Already, these accessory proteins have been identified for VGCCs (Campiglio and Flucher, 2015), AMPARs (Kamalova and Nakagawa, 2021), nAChRs (Boulin et al., 2012), and most recently $GABA_A$Rs (Castellano et al., 2020; Han et al., 2020). Indeed, targeting of auxiliary subunits has already succeeded with gabapentin being used for the treatment of epilepsy and pain. Currently, the transmembrane AMPAR regulatory protein (TARP) auxiliary subunit γ8 is in clinical trials for the treatment of pain and epilepsy (Kato et al., 2016; Maher et al., 2017). Targeting receptor complexes offer an exciting opportunity for more precision-based pharmacology and to mitigate off-target effects associated with numerous drugs.

Like other receptors, CB1Rs are expressed ubiquitously in the CNS and targeting CB1R-associated proteins to modify downstream intracellular signaling could perhaps yield novel drug targets for development, such as cannabinoid receptor-interacting protein 1a and 1b (CRIP1a and CRIP1b, respectively). For example, CRIP1a is a CB1R-specific accessory protein (Niehaus et al., 2007) which has been demonstrated to compete with b-arrestin binding which prevents desensitization and internalization of the receptor (Blume et al., 2017). Additionally, CRIP1a can alter CB1R signaling by altering GPCR signaling pathways (Blume et al., 2016) and stopping CB1R-mediated closure of VGCCs (Niehaus et al., 2007). Within this context, it is perhaps possible that our MD-induced decreases in CB1R in adult rat LHb (**Figure 1**) is due to decreased CRIP1A which allows b-arrestin-mediated endocytosis of CB1Rs; however, this is pure speculation and would need to be verified by both CRIP1a expression, as well as biochemical studies demonstrating the association of both CRIP1a and CB1R in the LHb. Therefore, further research regarding the role of CRIP1a and CRIP1b signaling in a cell type-and circuit-specific manner, in preclinical animal models would provide critical data on the physiological role of these accessory proteins as well as whether they are dysregulated in ELS animal models.

CONCLUSION

In summary, given the robust role of the LHb in psychiatric disorders as well as the role of CB1R-mediated regulation of LHb activity, targeting the eCB system for ELS-induced psychiatric disorders is a potential therapeutic option. Although a majority of the clinical data has been conducted in adults, greater observations to the role of eCB signaling in adolescents, as well as possible clinical administration of eCB-targeting compounds could provide data as to their safety and efficacy in younger individuals. Lastly, targeting receptor complexes (Rosenbaum et al., 2020) might be a more precise therapy that can reduce the likelihood of adverse off-target effects and mitigate the aforementioned concerns.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Uniformed Services University IACUC committee.

AUTHOR CONTRIBUTIONS

FN and RS designed the experiments and wrote the manuscript. RS performed the experiments, analyzed the data, and prepared the figures. All authors contributed to the article and approved the submitted version.

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frontiers in Synaptic Neuroscience

REVIEW
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Cannabinoid Modulation of Dopamine Release During Motivation, Periodic Reinforcement, Exploratory Behavior, Habit Formation, and Attention

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Motivational and attentional processes energize action sequences to facilitate evolutionary competition and promote behavioral fitness. Decades of neuropharmacology, electrophysiology and electrochemistry research indicate that the mesocorticolimbic DA pathway modulates both motivation and attention. More recently, it was realized that mesocorticolimbic DA function is tightly regulated by the brain's endocannabinoid system and greatly influenced by exogenous cannabinoids—which have been harnessed by humanity for medicinal, ritualistic, and recreational uses for 12,000 years. Exogenous cannabinoids, like the primary psychoactive component of cannabis, delta-9-tetrahydrocannabinol, produce their effects by acting at binding sites for naturally occurring endocannabinoids. The brain's endocannabinoid system consists of two G-protein coupled receptors, endogenous lipid ligands for these receptor targets, and several synthetic and metabolic enzymes involved in their production and degradation. Emerging evidence indicates that the endocannabinoid 2-arachidonoylglycerol is necessary to observe concurrent increases in DA release and motivated behavior. And the historical pharmacology literature indicates a role for cannabinoid signaling in both motivational and attentional processes. While both types of behaviors have been scrutinized under manipulation by either DA or cannabinoid agents, there is considerably less insight into prospective interactions between these two important signaling systems. This review attempts to summate the relevance of cannabinoid modulation of DA release during operant tasks designed to investigate either motivational or attentional control of behavior. We first describe how cannabinoids influence DA release and goal-directed action under a variety of reinforcement contingencies. Then we consider the role that endocannabinoids might play in switching an animal's motivation from a goal-directed action to the search for an alternative outcome, in addition to the formation of long-term habits. Finally, dissociable features of attentional behavior using both the 5-choice serial reaction time task and the attentional set-shifting task are discussed along with their distinct influences by DA and cannabinoids. We end with discussing potential targets for further research regarding DA-cannabinoid interactions within key substrates involved in motivation and attention.

Keywords: cannabinoids, dopamine, motivation, reinforcement, attention, habit, timing, adjunctive

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INTRODUCTION

Statement of Purpose

Because many reviews already exist that describe endocannabinoid (eCB) signaling (Toczek and Malinowska, 2018; Zou and Kumar, 2018; Cristino et al., 2020; Kaczocha and Haj-Dahmane, 2021), the risks of cannabis abuse (Ferland and Hurd, 2020; Hindley et al., 2020), and the potential cannabinoids may offer in psychiatric medicine (Amar, 2006; Black et al., 2019; Navarrete et al., 2020), our goal in the present manuscript is to describe how exogenous cannabinoids and eCBs influence dopamine (DA) signaling and behavior. While we will emphasize our own observations, we will also consider how they fit into the context of the general literature on reinforcement, appetitive behavior, adjunctive behavior, habit formation, and attentional processes. We conclude by considering how cannabinoid-induced changes in one neurobehavior might influence another if they share overlapping neural circuitry.

Phytocannabinoids, Synthetic Cannabinoids, Endocannabinoids

Approximately 535 chemicals and 90 different C_{21} terpenophenolic phytocannabinoids exist in the cannabis plant (Radwan et al., 2009; Andre et al., 2016). While these chemicals act synergistically to produce an entourage effect with delta-9-tetrahydrocannabinol (THC), the latter is principally responsible for cannabis's psychoactive effects by activating G protein-coupled receptors (GPCR) in the brain (e.g., cannabinoid receptor type 1; CB1) (Casajuana Kögel et al., 2018; Russo, 2019). The first synthetic cannabinoids (e.g., CP-55,940) were developed by Pfizer and found to be more potent and effective at activating the CB1 than THC (Matsuda et al., 1990; Marzo and Petrocellis, 2006). The Sterling research group then discovered that uniquely structured aminoalkylindole agonists also activate the CB1 with high potency and efficacy (D'Ambra et al., 1992). The aminoalkylindole synthetic cannabinoid WIN 55,212-2 (WIN) is particularly noteworthy because it has been employed extensively in psychopharmacology research (D'Ambra et al., 1992)—including several studies that will be described herein. However, it should be noted that WIN is about 80% more effective at activating the CB1 than the phytocannabinoid THC (Sim et al., 1996). The discovery of a brain cannabinoid receptor led to an exploration for its endogenous ligands, or eCBs (Marzo and Petrocellis, 2006). The best characterized eCBs are 2-arachidonoylglycerol (2AG) (Mechoulam et al., 1995; Sugiura et al., 1995) and *N*-arachidonoylethanolamine (anandamide; AEA) (Devane et al., 1992). It is now recognized that 2AG and AEA have different synthetic and metabolic pathways (Lu and Mackie, 2016). 2AG is predominantly synthesized from 2-arachidonoyl-containing phospholipids (e.g., diacylglycerol; DAG) by DAG lipase (DAGL) and metabolized by monoacylglycerol lipase (MAGL); AEA is predominantly synthesized from *N*-acyl-phosphatidylethanolamine (NAPE) by NAPE-specific phospholipase D (NAPE-PLD) and metabolized by fatty acid amidohydrolase (FAAH) (Lu and Mackie, 2016; Toczek and Malinowska, 2018; Zou and Kumar, 2018). In this review, we will describe several studies that manipulate 2AG levels by inhibiting either DAGL or MAGL. We attempt to specify when we are describing the specific effects of phytocannabinoids, synthetic cannabinoids, or eCBs on neurobiology and behavior. When making broader conclusions we use the term cannabinoid, which we define as any ligand that interacts with the cannabinoid receptors or their associated machinery.

Cannabinoid Receptors

In addition to the aforementioned CB1 it is necessary to acknowledge several other cannabinoid receptor targets, most notably the cannabinoid receptor type 2 (CB2). While once thought to be relegated to the immune system and spleen, recent evidence suggest that CB2 is expressed in both neurons and glial cells of the brain as a unique isoform (Jordan and Xi, 2019). Specifically, mRNA for the CB_{2A} variant was found to be expressed in the brain and the testis, whereas mRNA for the CB_{2B} variant was found in the spleen and immune cells (Liu et al., 2009). The exact role that the CB_{2A} variant plays in modulating operant behavior remains to be fully elucidated, but it appears to be involved in multiple cellular and behavioral functions (Jordan and Xi, 2019). There is also evidence that AEA activates TRPV1 ion channels (van der Stelt et al., 2005), which have been shown to modulate habitual behavior (Shan et al., 2015). But also see (Gianessi et al., 2019), who recently reported that antagonism of TRPV1 does not influence habit formation. The GPR55 orphan receptor, which is thought to be activated by both eCBs and synthetic cannabinoids (Marichal-Cancino et al., 2017), was also reported to influence learning in a T-maze (Marichal-Cancino et al., 2016). Peroxisome proliferator-activated receptors (PPAR) are yet another target worth considering. PPARs are activated by various lipids, including eCBs (Iannotti and Vitale, 2021), and are thought to influence DA release (Melis et al., 2013a). In all, at least 12 different receptors are known to be activated by eCBs (Maccarrone, 2020), suggesting that the scope of mechanisms through which phytocannabinoids, synthetic cannabinoids, and eCBs regulate behavior are considerably more complex than our current conception.

eCBs, DSI, and a Model of DA Release

A unique feature of eCB signaling is that these molecules are not stored in vesicles like classical neurotransmitters but are instead synthesized *de novo* and released from post-synaptic neurons in times of sustained neuronal activity (Freund et al., 2003; Castillo et al., 2012; Ohno-Shosaku and Kano, 2014). Heightened neural activity results in increased intracellular Ca^{2+} that leads to the activation of synthetic enzymes (DAGL, NAPE-PLD) responsible for the rapid synthesis of eCBs (Marsicano et al., 2003; Lu and Mackie, 2016). Following their release from the postsynaptic neuron into the synaptic cleft, eCBs retrogradely activate CB1s located on presynaptic terminals of both GABA and glutamate neurons (Wilson and Nicoll, 2002; Melis et al., 2004; Alger and Kim, 2011). Retrograde eCB modulation of GABA terminals can produce depolarization-induced suppression of inhibition (DSI),

whereas retrograde eCB modulation of glutamate terminals can produce depolarization-induced suppression of excitation (DSE) (Fortin and Levine, 2007; Lange et al., 2017). In DSI, eCB activation of CB1s on GABA terminals is thought to produce a transient suppression of GABA release onto the postsynaptic neuron, thereby disinhibiting it. By contrast, during DSE, eCB activation of CB1s on glutamate terminals is thought to produce a transient suppression of glutamate release onto the postsynaptic neuron, thereby inhibiting it. While CB1 activation mediates both DSI and DSE, DSI is believed to be much more prominent than DSE due to differences in CB1 sensitivity between inhibitory and excitatory synapses (Ohno-Shosaku et al., 2002).

Cannabinoid receptor type 1-mediated DSI provides a model that might explain how phytocannabinoids, synthetic cannabinoids, and eCBs increase DA release from the midbrain. For a thorough description of how DSI is thought to modulate DA release, we refer the reader to a previously published review clarifying the mechanisms involved (Covey et al., 2017). In the midbrain, CB1s are thought to occur on GABAergic and glutamatergic terminals rather than on DA neurons (Julian et al., 2003; Mátyás et al., 2008). In the awake and behaving animal, midbrain DA neurons fire in one of two distinct patterns: tonic and phasic (Grace and Bunney, 1984; Grace, 1991; Grace et al., 2007). At rest, DA neurons are tonically active and exhibit steady pacemaker activity, firing at an average rate of 5 Hz. By contrast, DA neurons fire in phasic bursts of 10–20 Hz when an animal is presented with a motivationally salient stimulus (Grace et al., 2007). These phasic bursts are thought to give rise to high-concentration transient DA release events in the NAc that encode the value of motivationally salient stimuli and actuate goal seeking (Wise, 2004; Grace et al., 2007; Schultz et al., 2015; Stauffer et al., 2016). Burst firing of DA neurons also leads to eCB synthesis, retrograde signaling, and activation of CB1s on GABA and glutamate terminals (Szabo et al., 2002; Riegel and Lupica, 2004; Melis et al., 2013b; Wang et al., 2015). If DSI is more prevalent than DSE in the midbrain, the result would be disinhibition of DA neurons and the subsequent release of DA at terminal sites of the mesocorticolimbic and nigrostriatal DA pathways. In support of this model, a growing body of evidence using a multitude of techniques report that eCBs (Solinas et al., 2006; Oleson et al., 2012), THC (Chen et al., 1990, 1993; Diana et al., 1998; Gessa et al., 1998; Voruganti et al., 2001; Pistis et al., 2002; Bossong et al., 2009), and synthetic cannabinoids (Tanda et al., 1997; Diana et al., 1998; Gessa et al., 1998; Fadda et al., 2006; Oleson et al., 2014) increase striatal brain DA levels in both rodents (Gessa et al., 1998; Pistis et al., 2002; Fadda et al., 2006) and humans (Voruganti et al., 2001; Bossong et al., 2009, 2015). Using *in vitro* electrophysiology, Melis et al. (2013b) demonstrated that this DSI-induced disinhibition of DA release is principally mediated by 2AG activating CB1s. Indeed, several studies will be presented herein demonstrating that the eCB 2AG effectively modulates DA-associated behavior in a CB1 dependent manner. However, while this model may explain the effects of synthetic cannabinoids and eCBs on DA release presented within this review, we acknowledge that it is incomplete because it does not account for the role of CB2 or other receptor targets (e.g., PPARs) that likely modulate DA release as well.

eCB Modulation of the Mesocorticolimbic System

The mesocorticolimbic DA system originates from DA neurons in the ventral tegmental area (VTA) that project to a variety of brain regions. Its most prominent target is the ventral portion of the striatum, or nucleus accumbens (NAc) (Morales and Margolis, 2017). While the VTA is primarily composed of DA neurons (∼60%), GABA (∼25%) and glutamate (∼15%) neurons also exist and are capable of modulating DA neural activity, mesocorticolimbic output, and behavior (Swanson, 1982; Morales and Root, 2014; Yoo et al., 2016). It is theorized that these neurons form subpopulations that then receive disproportionate afferent input from distinct brain structures (e.g., periaqueductal gray, lateral hypothalamus, raphe nuclei, rostromedial tegmental nucleus) to form dissociable microcircuits that may subserve unique behavioral functions (Lammel et al., 2014; Breton et al., 2019). Thus, in addition to disinhibiting DA release in the VTA, it is likely that eCBs also modulate DA-associated behavior by acting on distinct afferents that then synapse onto DA neurons. It is also worth considering eCB modulation of neural activity at terminal fields of the mesocorticolimbic system. Like other monoamines, DA functions as a relatively slow neuromodulator of fast glutamate- and GABA-mediated neurotransmission and, in the awake and behaving rat, the effect that DA exerts on postsynaptic potentials is greatly influenced by these converging inputs into a given terminal field (O'Donnell et al., 1999; Brady and O'Donnell, 2004). As a prominent mesocorticolimbic hub, the effect DA exerts in the NAc can therefore be influenced by eCB modulation of amygdalar, hippocampal, and cortical input into it. The neuromodulatory effects of DA in the NAc can also be influenced by co-release of GABA and glutamate from VTA DA neurons. Emerging evidence suggests that VTA DA neurons are capable of co-releasing GABA and glutamate in a manner that regulates motivational drive along with DA (Tritsch et al., 2012; Zhang et al., 2015; Yoo et al., 2016).

Dopamine signaling in the NAc is primarily mediated through D1- and D2-like receptors. D1 receptors generally exhibit low binding affinity for DA and preferentially couple to G_s protein subunits. D2 receptors generally exhibit high binding affinity for DA and preferential coupling to G_i or G_o protein subunits (Beaulieu and Gainetdinov, 2011). Both D1 and D2 DA receptors are expressed as heteroreceptors on dendritic spines of medium spiny GABA neurons (MSNs) within the NAc (Levey et al., 1993; Monory et al., 2007), though D2s are also expressed on presynaptic DA terminals where they function as autoreceptors to attenuate DA release (Bello et al., 2011; Budygin et al., 2017). Notably, CB1s form heterodimeric complex with D2s where colocalization exists, suggesting CB1 may interact with D2 autoreceptors to modulate DA release (Khan and Lee, 2014). It is also

noteworthy that CB1s are expressed in a subpopulation of fast-spiking interneurons (FSI) within the NAc (Winters et al., 2012). Despite only composing 2–3% of striatal neurons, FSI are thought to powerfully orchestrate the activity of the more predominate MSNs and, possibly, control gamma frequency oscillations originating from this region (Tepper et al., 2010). Individual FSIs innervate hundreds of MSNs and, when excited, inhibit their collective output (Tepper et al., 2008). Within the NAc, eCBs are synthesized and released from MSNs following activation of either D1 (Shonesy et al., 2020) or D2 (Lerner and Kreitzer, 2012) receptors. Also noteworthy is recent evidence suggesting that tyrosine receptor kinase B activation augments intracellular calcium transients to promote eCB synthesis and spike-timing dependent plasticity in the striatum (Gangarossa et al., 2020). Upon release from MSNs, the eCBs then travel retrogradely before acting upon CB1s located on FSIs within the NAc (Mateo et al., 2017; Wright et al., 2017). Wright et al. (2017) recently used electrophysiology to demonstrate that the inhibitory control that CB1-expressing FSIs exert over MSNs is suppressed by eCB signaling. In addition, these authors (Wright et al., 2017) found that the CB1 expressing FSIs that synapse onto MSNs facilitate a long-term form of eCB-mediated synaptic plasticity (i.e., long-term depression, LTD) that might be important for learning and memory. While previous immunohistochemical studies suggested CB1s are also expressed on cholinergic interneurons within the striatum (Fusco et al., 2004), a more recent study using CB1 radioactive antisense riboprobes found no evidence of CB1 mRNA expression within cholinergic interneurons of the NAc (Mateo et al., 2017). This latter finding is particularly relevant for the current review because we primarily focus on transient DA release events in the NAc. And, it is now recognized that these transient release events can be promoted by either local cholinergic interneurons that activate acetylcholine receptors on adjacent DA terminals (Cachope et al., 2012) or by VTA DA cell activation, both of which are modulated by eCB signaling (Mateo et al., 2017). However, the lack of CB1s on cholinergic interneurons in the NAc suggests that eCBs modulate the influence local cholinergic interneurons exert over terminal dopamine release indirectly. In support of this notion, Mateo et al. (2017) recently reported that eCB modulation of cholinergic-induced terminal dopamine release results from CB1 activation on cortical glutamate afferents into the NAc. In addition to the aforementioned findings by (Wright et al., 2017), this latter observation is highly relevant for eCB- and DA-modulation of learning and memory. D2-dependent eCB-LTD has been verified in glutamatergic corticostriatal projections within the indirect pathway of the basal ganglia—a group of subcortical nuclei including the striatum that modulate behavioral action, procedural learning, and working memory (Lerner and Kreitzer, 2012; Simonyan, 2019). Thus, eCBs likely influence skill learning and memory by indirectly modulating terminal DA release and by gating FSI-control of MSN feedforward inhibition. These separate mechanisms—comprising eCB-mediated DSI/DSE within the VTA, eCB modulation of neural signaling with the NAc, and eCB modulation of afferent input into the NAc and VTA—may all converge to influence mesolimbic DA neurotransmission (Covey et al., 2017).

A Synthetic Cannabinoid Dose-Dependently Increases DA Release and Tolerance Develops to This Effect Following Chronic Exposure

Abused drugs are theorized to exert their reinforcing effects by mimicking these endogenous patterns of DA release in the NAc that normally strengthen goal-directed behavior (Volkow and Morales, 2015; Volkow et al., 2017), and cannabinoids are no exception. We recently confirmed that the synthetic cannabinoid WIN increases accumbal transient DA release events in a dose-dependent manner and further investigated whether tolerance develops to this effect. We used fast-scan cyclic voltammetry (FSCV) to measure sub-second DA transients using NAc-implanted electrodes while treating awake and behaving rats with increasing, cumulative doses of intravenous (IV) WIN. As illustrated in **Figures 1A–C**, WIN dose-dependently increased both the frequency and amplitude of transient DA release events in the NAc shell (Gomez et al., 2020). We next wanted to assess whether chronic WIN exposure produces tolerance to the DA releasing effects of WIN. Although chronic treatment with synthetic or phytocannabinoids is known to produce tolerance to a tetrad of behavioral/physiological effects that is used to screen whether a drug functions as a cannabinoid (i.e., antinociception, catalepsy, hyopthermia, and hypomotility) (Little et al., 1988; Wiley and Martin, 2003; Hama and Sagen, 2009; Nealon et al., 2019)—it remains unclear whether tolerance develops to the rewarding/reinforcing and the DA releasing effects of cannabinoids. Because the degree of tolerance that develops to specific cannabimimetic effects varies as a result of CB1 desensitization occurring in a brain region-dependent manner (Breivogel et al., 1997; Whitlow et al., 2003), it is possible that midbrain CB1s show resistance to tolerance. Supporting this notion, Frau et al. (2019) found that prenatal exposure to THC produces a hyperDAergic rather than a hypoDAergic phenotype, Mavrikaki et al. (2010) found that chronic WIN exposure does not alter brain-reward thresholds, Hirvonen et al. (2012) found that CB1s are downregulated in cortical but not subcortical regions of cannabis smokers, and Wu and French (2000) found that chronic THC treatment does not influence its ability to induce burst firing in putative DA neurons.

To test whether tolerance develops to the DA-releasing effects of WIN, we treated rats with either vehicle or intravenous (IV) WIN using an escalating dosing regimen. To determine if this dosing regimen produced tolerance to standard cannabimimetic effects, we first tested the consequences of it using the tetrad test. As expected, we found that WIN-treated rats displayed a rightward shift in the dose-response relationship (0.002–0.8 mg/kg IV) across all behavioral/physiological measures when compared to vehicle-treated controls. We then used FSCV to investigate whether the same pharmacological history produced tolerance to the DA releasing effects of WIN and cross-tolerance to the DA releasing effects of heroin. We additionally characterized whether this dosing regimen produces

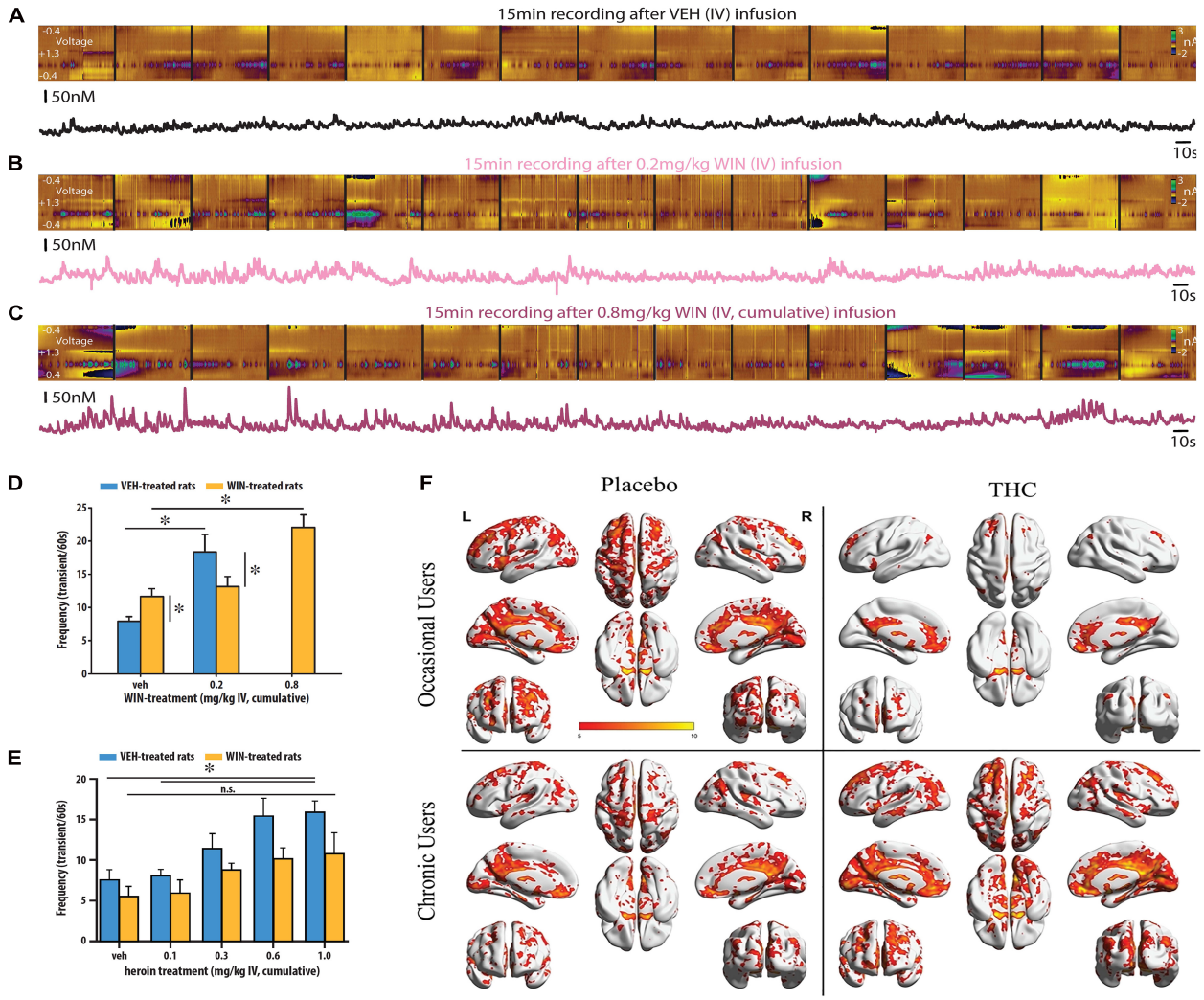

FIGURE 1 | Cannabinoids increase the frequency and amplitude of DA transients. Illustrative recording session in which the synthetic cannabinoid WIN was administered to an awake and freely moving rat. Stitched color plots [voltammetric current (*z*-axis) × applied scan potential (*y*-axis) × time (*x*-axis)] are shown above corresponding DA concentration traces. Vehicle **(A)**, 0.2 mg/kg **(B),** and 0.8 mg/kg WIN **(C)** were administered in cumulative, ascending IV doses while FSCV measurements of DA release events occurred in the NAc shell in near real-time. Dose dependent increases in the frequency and amplitude of DA release events can be observed by the larger and more frequent green dots at a potential of +0.6 V in the color plots and the more frequent and pronounced transient peaks in the corresponding DA concentration traces. **(D)** WIN increased the frequency of DA release events but was less potent in chronically WIN-treated rats. A higher dose of WIN (0.8 vs. 0.2 mg/kg IV) was required to produce a significant increase in DA release vs. vehicle treated rats. **(E)** Heroin dose-dependently increased the frequency of DA release events but was less effective in chronically WIN-treated rats. In WIN-treated rats, heroin did not significantly increase the frequency of DA transients vs. vehicle at any dose tested. Republished from Gomez et al. (2020). **(F)** NAc–related functional connectivity in the left hemispheres. Shown are thresholded *Z*–score maps of functional connectivity for each group and each condition. Smoked THC reduced functional connectivity between the NAc and broad areas of the frontal, temporal, parietal and occipital lobes in occasional, but not chronic cannabis users. Republished from Gomez et al. (2020). *$p < 0.05$.

cross-tolerance to the DA releasing effects of heroin because Cadoni et al. (2008) observed this effect using microdialysis. In addition, synthetic cannabinoids/phytocannabinoids and opioids are well known to produce cross-tolerance to several shared neurobehavioral effects (Hine, 1985; Thorat and Bhargava, 1994; Manzanares et al., 1999; Vigano et al., 2005; Gerak et al., 2015). We found that after chronic WIN exposure, both WIN (**Figure 1D**) and heroin (**Figure 1E**) were less effective at increasing the frequency of DA release events in the NAc shell of adult male rats. If DA is important for drug reward (Di Chiara et al., 2004) or to motivate drug seeking (Volkow et al., 2017) as is currently theorized, a diminished ability to evoke DA release could promote the use of larger quantities and more potent doses. These data support a recent PET imaging study demonstrating that cannabis-dependent patients show a deficit in striatal DA release after the investigators controlled for several comorbidities that may have influenced previous imaging studies (van de Giessen et al., 2017). In another noteworthy imaging study, Mason et al. (2021) used resting-state functional magnetic resonance imaging (fMRI) to determine functional connectivity

between the NAc and other brain regions of interest in occasional and chronic cannabis users. Both groups received placebo and 300-μg/kg THC on separate days. In occasional users, THC produced a marked reduction in functional connectivity between the NAc and broad areas of the frontal, temporal, parietal and occipital lobes (**Figure 1F**)—a pattern the authors note is typical of increased DA neurotransmission. In chronic users, THC did not produce changes in functional connectivity associated with the NAc (**Figure 1F**). The occasional, but not chronic cannabis users, also reported increases in subjective high and showed impairments in a sustained attention task. From these observations, the authors conclude that excessive cannabis use may result in neuroadaptations in accumbal circuitry that reduce the neurobiological and behavioral response to acute cannabis impairment.

However, further studies are necessary to compare how synthetic cannabinoids, eCBs, and phytocannabinoids produce tolerance, whether each produces tolerance to the DA releasing effects of a CB1 agonist, and whether these effects vary with age, sex, or species. It is possible that a synthetic aminoalkylindole cannabinoid like WIN produce distinct effects on molecular, cellular, and/or behavioral tolerance in comparison to a phytocannabinoid like THC. Two complimentary molecular mechanism are thought to contribute to CB1 desensitization and downregulation (Nguyen et al., 2012; Nealon et al., 2019). One involves the recruitment of beta-arrestin2 to GRK-phosphorylated CB1s (Jin et al., 1999; Nguyen et al., 2012). The other is a distinct JNK-mediated form of molecular tolerance that appears to occur in an agonist specific manner (Nealon et al., 2019). Of note, it was recently reported that disrupting JNK signaling prevents several forms of behavioral tolerance induced by THC, but not by WIN (Henderson-Redmond et al., 2020). Thus, future studies are needed to determine how different cannabinoid ligands produce tolerance to distinct behavioral/physiological effects.

CANNABINOIDS AND MOTIVATED ACTION UNDER A VARIETY OF REINFORCEMENT CONTINGENCIES

Response Reinforcement and Schedule-Controlled Behavior

While many different behavioral approaches exist to study the effects of cannabinoids on behavior, this review will primarily focus on response reinforcement and operant behavior. Response reinforcement was first described by Thorndike (1927) as a law of effect—meaning that responses following a satisfying connection act upon it to alter its strength. Concepts associated with the law of effect were further explored in great detail following Skinner's inventions of the operant conditioning chamber and the cumulative recorder (Ferster and Skinner, 1957). The operant conditioning chamber allows experimenters to measure repeatable responses in the face of changing conditions. The cumulative recorder produced a graphical record of the animal's responses, allowing experimenters to study how changing conditions influence the probability of a response. Using this new technology, Ferster and Skinner (1957) reported that the pattern of responses can be greatly influenced by the reinforcement schedule. In the operant context, schedules can be thought of as the rules under which reinforcement is made available, or the contingencies of reinforcement. The observation that reinforcement schedules powerfully modify operant behavior had profound implications for our understanding of the phylogeny of behavior and neurobiology. In an evolutionary context, it is likely that patterns of behaviors were neurobiologically stamped-in when they maximized the receipt of an advantageous outcome (e.g., food) in the face of changing environmental conditions (e.g., the periodic availability of food). Because the environment changes in recurring patterns, it would therefore be advantageous for the brain to produce complex patterns of behavior that adapt to the environmental rules governing reinforcement (Skinner, 1966). In the context of cannabinoid effects on the brain and behavior, it is equally important to recognize that a drug or neurochemical can produce unique effects on operant behavior under different schedules of reinforcement. This phenomenon was first described by Peter Dews, who used an operant conditioning chamber and cumulative recorder to demonstrate that injecting pigeons with the same dose of pentobarbital increased responding for food under a fixed-ratio scheduled but decreased responding for food under a fixed-interval schedule (Dews, 1955). Under a fixed ratio (FR) schedule, behavior is reinforced after the animal responds a pre-defined number of times. This contingency of reinforcement produces a bimodal step-like pattern in which the animal is either responding at a constant rate or at zero (Ferster and Skinner, 1957) (**Figure 2A**). Under a fixed interval (FI) schedule, behavior is reinforced after the animal responds after a pre-defined period of time. This contingency of reinforcement produces a scalloped-like pattern of responding (Dews, 1978) (**Figure 2A**). Because this review will focus on the interaction between cannabinoids and DA signaling in particular, it is also worth noting that DA pharmacology is well known to produce divergent behavioral effects under these two schedules of reinforcement. Equivalent doses of the DA releasers amphetamine and methamphetamine (Cho, 1990; Jones et al., 1998) both decrease response rate under an FR1 schedule and increase response rate under a FI schedule (Dews, 1958; McKearney and Barrett, 1978).

DA Value Signals in Reinforcement and Goal-Directed Action

In the awake and behaving animal, midbrain DA neurons fire in phasic bursts (>20 Hz) under a variety of conditions (Redgrave et al., 2016; Sharpe and Schoenbaum, 2018), including the presentation of rewarding stimuli (Stauffer et al., 2016). These phasic bursts of neural activity contribute to transient DA release events in the primary terminal field of the mesolimbic pathway, the nucleus accumbens (Dreyer et al., 2010). Currently, it is thought that transient DA signals within this brain region encode value as positive or negative reward prediction errors. In support of this theory, a series of *in vivo* electrophysiology studies demonstrated that phasic bursts of DA neural activity

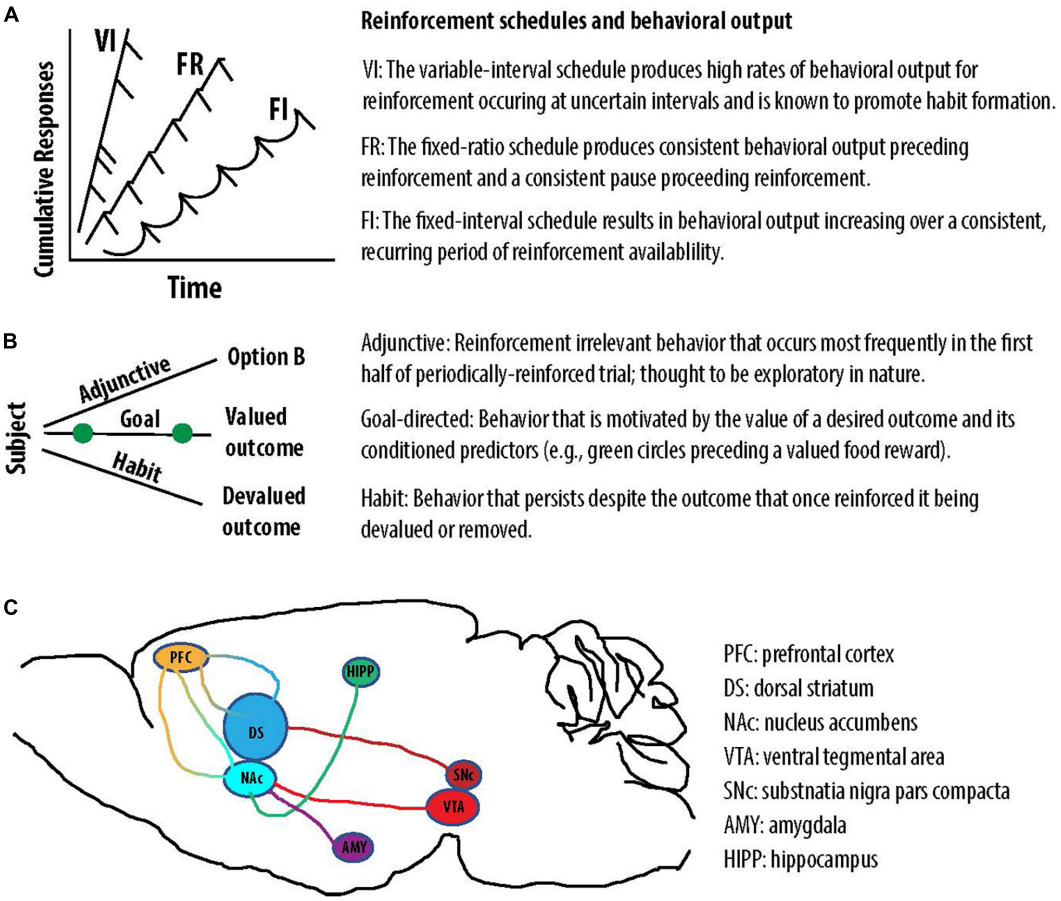

FIGURE 2 | Reinforcement schedules engender distinct behaviors and a depiction of two DA pathways. **(A)** The VI, FR, and FI schedules produce unique patterns of reinforced behavior. **(B)** The contingencies of reinforcement can produce adjunctive, goal-directed, or habitual behavior. **(C)** Illustrative projections associated with the nigrostriatal (dark red) and mesocorticolimbic (light red) DA pathways. DA in the NAc is thought to modulate converging input from brain regions including the amygdala (AMY), hippocampus (HIPP), and prefrontal cortex (PFC). Cortico-striatal loops are depicted in multicolor (orange-blue).

respond to gambles that guide economic decision making and integrate various factors that underlie value representations to influence choice (Lak et al., 2014, 2016; Stauffer et al., 2014, 2016). We recently tested the notion that transient DA value signals represent value and influence valuation during both positive and negative reinforcement (**Figure 3**) (Schelp et al., 2017; Pultorak et al., 2018; Oleson and Roberts, 2019). Positive reinforcement refers to an increase in behavior to receive an outcome (e.g., appetitive sugar pellet); negative reinforcement refers to an increase in behavior to avoid an outcome (e.g., electrical footshock). Using FSCV, we first demonstrated that the concentration of transient DA release events evoked by an appetitive sugar pellet or its conditioned predictor decreased with the price required to obtain it (**Figure 3A**) (Schelp et al., 2017). DA release events and behavioral output were measured as rats responded in a within-session behavioral economics-based operant task. In this task, the unit-price (responses/mg sugar) to obtain reinforcement increased in fixed epochs over the course of each session. As illustrated by the representative cumulative response records in **Figure 3B**, under these response contingencies lever pressing increases across the fixed epochs (as price increases) until a maximal price is reached at which the animal is no longer willing to pay the required opportunity cost to obtain reinforcement. We then used optogenetics to augment DA release and found that increasing DA release at the reward predictive stimulus rendered animals more sensitive to price and decreased DA concentration at reward delivery, consistent with a negative reward prediction error (Schelp et al., 2017). Optogenetics is a neuroscientific technique that allows the experimenter to transiently turn on/off a neural population of interest by activating genetically introduced light sensitive ion channels (i.e., opsins) with a laser (Vlasov et al., 2018). In comparison to this animal's baseline cumulative record (light orange line) increasing DA release at reward delivery (purple line) resulted in the animal paying a higher price to continue seeking sugar; whereas, increasing DA release at the reward predictive cue (dark orange line) resulted in the animal giving up at a lower price (**Figure 3B**). We then converted the behavioral data into demand curves by calculating total sugar in each epoch and plotting it against the corresponding unit-price. Demand curves

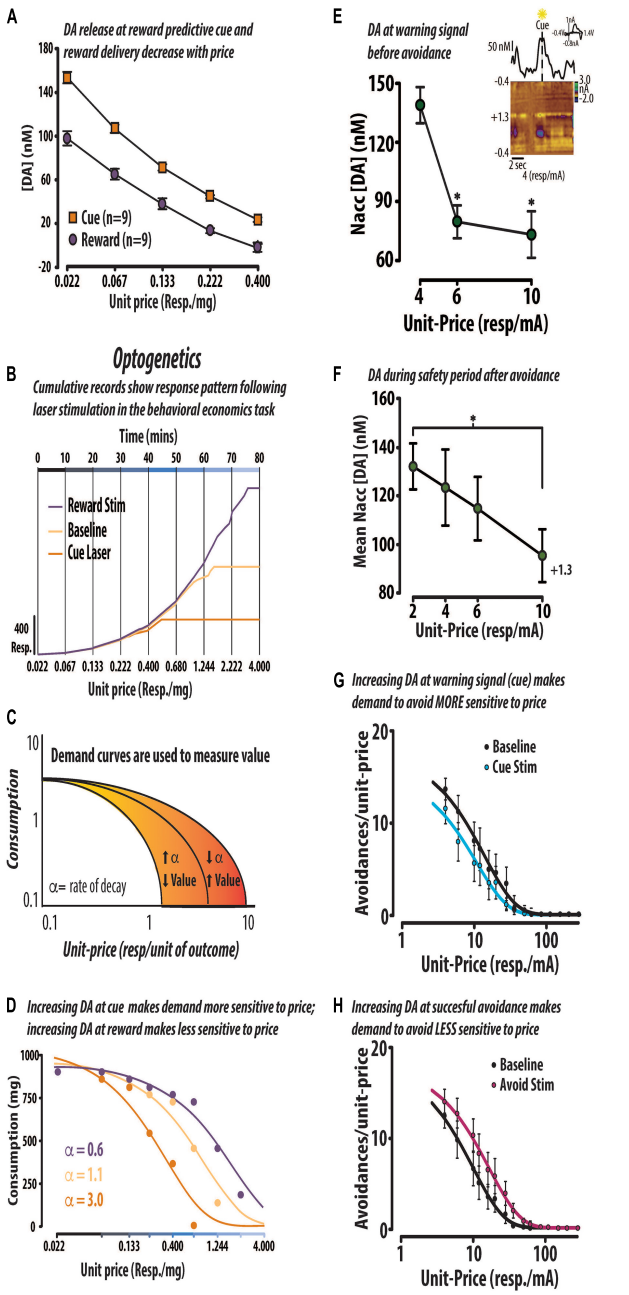

FIGURE 3 | DA value signals encode price and modify the maximal price rats will pay for positive or negative reinforcement. *Positive reinforcement:* DA (DA) concentration (mean ± SEM) evoked by a reward predictive cue and delivery of a 45 mg sugar pellet decreased across the first five prices in a within-session behavioral economics-based task. In this task, the unit-price (responses/mg sugar) increased across fixed epochs of time **(A)**. Optogenetic stimulation alters price sensitivity in a representative rat. Cumulative response records from one animal responding in the behavioral economic task under baseline conditions (light orange), and those in which DA release is amplified at cue presentation (dark orange) and at reward delivery (purple) **(B)**. Changes in value were assessed using demand curves which measure changes in consumption in response to changes in unit-price. We formally extracted a dependent measure of value (i.e., α) which, represents the rate at which demand curve decay. Demand decays at a faster rate when the animal

(Continued)

FIGURE 3 | Continued

becomes more sensitive to price. As the animal is willing to pay less for the commodity, we would interpret the resulting increase in α as a decrease in value **(C)**. The same data from the cumulative records in panel **(B)** are replotted in the form of demand curves to illustrate the optogenetic-induced shifts in value **(D)**. *Negative reinforcement:* The concentration of DA evoked by a warning signal that predicted the opportunity to avoid decreased with the price to avoid. Inset: Representative avoidance trial shows that DA concentration began increasing in anticipation of warning signal presentation **(E)**. The concentration of DA release events during the safety period decreased with price in trials in which the rat successfully avoided electrical foot shock **(F)**. Optogenetic activation of VTA DA neurons at the warning signal made animals more sensitive to price, consistent with a negative reward prediction error **(G)**. In contrast, optically stimulating DA neurons at successful avoidance made animals less sensitive to price, consistent with a positive reward prediction error **(H)**. Republished from Schelp et al. (2017) and Pultorak et al. (2018). *$p < 0.05$.

are a common tool used by economists to measure changes in valuation. If demand becomes more sensitive to price it is said to be more elastic, suggesting diminished value; if demand becomes less sensitive to price it is said to be more inelastic, suggesting enhanced value (**Figure 3C**). Replotting the same data from the aforementioned cumulative records revealed that enhancing DA release at cue presentation made demand for sugar more elastic, while enhancing DA release at reward delivery made demand for sugar more inelastic (**Figure 3D**). From these observations, we infer that valuation of the sugar pellet was decreased when the DA value signal was amplified at the reward predictive cue because the animal perceived that they received less than expected upon receiving the standard 45 mg sugar pellet. By contrast, an amplified DA value signal at the receipt of the 45 mg sugar pellet following a standard prediction might suggest to the animal that they received a better bargain than expected. Similar observations were observed during operant behavior maintained by the avoidance of electrical footshock (Wenzel et al., 2015; Pultorak et al., 2018). The concentration of DA release events—evoked by both a warning signal predicting the delivery of electrical footshock and by the successful avoidance of footshock—decreased with the price required to avoid it (**Figures 3E,F**) (Pultorak et al., 2018). Furthermore, optogenetically increasing DA release at the warning signal made the demand to avoid more sensitive to price (**Figure 3G**) whereas, increasing DA release at successful avoidance made demand for avoidance less sensitive to price (**Figure 3H**) (Pultorak et al., 2018). Taken together, these findings support the notion that transient DA signals can represent subjective value during both positive and negative reinforcement and causally modify reinforcement processes.

ECB Signaling Modulates DA Value Signals and Reinforcement Under a Fixed Ratio Schedule

Given the well-established role DA value signals play in reinforcement and motivating action (Schultz et al., 2015), we next began to question whether the brain's endogenous cannabinoid system capably modulates transient DA release events during goal-directed behavior. Inspiration for this research

question originated from early psychopharmacological studies. It was reported that disrupting eCB signaling by treating rats with CB1 antagonists reduced food seeking (Ward and Dykstra, 2005) and generally diminished the effects that conditioned stimuli exert over goal-directed behavior (Stiglick and Kalant, 1982; Le Foll and Goldberg, 2005; Ward et al., 2007). Thus, we first assessed whether treating rats with a CB1 antagonist reduced conditioned DA release events during positive reinforcement. Two reinforcers were assessed: brain stimulation reward and appetitive food. In the case of brain stimulation reward, rats responded for electrical currents delivered to the origin of the mesolimbic DA pathway—the VTA, under a FR1 schedule of reinforcement. The availability of reinforcement was signaled to the rat by a cue light placed above the lever, which began to function as a conditioned stimulus. Under these conditions, the concentration of DA value signals evoked by the cue light increased across trials as reinforcement was strengthened (Day et al., 2007; Oleson et al., 2012). Once DA value signals were determined to be stable, we intravenously treated rats with vehicle and then a CB1 antagonist (SR141716; AKA, rimonabant). In comparison to vehicle, systemic administration of the CB1 antagonist rimonabant significantly decreased the DA value signal while concurrently delaying reinforced responding (**Figure 4A**). Identical trends were found when we measured DA value signals while rats responded for 45 mg sugar pellets under a FR1 reinforcement schedule, demonstrating the reliability of these results during positive reinforcement (Oleson et al., 2012). And identical trends were found when we infused rimonabant directly into the VTA during brain stimulation reward (**Figure 4B**), suggesting that local eCB modulation of DA release in the midbrain is alone sufficient to modulate DA value signals and reward seeking. To assess whether increasing eCB signaling facilitates positive reinforcement, we then treated rats with an enzymatic inhibitor that prevents metabolic degradation. We focused on MAGL inhibitors because FAAH inhibitors failed to influence reinforcement in our initial studies (Oleson et al., 2012, 2014) and 2AG is thought to be the principle eCB that augments DA release by activating CB1s on GABA terminals (Covey et al., 2017). We replicated our aforementioned approach by intravenously administered the MAGL inhibitor JZL184 while rats responded for brain stimulation reward under a FR1 schedule during ongoing FSCV measurements of DA value signals. In contrast to rimonabant, intravenous JZL184 amplified DA value signals while concurrently reducing response latencies (**Figure 4C**). The same trends were observed when JZL184 was infused directly in the VTA (**Figure 4D**). Using a new and improved iteration of MAGL inhibitor called MJN110, the Bass lab recently replicated these findings by demonstrating increasing 2AG facilitates cue-motivated reward seeking (Feja et al., 2020). To determine whether eCBs modulate DA value signals during negative reinforcement we also assessed whether systemic administration of a CB1 antagonist influences DA value signals during avoidance. Using a signaled active avoidance operant approach, we treated rats with the CB1 antagonist rimonabant while conducting FSCV. Avoidance was maintained under a FR1 schedule. A warning signal was provided 2s prior to the occurrence of electrical foot shock by illuminating a cue light placed directly above the lever. In comparison to vehicle treatment, intravenous rimonabant significantly decreased DA release time-locked to the warning signal while concurrently decreasing avoidance (**Figure 4E**) (Wenzel et al., 2018). We next sought to assess whether 2AG manipulations specifically modify the influence of DA value signals on negative reinforcement. To do this we administered microinfusions of either vehicle or tetrahydrolipstatin (THL) into the VTA of rats. THL is a potent inhibitor of the synthetic enzyme responsible for generating 2AG, DAGL (Ortar et al., 2008). As predicted, intrategmental THL significantly reduced avoidance and 2AG tissue content in comparison to vehicle treated rats (**Figure 4F**) (Wenzel et al., 2018). Finally, we used optogenetics to stimulate DA neurons during avoidance and found that restoring DA value signals in the presence of THL was sufficient to rescue avoidance (Wenzel et al., 2018). Together, these findings suggest that the eCB 2AG facilitates cue-motivated action by amplifying DA value signals originating from the VTA (**Figure 4G**) (Oleson and Cheer, 2014; Covey et al., 2017; Wenzel et al., 2018; Peters et al., 2021). These 2AG-modulated patterns of DA release and behavior are apparent during both positive and negative reinforcement when a conditioned stimulus signals the availability of a goal-directed outcome and reinforcement is available under a FR schedule.

Increasing Cannabinoids Amplifies Temporally Engendered Patterns of DA Release and Accelerates Responding Under a Fixed Interval Schedule of Reinforcement

Whereas FR schedules engender a bimodal response pattern consisting of recurring response-pause successions, the FI schedule engenders a scalloped response pattern. Rather than receiving reinforcement after meeting a fixed response requirement, on a FI schedule, reinforcement occurs at the end of a defined period of time. The lever does not retract during the interval, allowing the experimenter to observe the emergence of a scalloped temporal response pattern using a cumulative response recorder (**Figure 5A**). The scalloped response pattern results from the animal's lever pressing accelerating across the interval until a maximum terminal rate is reached at the interval terminus (Ferster and Skinner, 1957). In addition to engendering a unique pattern of behavior relative to the FR schedule, the FI schedule also produces a unique pattern of accumbal DA release. As illustrated in **Figure 5C**, a first peak of DA release can be observed when reinforcement (an appetitive food pellet) is delivered. After a brief pause in release, DA concentration then begins to rise with the onset of the FI before gradually decaying over its duration (Oleson and Cheer, 2014; Oleson et al., 2014). As DA concentration is inversely related to local response rate, we infer that under the contingencies of a FI schedule, DA concentration represents the primary interoceptive cue driving reinforcement: time (Oleson et al., 2014; Everett et al., 2020). To investigate how cannabinoids alter both the patterns of behavior and DA release engendered by the FI schedule, we applied FSCV while treating mice with the cannabinoid agonist WIN as they responded for appetitive food pellets (Oleson et al., 2014). To analyze

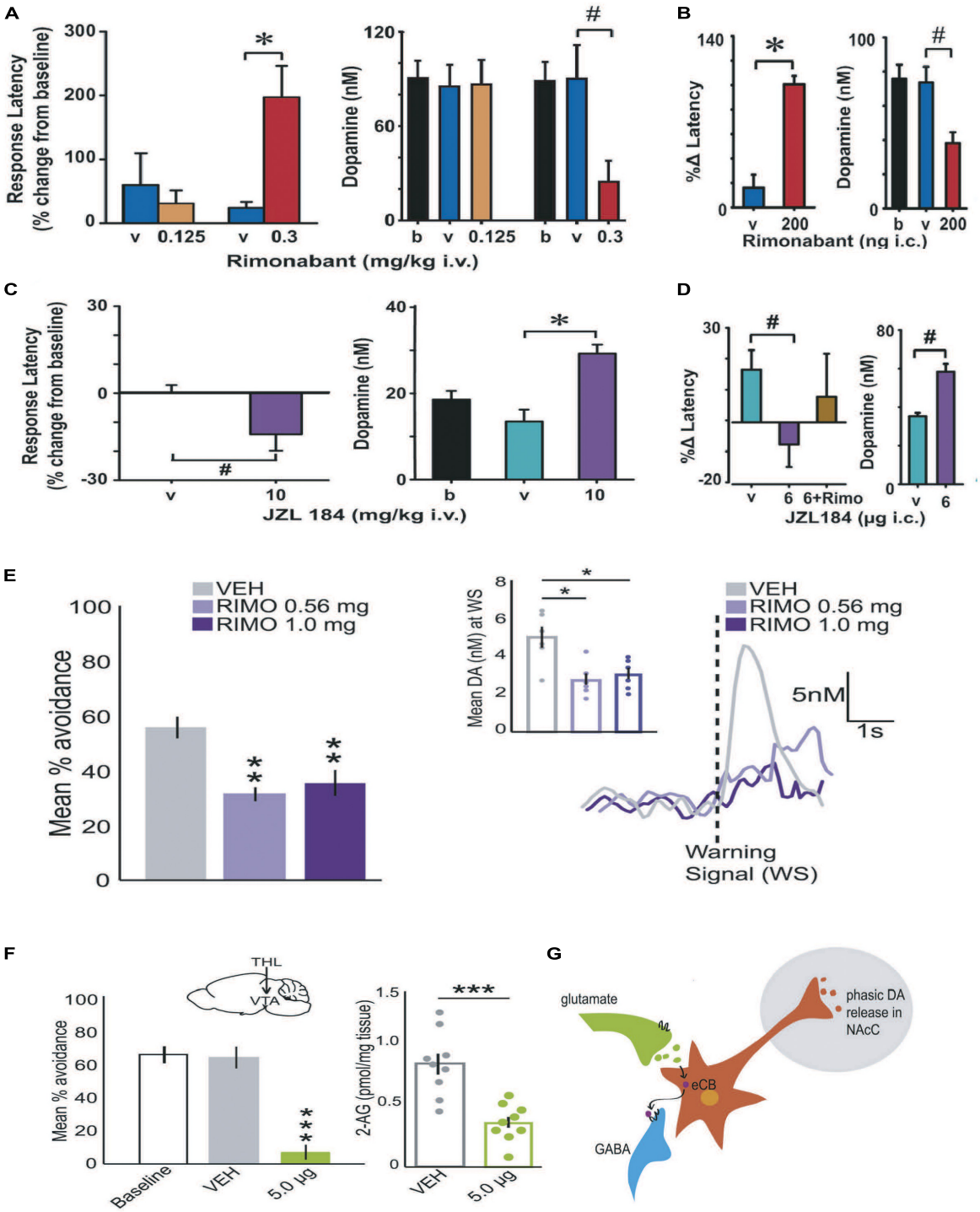

FIGURE 4 | Cannabinoids modulate DA value signals during positive and negative reinforcement maintained under an FR schedule. *Positive reinforcement:* Systemically treating (intravenous; IV) rats with the cannabinoid receptor antagonist rimonabant increased the latency to respond for brain stimulation reward and decreased the concentration of cue-evoked DA value signals **(A).** Intragemental infusions (IC) of rimonabant recapitulated these effects on reward seeking and DA release, demonstrating that eCB modulation of DA neural activity in the VTA is alone sufficient to modulate DA release and positive reinforcement **(B)**. Systemically increasing 2AG levels by pre-treating rats with JZL184 (IV) reduced the latency to respond for brain stimulation reward and increased the concentration of cue-evoked DA value signals **(C)**. Intragemental infusions (IC) of JZL184 recapitulated these effects, suggesting that the action of 2AG in the VTA is alone sufficient to modulate DA release and positive reinforcement **(D)**. *Negative reinforcement:* Systemic rimonabant administration (IV) reduced the number of successful avoidance responses and the concentration of DA evoked by the warning signal **(E)**. Inhibiting DAGL-induced synthesis of 2AG by infusing THL into the VTA decreased avoidance and reduced 2AG tissue content in the VTA **(F)**. Taken together, these observations generally support a DSI-model of 2AG-modulation of DA value signals **(G)** during positive and negative reinforcement maintained under an FR schedule. Republished from Oleson et al. (2012) and Wenzel et al. (2018). # < 0.05; **$p < 0.001$; ***$p \leq 0.001$.

how responding changed across the interval, we first calculated rate/terminal rate values by dividing the local response rate into five fixed epochs and then dividing each by the terminal rate (i.e., the maximal local response rate in the final epoch). We found that WIN accelerated local response rates across the interval in a dose- and CB1-dependent manner (**Figure 5B**). Similarly, WIN dose-dependently increased DA concentration across the duration of the interval in a CB1-dependent manner (**Figure 5C**). We also performed a more refined behavioral analysis by assessing the index of curvature of individual scalloped response patterns (**Figure 5D**) (Fry et al., 1960; Narayanan et al., 2012). Using the index of curvature analysis, a negative index of curvature is detected when the animal's scalloped response pattern accelerates prematurely; thereby suggesting that timing behavior is accelerated. By contrast, a slower acceleration of responding across the interval produces a positive index of curvature, suggesting that timing behavior is slowed (Fry et al., 1960; Narayanan et al., 2012). This additional analysis confirmed that WIN accelerated the timing of reinforced responding under a FI schedule while concurrently accelerating the temporally engendered pattern of DA release (**Figure 5E**). We then treated mice with enzymatic inhibitors to investigate whether specifically increasing the eCBs 2AG or anandamide modulate the scalloped response pattern observed during fixed interval reinforcement. We found that systemic treatment with the MAGL inhibitor JZL184, but not the FAAH inhibitor URB597 accelerated the temporal response pattern similarly to WIN (cf. **Figures 5F,G,H** vs. **E,B**) (Oleson et al., 2014). These data suggest that the eCB 2AG modulates goal-directed action under a variety of contingencies, including periodically reinforced behavior.

ENDOCANNABINOIDS, EXPLORATORY/ADJUNCTIVE BEHAVIOR, AND HABITS

Endocannabinoids and Exploratory/Adjunctive Behavior From an Ethological Perspective

We next consider these observations from a phylogenetic and ethological perspective. If cannabinoids amplify patterns of DA release and accelerate timing behavior under conditions of fixed periodic reinforcement, it is possible that they contribute to motivational switching in response to changing environmental conditions. After waiting a lengthy period of time for a primary food source, it may become advantageous to switch from seeking the desired option to foraging for alternative options. In the operant chamber, these foraging-like actions can be noted as reinforcement-irrelevant, or adjunctive behaviors (Falk, 1971, 1977). One proposed way to quantify adjunctive behavior in the operant chamber is the measure responding on a secondary inactive lever (Killeen and Fetterman, 1988). To assess whether cannabinoids influence adjunctive behavior, we reanalyzed the FI data and found that increasing 2AG using the MAGL inhibitor JZL184 (**Figure 5I**) or antagonizing CB1 with AM251 (Oleson et al., 2014) significantly reduced inactive lever presses. We interpret these findings to suggest that a basal eCB tone and a moderate concentration of accumbal DA provide the sweet spot of intermittency necessary to switch an animal's incentive to obtain a primary goal (e.g., food) to the pursuit of alternative options (e.g., foraging for an alternative food source) (Oleson et al., 2014). Additionally, sudden increases or decreases in eCB signaling can lead to perseverative goal seeking. In agreement with this supposition, cannabinoids have been reported to promote perseverative action and infiexibility (Hill et al., 2006; Jiao et al., 2011).

The Variable Interval Schedule and Habit Formation

Recent studies utilizing the variable interval (VI) schedule demonstrate that eCBs are critically involved in habit formation. As previously described, when reinforcement is delivered in fixed intervals, the animal learns to time the interval and accelerate their responding toward its culmination. By contrast, under a VI schedule, responding is reinforced after a random period of time has elapsed since the first response. Under these conditions, the cumulative response pattern is maintained at a high, constant rate—presumably because the animal is uncertain about the time of reward availability (Ferster and Skinner, 1957) (**Figure 2A**). In comparison to ratio schedules or the FI schedule, the VI schedule is known to produce habitual behavior (DeRusso et al., 2010). To determine if a behavior is habitual rather than goal-directed, experimenters determine if the instrumental action is driven by a valued outcome or devoid of its consequences (**Figure 2B**). To characterize and parse the purpose of action, Adams and Dickinson developed what is known as the devaluation test (Adams and Dickinson, 1981). After training an animal to respond for what was originally a valued outcome, the outcome is then devalued. In the case of food-maintained responding, the animal is either over-fed or subjected to food poisoning. If the animal's responding is significantly affected by devaluation, it is inferred that action is still directed toward a valued goal; however, if the animal's responding is insensitive to devaluation, it is inferred that action has become habitual. In the latter scenario, the habitual behavior is believed to be unresponsive to changes in outcome value and the contingency between action and outcome (Dickinson and Balleine, 1994).

eCBs May Be Involved in Habit Formation

Growing evidence suggests that eCB signaling is crucial for habit formation, although the precise roles each eCB play in habit formation and whether these roles differ at distinct loci in the brain remains to be determined. Hilário et al. (2007) first demonstrated a role for eCBs in habit formation. First, these authors confirmed that a history of responding under a VI-reinforcement schedule is particularly suited for establishing habitual responding. After providing mice with a history of responding for a sugar solution under either a VI schedule or a variable-ratio (i.e., VR) schedule, they were tested in a devaluation test. In this test all mice were given access to either a sugar solution (i.e., reinforcer from operant training) or

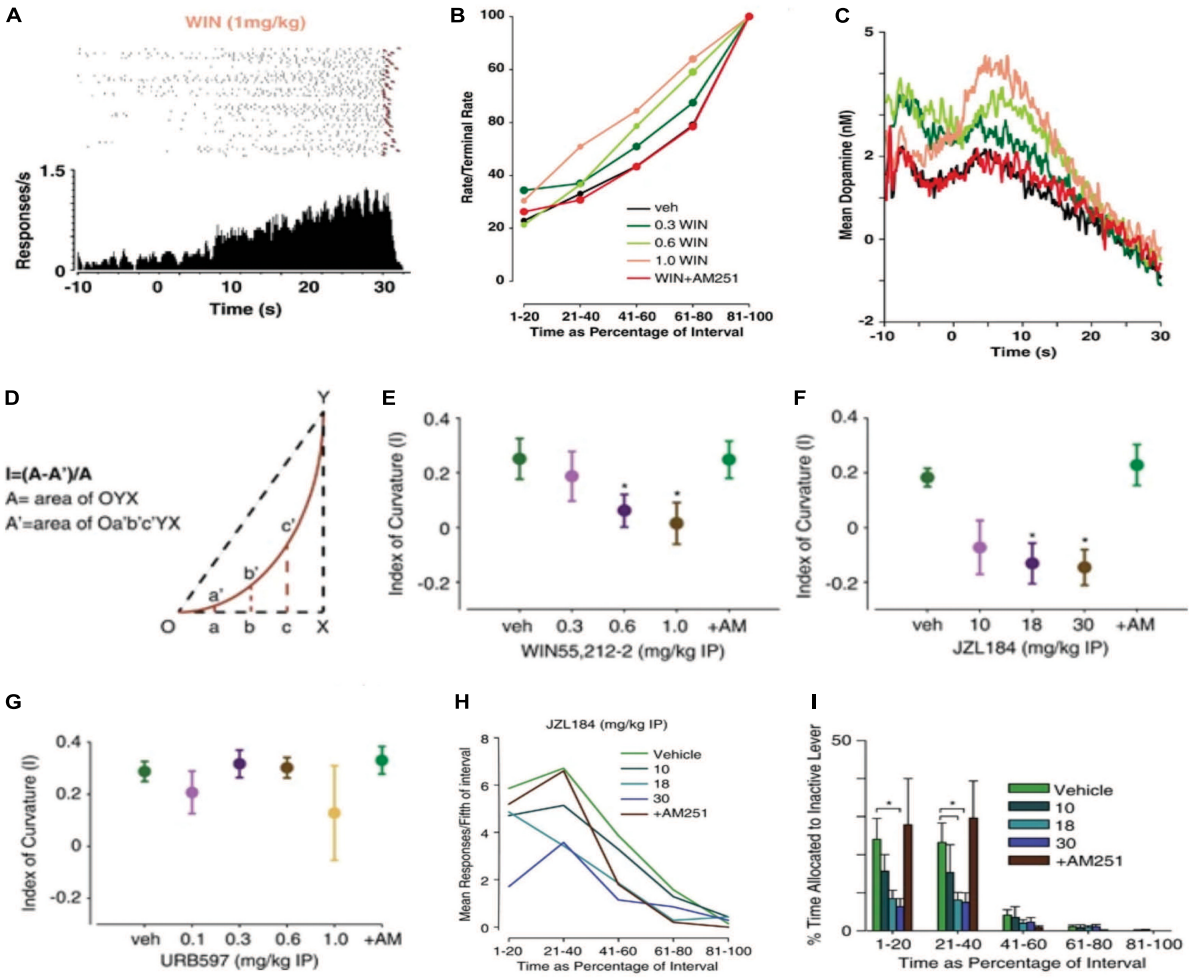

FIGURE 5 | Cannabinoids modulate a temporally engendered pattern DA release during reinforcement maintained under an FI schedule and adjunctive behavior. An illustrative cumulative response pattern (top: raster plots; bottom: corresponding peri-event histograms) of a WIN-treated mouse responding for food reinforcement under a FI schedule. The pattern of lever pressing lawfully increases in the FI task to form a scalloped response pattern. The raster plot shows responses (black ticks) preceding food reinforcement (red triangle) across the 30 s interval. All trials are shown in chronological order as they occurred in a representative experimental session. The peri-event histogram shows the summation of responding under each corresponding raster plot. **(A)**. WIN 55,212-2 accelerated the timing of scallop response pattern in a dose- and CB1-dependent manner. Mean behavioral response patterns following cannabinoid administration are plotted as a function of the interval duration **(B)**. WIN amplified a temporally engendered pattern of DA release in a dose- and CB1-dependent manner. Mean DA concentration traces for each drug treatment conditions are plotted as a function of the interval **(C)**. Cannabinoid-induced changes in interval timing were quantified by assessing the index of curvature—a computational measure of the extent and direction of change in the temporal response pattern produced by the FI schedule **(D)**. WIN produced a negative index of curvature, suggesting an acceleration of timing behavior **(E)**. Increasing 2AG with JZL184, but not increasing anandamide with URB597, accelerated interval timing **(F,G)**. eCB-induced changes in reinforcement irrelevant or adjunctive behavior were assessed by quantifying responses on an inactive lever. Mean responses on the inactive lever initially increase before declining through the interval **(H)**. JZL184 significantly decreased the percentage of time spent responding on the inactive lever, suggesting that adjunctive behavior was reduced by elevating 2AG levels **(I)**. These data show that cannabinoids module periodically reinforced behavior and DA release under an FI schedule and, might suggest that a delicate balance of 2AG and DA release are necessary to produce the sweet-spot of intermittency that produces adjunctive behavior. Reproduced from Oleson et al. (2014).

standard chow (home cage food) for 1hr preceding an extinction session. During the extinction session, mice were given access to the sugar-paired lever used in operant training; however, no scheduled consequences occurred when it was pressed. As predicted, they found that a history of responding under VI schedule, but not the VR schedule, resulted in sugar-sated mice persevering in their responses on the sugar-paired lever. The authors also conducted a separate exploration test in which mice were given access to the previously active lever and a novel level. They found that in comparison to mice with a history under the VR schedule, mice with a history of responding under the VI schedule were more likely to engage with it. To test the effects of eCB signaling, the authors replicated their experimental approach using CB1 mutant mice and their wild-type littermates. After a history of responding for sugar under a VI schedule, wild-type (WT), heterozygous $CB^{+/-}$ (HET) and homozygous $CB^{+/-}$ (HO) mice were given access to the regularly active lever in either a sugar-sated or non-sated state. As shown in **Figure 6A**,

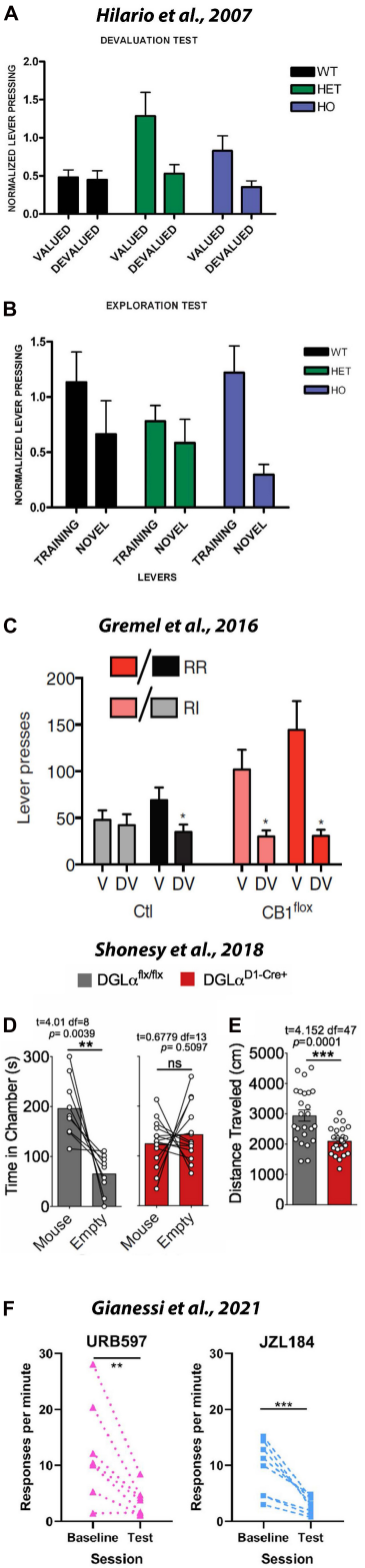

FIGURE 6 | CB1s are necessary for habit formation and adjunctive/exploratory behavior. To investigate the role of eCBs in habit formation and adjunctive behavior WT, CB1$^{+/-}$, and CB1$^{-/-}$ were trained on *(Continued)*

FIGURE 6 | Continued
a variable interval schedule and then tested in devaluation and exploration tests. **(A)** Normalized lever pressing during the valued versus the devalued condition for WT, CB1$^{+/-}$, and CB1$^{-/-}$ mice. CB1 mutants showed sensitivity to sensory-specific satiety, suggesting that their actions were goal-directed rather than habitual. These data suggest that the CB1 and eCBs are necessary for habit formation. **(B)** Lever pressing (normalized) on the training lever versus a novel lever in WT, CB1$^{+/-}$, and CB1$^{-/-}$ mice. Relative to other groups, CB1$^{-/-}$ mice responded less on the novel lever, suggesting that the CB1 and eCBs may be involved in adjunctive behavior. (Republished from Hilário et al., 2007). **(C)** Graph shows responses in the valued (V) and devalued (DV) states in RI and RR training contexts. RR, random ratio (aka FR); RI, random interval (aka VI). During outcome devaluation procedures, control mice showed reduced lever pressing in the devalued state in the RR context but not the RI context. However, mice that lacked CB1s on OFC projection neurons into the striatum responded less in the devalued state under both RR and RI conditions. These data suggest that CB1s in cortical-striatal loops are necessary for habit formation (Republished from Gremel et al., 2016). Mice lacking the enzyme for the synthesis of 2AG from D1 MSNs (D1-Cre+) showed decreased exploration of a novel conspecific **(D)** and a novel environment **(E)**. These data suggest that 2AG in the striatum plays an important role during adjunctive behavior (Republished from Shonesy et al., 2018). **(F)** Surprisingly, blocking metabolism of AEA with URB597 and 2AG with JZL184 disrupted rather than promote habit formation. These latter findings might suggest that AEA and 2AG are not important in habit formation or that non-specific behavioral effects (e.g., increased motivation for food) can confound tests of habitual behavior. $^{**}p < 0.01$; $^{***}p \leq 0.001$.

devaluing the sugar solution failed to affect responding on the previously sugar-paired lever. This finding supports the notion that a history of responding under the VI schedule produces habitual responding. However, both the HET and HO groups CB1 mutant mice showed sensitivity to sugar devaluation. As evidenced by the green and blue bars, providing *ad libitum* access to the sugar solution before the devaluation test resulted in both CB1 mutant groups responding less in the sated state, suggesting that habit formation is impaired in CB1 mutant mice. As illustrated in **Figure 6B**, in the exploration test they found that HO mice, but not HET or WT mice, failed to explore the novel level. Taken together, these data suggest that CB1 signaling may play an important role in habit formation and exploring novel options. These data are in agreement with our aforementioned finding that pretreating mice with AM251 reduced inactive lever responses; although, it remains unclear why increasing 2AG levels with JZL184 also reduced adjunctive, or exploratory behavior. One likely possibility is that systemically increasing 2AG produces an array of physiological and behavioral effects at different levels of distinct neural networks. By harnessing recent technical advances, investigators are beginning to target specific cellular populations and neural circuits responsible for habit formation, but many additional studies are required to completely understand the mechanisms involve.

A Brief Introduction to the Nigrostriatal Habit Circuitry

The majority of aforementioned DA studies measured its release in the primary terminal field of the mesocorticolimbic pathway (**Figure 2C**), the NAc. The NAc is typically thought of as a Pavlovian-motor interface that guides model-based goal-directed

actions (Yin and Knowlton, 2006; Bornstein and Daw, 2011)—including reward seeking, conditioned active avoidance, and periodically reinforced behavior. In contrast, habits are thought to mediated by interactions between the dorsal striatum and the cortex, or cortico-striatal loops (**Figure 2C**). The dorsal striatum is often divided into the dorsomedial (caudate in primates) and the dorsolateral (putamen in primates) striatum (Yin and Knowlton, 2006). The dorsomedial striatum is thought to guide model-based, goal-directed actions using environmental rules that dictate the contingencies of reinforcement. By contrast, the dorsolateral striatum is thought to play a role in guiding model-free, habitual action using previously learned associations (Yin and Knowlton, 2006; Lee et al., 2014). This form of model-free habitual action is thought to arise from sensorimotor loops that can be modified by eCB signaling and DA release at the level of the dorsal striatum. Whereas the NAc receives DAergic input from mesocorticolimbic pathway originating in the VTA, the dorsal striatum primarily receives DAergic input from the nigrostriatal pathway originating in the substantia nigra pars compacta (**Figure 2C**).

eCB Modulation of Cortico-Striatal Input Into the Dorsal Striatum Gates Goal-Directed and Habitual Behavior

Gremel et al. (2016) provided incisive insight into the role eCBs might play in orbitostriatal input into the dorsal striatum. The orbitofrontal cortex (OFC) is thought to contribute to cortico-striatal loops that may gate behavior between being goal-directed and habitual. Using viral technology to selectively knock-out CB1 from OFC neurons, they first demonstrated that OFC neurons projecting to the dorsomedial striatum exhibit greater activity during goal-directed behavior in a VR than in a VI task. Similar to Hilario, they further confirmed that VI training produced more habitual responding than VR training. They then used a retrograde virus and chemogenetics to selectively inhibit OFC projections into the dorsomedial striatum. While control mice reduced responding on both the VR and VI task when in the devalued (i.e., sugar-sated) state, chemogenetic inhibition of OFC input into the dorsomedial striatum did not reduce responding in either the VR or VI task (**Figure 6C**). To better assess the local contribution of OFC input in the dorsomedial striatum, they repeated their experiment but injected the clozapine-n-oxide used to induce chemogenetic suppression directly into the dorsal striatum rather than into the intraperitoneal space. They found that handling the WT mice during microinfusions abolished habitual responding; specifically, mice only responded on the sucrose-paired lever when in a sucrose-sated, or devalued state. However, when the microinfusion produced chemogenetic inhibition of OFC input into the dorsal striatum, responding on the previously sucrose-paired lever persisted in-spite of sucrose devaluation. Taken together, these data suggest that eCB-modulation of OFC input into the dorsal striatum might gate behavior between dorsomedial-mediated goal-directed behavior and dorsolateral-mediated habitual behavior. Future studies are necessary to clarify the specific roles distinct eCBs play in modulating behavior; the specific eCBs involved, the circuit they are acting in, and the specific cell-type they are acting on are all important variables to consider in future studies.

2AG From D1-Expressing MSNs Mediates Exploratory Behavior and Perseverative Responding

To provide cell-type and eCB specific data, Shonesy et al. (2018) investigated the effects of conditionally knocking down the primary synthetic enzyme of 2AG (i.e., DAGL) from striatal MSNs. The majority of MSNs in the dorsal striatum can be segregated into one of two populations. The D1-expressing neurons of the direct pathway are thought to promote action during reinforcement, whereas the D2-expressing neurons of the indirect pathway are thought to inhibit action during reinforcement (Kravitz et al., 2012). Shonesy et al. (2018) found that knocking down DAGL from the D2-expressing neurons of the indirect pathway failed to influence any of the behavioral outcomes they assessed. In contrast, they found that removing 2AG signaling from D1-expressing neurons of the direct pathway produced distinct behavioral effects depending on whether the conditional knock down occurred in the dorsal or ventral striatum. Specifically, they found that removing 2AG from dorsal striatal D1-containing MSNs reduced both social (**Figure 6D**) and spatial (**Figure 6E**) exploration of novelty. However, it should be noted that repetitive grooming occurred following removal of 2AG signaling from the ventral rather than the dorsal striatum. The authors also found that removing 2AG signaling from MSNs failed to influence operant behavior maintained under either fixed-ratio or progressive-ratio schedule. These paradoxical behavioral findings notwithstanding, Shonesy et al. (2018) also provided important information regarding the role of 2AG in modulating synaptic plasticity in striatal circuits. Using electrophysiology, they found that removing 2AG signaling from D1-MSNs reduced feedback inhibition at both glutamatergic and GABAergic MSN synapses and increased basal glutamatergic release onto D1-MSNs. Specifically, they found that KO of DAGL from D1-MSNs significantly increased the frequency of their excitatory post-synaptic currents, suggesting an impairment of eCB mediated feedback inhibition on glutamate release (i.e., DSE). They also found that the KO of DAGL from D1-MSNs impaired DSI at these cells arising from GABAergic synapses, although GABAergic transmission was determined to be unchanged. Overall, these data suggest DAGL-KO from D1-MSNs excite dMSNs due to a loss of DSE. When this breakdown in feedback regulation occurs in the dorsal striatum, exploration of social and spatial novelty are impaired; when this breakdown occurs in the ventral striatum, perseverative grooming behavior is observed.

Surprising Findings and Important Considerations Regarding the Overlap Between Measures of Appetitive Goal-Seeking and Habitual Responding

While the aforementioned studies offer compelling evidence that increases in 2AG within the dorsal striatum act on CB1s

to modulate habitual behavior, a recent study by Gianessi et al. (2021), suggest there is considerable nuance to this story that still needs to be considered. These investigators trained mice to respond for sucrose-sweetened grain pellets under a VI schedule and then tested for habitual responding using contingency degradation. As opposed to sating the mice with *ad libitum* sugar, the reinforcement contingency was degraded by allowing the animal to respond as if in the VI-task but lever presses resulted in no programmed consequence. Rather, reinforcers were delivered at equal intervals, matching the total number of reinforcers earned the previous day. The FAAH inhibitor URB597 was administered to test the effects of elevated AEA levels on habitual responding; the MAGL inhibitor JZL184 was administered to test the effects of elevated 2AG levels on habitual responding. Contrary to their predictions, they found that both drugs reduced responding during the test day following contingency degradation (**Figure 6F**). These findings paradoxically suggest that elevating neither anandamide nor 2AG strengthens habitual responding. Furthermore, they demonstrated that the effects of the CB1 antagonist/inverse agonist AM251 on habitual responding varied depending on the vehicle used and the relative time of drug pre-treatment. The authors first note that solubility of prepared cannabinoid solutions varies greatly across labs because these lipophilic compounds are not easily dissolved in water. They demonstrate that dissolving AM251 in a mixture of DMSO and TWEEN produced dose-dependent reductions in operant responding, but dissolving AM251 in DMSO alone did not. Thus, it is important to note differences in vehicle and drug preparation may drastically impact bioavailability when comparing cannabinoid studies. For example, while the Gianessi et al., 2021 study reported that 1 mg/kg AM251 reduced operant responding, the Hilario study reported that neither 3 nor 6 mg/kg AM251 did. Perhaps more importantly, Gianessi et al., 2019., also found that the timing of habitual testing relative to drug-treatment is important to consider during experimental design. When they assessed for habitual behavior immediately after a series of AM251 treatments, they observed a significant increase in responding. However, when they assessed for habitual behavior after allowing for AM251 to clear the system, responding was found to be decreased. From this observation, and their finding that AM251 reduced responding for sugar pellets, the authors concluded that mice increased responding after the series of AM251 treatments because they had not been reaching satiety across the VI training sessions and were therefore showing an increase in goal-directed appetitive behavior rather than habitual responding during the first contingency degradation session. It is also worth noting that this group also reported that when administered alone, JZL184 does not alter the expression of food habits (Gianessi et al., 2019) or alcohol habits (Gianessi et al., 2020). However, in the latter study Gianessi et al. (2020) did find that JZL184 increased motivation for food as assessed using a progressive ratio schedule. Thus, while compelling evidence suggests that 2AG may be important in gating goal-directed to habitual action, many more studies are required to reconcile the nodes of the neural circuitry involved, the role of specific receptors and cell-types being acted upon within each node, and the contributions of distinct eCBs. Furthermore, the potential confound of CB1-mediated changes in appetitive behavior on habitual testing underscores the importance of concurrently considering the literature on eCB-modulation of appetitive behaviors, habitual responding, and attentional processes.

Transition From Reinforcement to Attentional Processes

The manifestation of motivationally switching from a primary reinforcer to an alternative outcome and habit-formation likely involve the additional recruitment of attentional processes. And, when considering the neural substrates involved in motivational switching, it became readily apparent that this circuitry often overlaps with the neural substrates of attention (e.g., OFC-dorsal striatum) (O'Hare et al., 2018). Furthermore, mesocorticolimbic DA signaling is believed to modulate value-driven goal-directed action, habit formation, and attentional processes. Thus, we next turn the focus of our review to the seemingly intertwined literature on cannabinoid and DAergic modulation of attentional processes.

CANNABINOIDS AND DA BOTH MODULATE ATTENTIONAL PROCESSES AS WELL

Introduction to the Study of Attention and Attentional Processes

The concept of attention has long historical roots in psychology and bears several definitions. While modern terminology surrounding attention may refer to disparate concepts such as arousal, vigilance, and distractibility, it may be broadly defined as selective activation of neural representations during information processing. Through this definition, attention may be best illustrated in relation to the highly related process of working memory. Whereas attention uploads information 'on-line' at any discrete timepoint, working memory stores and utilizes these activated representations during recall across small spans of time (Baddeley, 1986; Cowan, 1993; McElree, 2001; Oberauer, 2019). While attention has different aspects or components associated with it, including its most fundamental sensory-based component involuntarily elicited in response to salient environmental stimuli, the behavioral paradigms referenced below generally focus on attentional control. An executive function, attentional control incorporates top-down regulation of bottom-up sensory driven attentional processes to subserve appropriate attendance toward behaviorally relevant stimuli (Posner and Petersen, 1990; Cohen et al., 1993; Hopfinger et al., 2000; Fan et al., 2002). Proper allocation of attention within complex, changing environments is an evolutionarily conserved trait crucial for effective information processing (Matzel and Kolata, 2010; Chun et al., 2011), allowing an animal's behavior to be adaptively modified by external contingencies in order to successfully engage in signal detection and goal-directed decision making (Broadbent and Gregory, 1963; Endler, 1992;

Verghese, 2001; Smith and Ratcliff, 2009; Asplund et al., 2010; Voloh et al., 2015). So, dysfunctions in attention weaken an individual's ability to allocate cognitive resources effectively to the task at hand. Therefore, deficits in attentional control are potential barriers to adaptive behavior and overall survivability of the organism, with pathologies affecting this executive function leading to maladaptive traits that negatively impact quality of life (Baddeley et al., 2001; Rueda et al., 2004; Williams-Gray et al., 2008; Burgess et al., 2010; Fajkowska and Derryberry, 2010; Schoorl et al., 2014; Stefanopoulou et al., 2014; Heeren and McNally, 2016).

Cortical Regulation of Attentional Control

Although the neuroanatomical loci of attention are many and work as an integrated network of multiple brain regions, attentional control is largely mediated by cortical regions. Spatial and visual attentional control, for instance, have been evidenced to be strongly regulated by frontoparietal regions that filter sensory information in a top-down fashion, with injury to these areas resulting in spatial neglect despite intact bottom-up, sensory-driven networks (Jeannerod, 1987; Karnath et al., 2001; Mort et al., 2003; Corbetta et al., 2005; Fiebelkorn et al., 2018). In terms of attentional command and action selection, the PFC and OFC have been shown to mediate selective attentional control during cognitive tasks, with the PFC regulating attentional focus during interference (Milham et al., 2001), redirection of attention based on task demands (Rossi et al., 2007), and attentional shifting across perceptual features (Owen et al., 1991; Birrell and Brown, 2000; Liston et al., 2009), while the OFC primarily serves redirecting attention during reinforcement switching within reversal learning (Hampshire and Owen, 2006). As the PFC is fundamental to cognitive control in general and regulates working memory, decision making, and other processes crucial to goal-directed behavior (Fuster, 2015), its involvement in attentional processes is perhaps self-evident. The OFC, on the other hand, has a more indirect relationship to attention as it is more associated with value encoding and behavioral inhibition (Teitelbaum, 1964; Gallagher et al., 1999; Izquierdo et al., 2004; Kim and Ragozzino, 2005; Jonker et al., 2015). Nonetheless, attention-based modulation of value encoding in the OFC has been recently supported, leaving an interesting role for the OFC in value-based decision making that may be under the control of attentional focus (Xie et al., 2018).

DA and eCB Regulation of Cortical Function

The multifaceted cortical functions of cognitive control are tightly regulated by both intra- and intercortical activity states mediated greatly by pyramidal cells, the principal neurons of the cortex. Far from being self-contained, pyramidal cell activity is impinged by numerous signaling molecules, including DA, which is projected in the cortex by rich innervations arising from the VTA (Lewis et al., 1986). DA regulates pyramidal cell function through numerous ways to primarily modulate glutamatergic and GABAergic signaling in the cortex (Law-Tho et al., 1994; Zheng et al., 1999; Gao et al., 2001; Seamans et al., 2001a; Flores-Hernandez et al., 2002; Gao and Goldman-Rakic, 2003; Wang et al., 2003; Beazely et al., 2006; Liu et al., 2006; Onn et al., 2006; Li et al., 2009; Hu et al., 2010; Tritsch and Sabatini, 2012). Overall DA has a dampening effect on excitatory transmission in the PFC through a presynaptic mechanism, reducing the probability of glutamate release (Gao et al., 2001). DA also modulates inhibitory signaling in the PFC, biphasically altering inhibition of pyramidal cells via G_i-coupled D2 DA receptor activation on presynaptic GABA cells and a complex interplay between signaling of postsynaptic pyramidal cell DA receptors D1 (G_s-coupled), D2, and D4 (Gi-coupled) (Seamans et al., 2001b; Wang et al., 2002; Trantham-Davidson et al., 2004). Pyramidal cell activity is modulated by DA in more direct ways too; postsynaptic mechanisms of intrinsic excitability have been shown to be adjusted in rats by VTA DA projections that modify spike frequency adaption and afterhyperpolarization potentials in the PFC (Buchta et al., 2017).

Cortical function is also mediated by eCB signaling. In the PFC, CB1 expression has been found to be preferential to GABAergic presynaptic terminals adjacent to glutamatergic ones, both synapsing onto dendrites of mGluR5-containing pyramidal cells. This places CB1 in a position to integrate and balance excitatory and inhibitory signaling during activity-dependent eCB mobilization (Fitzgerald et al., 2019). This mGluR5-mediated integration of PFC pyramidal signaling may take place post-synaptically to directly increase pyramidal cell excitability and synaptic drive, or pre-synaptically as this ligand gated Gq protein-coupled receptor is capable of stimulating eCB production to induce DSI-mediated disinhibition of pyramidal cells via CB1 signaling (Kiritoshi et al., 2013). eCB signaling may also simultaneously modulate glutamate and DA in the PFC as systemic administration of the CB1 agonist WIN has been shown to increase transmission of both within this region (Polissidis et al., 2013). Furthermore, intra-PFC WIN administration induces bi-phasic functional effects in VTA DA cell activity, with low doses increasing and high doses decreasing spontaneous DA cell firing (Draycott et al., 2014). Although less characterized, eCB signaling within the OFC influences pyramidal function too. Similar to the PFC, postsynaptic mGluR5 activation has been shown to increase local eCB release and enhance CB1 signaling within GABAergic presynaptic terminals of the OFC (Lau et al., 2020). Interestingly, in the lateral aspect of the OFC, impaired astrocytic glutamate transport has been found to result in aberrant eCB tone and subsequent LTD of inhibition onto pyramidal cells, presumably via increased mGluR5 activation from excess extrasynaptic glutamate. Whether this eCB-mediated astrocytic regulation of mGluR5 activation is shared by PFC synapses remains to be investigated.

DA and eCB Regulation of Attentional Processes

The influence of cortical DA on cognition, including attentional processes, is a well-researched subject that has been intensely studied by neurobiologists and computational neuroscientists alike. Broadly, DA in the PFC facilitates integration of complex

signals between sensorimotor networks by synchronizing different brain networks in response to both external signals and internal representations (Ott and Nieder, 2019). This is enabled by stabilizing neural representations in the cortex through gating sensory signals at the level of the PFC and gain changes of different pyramidal cell subpopulations, which support action selection and goal-directed behavior in stimuli-rich environments (Foote et al., 1975; Durstewitz et al., 2000; Mehta et al., 2000; Assad, 2003; Yantis and Serences, 2003; Maunsell and Treue, 2006; Scolari and Serences, 2009; Dang et al., 2012; Byers and Serences, 2014; Shafiei et al., 2019). As a neuromodulator, DA's influence via the signaling dynamics referenced above are tightly regulated at both the synaptic and systems level and are subject to the classic Yerkes-Dodson (inverted U-shaped curve) dose-response relationship, with hyper- or hypoDAergic levels resulting in cognitive dysfunction (Yerkes and Dodson, 1908; Vijayraghavan et al., 2007). This DA-sensitive nature of attentional control has been demonstrated by both human and rodent studies showing measures of inattentiveness correlated with low levels of DA release may be repaired by increasing DA transmission by means of neural stimulation or pharmacological manipulation (Turner et al., 2017; Fukai et al., 2019). In contrast, administering the D2 antagonist haloperidol to healthy human subjects increases involuntary directing of attention toward task-irrelevant events (Kähkönen et al., 2002). eCB signaling within the cortex must also walk a fine line to sustain attentional control and while local cortical CB1 dynamics are less studied than those within cortico-accumbens projections within this context, their effect on cognition is duly noted. In the PFC, viral-induced overexpression of CB1 results in impaired cognitive flexibility in the form of decreased reversal learning in rats (Klugmann et al., 2011). Within the OFC, the medial but not the lateral aspect has been found to display low levels of CB1 gene expression in rats with high impulsivity (Ucha et al., 2019). And, goal-directed behavior in mice has been shown to be regulated by a CB1-dependent mechanism in OFC projections to the dorsal striatum, with genetic knock out of CB1 here preventing habit formation of instrumental responding (Gremel et al., 2016).

Common Methods to Investigate the Components of Attention

While there are many components of attentional control, this review will focus on sustained attention, response control (impulsivity), attentional set-shifting and reversal learning as indices of attentional control as well as their respective deficits. Of the many factors that may influence attentional control, reversal learning – instrumental responding to swapped outcome contingencies between manipulanda – and impulsivity are both affective state-sensitive, pathology-related variables readily examined in the operant setting as adjuncts to more direct measures of attention itself (Puumala and Sirviö, 1998; Kenemans et al., 2005; Izquierdo and Jentsch, 2012; Linley et al., 2016; Paret and Bublatzky, 2020). While these two measures remain technically distinct from those of attention *per se*, they index prioritization of attentional demand to reward-associated stimuli (Mackintosh and Little, 1969; Oemisch et al., 2017). Accordingly, their relationship to attention and its operant tests are discussed alongside attention itself. Because performance inconsistencies are more informative than absolute performance, and because anatomical and neurochemical specificity is more readily correlated to specific measures, focus will be given to impairments and enhancements of these measures under different pharmacological conditions. Limitations are inherent in each reported finding as no pure test of attention is currently accepted, although evidence supporting correlations between certain pathologies and specific attentional dysfunctions will be highlighted. As attention is both inherently sensitive and limited, unique internal (e.g., neurofunctional) and external (e.g., experiential) factors may affect its processing to either enhance or constrain different attentional components. Furthermore, individual differences in attentional control may result in differing perceptions and behavioral outputs across samples under identical environmental conditions (Dukas and Kamil, 2000; Derryberry and Reed, 2002; Mathews et al., 2004; Ólafsson et al., 2011; Sali et al., 2015; Yuan et al., 2019). Such factors will be considered here, focusing on how key mesocorticolimbic regions regulate commonly investigated attentional control processes while also relating changes in functional activity to pathology. The review will then culminate with DA/eCB interactions evidenced to modulate these processes with special consideration toward gaps in the literature. To best frame the aforementioned components of attention, the behavioral tests most popularly used for their measurement will be introduced below, with the 5-Choice Serial Reaction Time Test (5-CSRTT) used to assess sustained attention and impulsivity and the Attentional Set Shifting Test (ASST) used to measure shifting attentional set and reversal learning.

Operant Methods to Assess for Changes in Attentional Processes

The 5-Choice Serial Reaction Time Test (5-CSRTT)

The 5-CSRTT for rodents was refurbished from a similar test of attentional processing during discrimination of visual stimuli in humans (Wilkinson, 1963; Carli et al., 1983). The paradigm consists of a food cup positioned in front of a hinged window, which once pushed open by the rodent initiates the behavioral session and delivery of the first food-based reward, usually a food pellet or measured amount of liquid sucrose. Additional manipulanda consists of five nose poke ports, each with their own cue lights positioned behind them as well as photobeams to detect individual nose pokes. After initial reward delivery following the opening of the food cup window, each successive delivery of reward is contingent upon a successful, exclusive nose poke through the port in which a cue light is randomly illuminated per trial. Responses for any port not signaled with an illuminated cue light may either terminate the trial without reward delivery or be tallied as non-rewarding errors within a lengthened response period (depending on the behavioral script ran at the time), after which the next trial begins. As the cue light is only briefly illuminated and responding via nose pokes is only allowed during a confined time period, a temporal domain is imposed onto the spatial

domain defined by the five different manipulanda separated by the apparatus. This dual-domain aspect of the paradigm demands attention be afforded to both domains simultaneously but also allows experimenters to dissociate each as they see fit, for instance by expanding the spatial separation of cues by exclusively illuminating peripheral ports or modifying time periods of cue illumination and/or delay periods. The dual-domain component of the task also allows multiple aspects of attention to be measured within a single experimental session. For instance, errors counted across nose-poke responses within unilluminated ports, considered inaccurate responses, are interpreted as lapses in sustained attention. Additional demands of 'attentional load' placed on the animal may be measured by modifying the temporal domain to increase uncertainty and/or duration of cue illumination. Another type of error may also be measured by tallying responses made during a brief inter-trial interval period programed before cue illumination at the onset of each trial (**Figure 7A**). These premature responses are interpreted as lapses in inhibitory control, or impulsivity. Other types of errors may also be measured by additional modifications programmed into the paradigm, though this review will focus on those of inaccurate and premature responding as a bulk of literature supports both DA and eCBs mediate these aspects of the task as commonly used with rodents.

The Attentional Set Shifting Test (ASST)

Like the 5-CSRTT, the ASST for rodents was adapted from behavioral assessments originally designed for human subjects. The most commonly cited comparison is with the Wisconsin Card Sorting Test (WCST), though a more direct comparison may be made with the Cambridge Neuropsychological Automated Testing Battery (CANTAB). Both are used to investigate 'behavioral flexibility' in healthy and abnormal neurological states by measuring the ability to shift attention from one reward-predictive perceptual feature to another following an unexpected switch (Berg, 1948; Grant and Berg, 1948; Weinberger et al., 1986; Sahakian and Owen, 1992; Paolo et al., 1995; West, 1996; Nieuwenstein et al., 2001; Barceló and Knight, 2002; Ridderinkhof et al., 2002; Romine et al., 2004; Nagahama et al., 2005). Specifically, the CANTAB design involves two-choice discriminations between either simple or complex exemplars to assess attentional bias toward a feature of perceptual stimuli, or dimension. One exemplar initially predicts reward faithfully at onset of the task and constitutes a single dimension (e.g., shape), while the other non-predictive exemplar is a presentation of a separate stimulus within the same dimension, in this case a separate shape. Once subjects learn this simple discrimination, a complex discrimination must be made after introduction of a second dimension (e.g., line segments) overlaying the first dimension in each exemplar that remains reward-predictive (shape). As both exemplars now consist of separate stimuli constituting two different dimensions (e.g., two different shapes with two superimposed line segments), the reward-predictive stimuli may be 'shifted' within the same dimension or to the other dimension. Changing stimuli while retaining reward-predictability to the initial dimension (shape) is labeled an 'intradimensional shift' (ID shift), while switching the reward-predictive dimension (shape→line segments) is labeled an 'extradimensional shift' (ED shift). Adding another level of analysis, each of these test components is followed by a reversal learning test, in which the reward-predictive stimuli of the two exemplars is reversed while the relevant dimension stays the same.

The ASST adapted for rodents has been designed as both a reward-digging task, in which exemplars comprise different combinations of digging materials and odors as dimensions (**Figure 7B**, panel A), and an instrumental operant task, in which reward delivery associations may be switched between two different levers and their respective cue light illumination settings (on/off) (**Figure 7B**, panel B). In these tasks, a bias toward one of the two dimensions is considered formation of an attentional set, expressed as relatively quicker and more accurate responding during intradimensional shifts than either extradimensional shifts or the initial simple discrimination test (Birrell and Brown, 2000). Attentional sets are therefore interpreted as information stores maintaining the reward-associative value of a perceptual feature that leads to relative ignorance toward other features (Folk et al., 1992).

In addition to assessing attentional sets, the separate reversal learning tests allow dissociation and detection of deficits relating to this ability alone. While similar to attentional set-shifting, reversal learning is considered a less complex but important process that relies on inhibition of previously rewarding actions (Jones and Mishkin, 1972). Because shifting attentional set requires a higher demand of attentional orientation and an aspect of learned irrelevance – accurately responding to rearrangement of complex, multidimensional stimulus pairings not correlated with reward – it is widely considered to be more cognitively challenging than reversal learning (Dias et al., 1996; Bissonette et al., 2008; Nilsson et al., 2015). Owing to its regulation of complex cognitive functions, it is logical that the PFC has been found to be critical to attentional set-shifting, with the non-human primate lateral PFC and homologous rodent medial PFC specifically evidenced to mediate this process (Dias et al., 1996; Birrell and Brown, 2000). Conversely, reversal learning is specifically impaired following damage to the OFC of both monkeys and rodents, perhaps owing to this region's encoding of reward value during decision-making processes that may influence response inhibition toward previously rewarding actions (Dias et al., 1997; McAlonan and Brown, 2003).

DA and eCB Signaling Effects on 5-CSRTT Performance

While both mesocorticolimbic DA and eCB signaling have been shown to affect 5-CSRTT performance, their influence on sustained attention and impulsivity may be separable. DA has been shown to play a crucial role in controlling inhibitory responding, with converging data indicating elevating synaptic DA increases impulsivity by activation of both D1 and D2 receptors (van Gaalen et al., 2006; Baarendse and Vanderschuren, 2012; Xue et al., 2018). In rodents, impulsivity has been linked to DA signaling specifically within the medial PFC and NAc

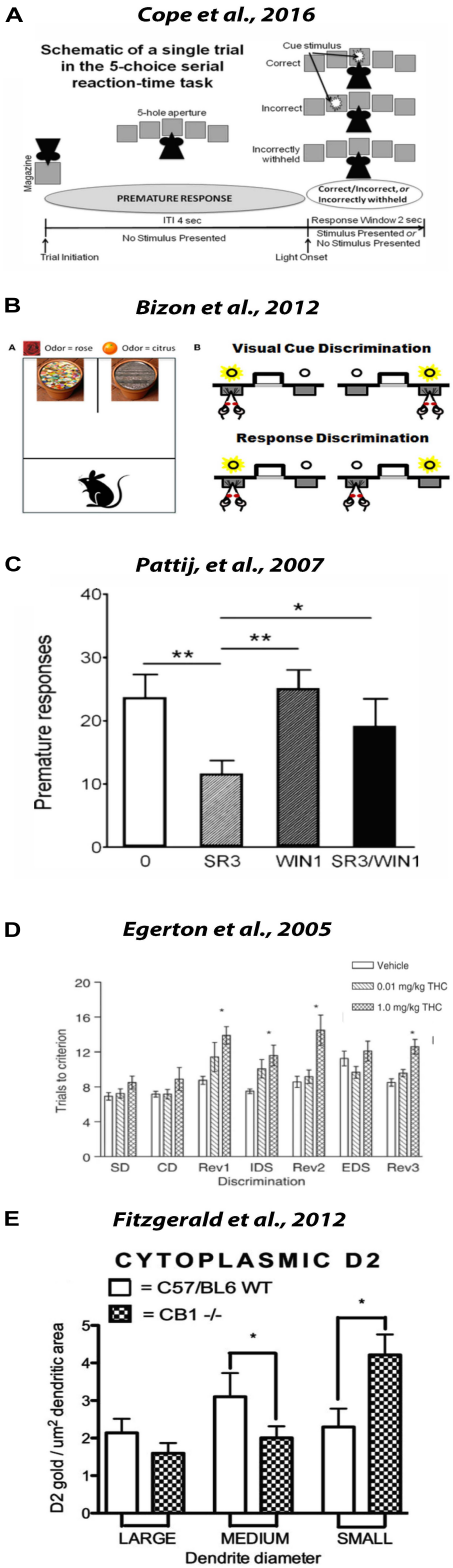

FIGURE 7 | Cannabinoids modulate attentional processes. **(A)** Schematic of a single trial in the 5-choice serial reaction time task (5-CSRTT). Animals are trained to perform a nose poke when one of five cue lights is presented in any *(Continued)*

FIGURE 7 | Continued
of the five nose-poke apertures following a fixed inter-trial interval (ITI), usually 4–6 s in length. Premature responses are tallied when made during this ITI. Responses made in one of the four nose pokes not illuminated is counted as an error of sustained attention. Reproduced from Cope et al. (2016). **(B)** Task schematics of two common set-shifting assessments for rodents. Panel **(A)** shows a schematic of the apparatus and examples of the stimuli used in the "dig" set-shifting task. Each pot has a unique odor (i.e., rose on left and citrus on right) and is filled with a unique digging medium (sequins on left, gravel on right). Only one stimulus feature is relevant to the location of a buried food reward in each phase of testing. Panel **(B)** shows a schematic of set-shift procedures performed in an operant version of the task. Rats are first trained to choose between two extended levers based on a light cue that is associated with one of the levers. After reaching criterion performance on that discrimination, there is an unsignaled change in rule and now the rat must ignore the light and choose levers based on their spatial location. Reproduced from Bizon et al. (2012). **(C)** Effect of CB1 antagonist/reverse agonist on impulsivity. Coadministration of WIN55,212-2 at 1.0 mg/kg (WIN1) prevents the effects of 3.0 mg/kg SR14716A (SR3) on inhibitory control in the 5-CSRTT. Reproduced from Pattij et al. (2007a). **(D)** Effect of acute THC administration on reversal learning. At 30 min before the start of the task, rats were administered vehicle, 0.01 mg/kg THC, or 1.0 mg/kg THC and the number of trials to reach criterion performance was recorded for a series of discriminations (SD, simple discrimination; CD, compound discrimination; Rev1,2,3, first, second, and third reversal stages; IDS, intradimensional shift; EDS, extradimensional shift). Animals in the 1 mg/kg THC treatment group exhibited marked deficits in performance at each of the reversal stages but not in the EDS stage. Reproduced from Egerton et al. (2005). **(E)** Altered compartmentalization of D2 immunogold stain in dendrites containing immunoperoxidase labeling for parvalbumin in the PL of the $CB1^{-/-}$ mice. Cluster analysis reveals a significant change in compartmental distribution of D2 immunogold in parvalbumin dendrites of $CB1^{-/-}$ mice. D2 immunogold density was assessed as particles of D2 immunogold/square μm dendritic area. In $CB1^{-/-}$ mice relative to $CB1^{+/+}$ controls, a significant ($p < 0.05$) increase in D2 immunogold was observed in small dendrites, while a decrease in D2 immunogold per μm dendritic area was observed in medium parvalbumin dendrites in $CB1^{-/-}$ mice relative to controls. Reproduced from Fitzgerald et al. (2012). $^{*}p < 0.01$; $^{**}p \leq 0.001$.

core and shell (Cole and Robbins, 1987; Miller and Cohen, 2001; Chudasama and Robbins, 2004; Dalley et al., 2004; Economidou et al., 2012). eCB tone may also be primed to modulate impulsivity as the synthetic CB1 antagonist rimonabant dose-dependently decrease it in rats, an effect occluded by co-administered WIN 55 (Pattij et al., 2007a) (**Figure 7C**). CB1 activity likely mediates its influence on impulsivity through regulating DA signaling as the CB1 antagonist rimonabant dose-dependently attenuates the impulsivity-inducing effects of the psychostimulants d-amphetamine and cocaine (Wiskerke et al., 2011; Hernandez et al., 2014). Additionally, THC and WIN administered twice daily across 2 weeks results in reduced DA turnover exclusively in the PFC and not in the dorsal nor ventral striatum of rats, with effects lasting up to at least 14 days post-abstinence (Verrico et al., 2003). This study is interesting in light of evidence supporting that response inhibition is regulated by cortical substrates and that protracted abstinence from chronic THC administration selectively impairs response inhibition in rats (Eagle and Baunez, 2010; Irimia et al., 2015). Collectively, these data suggest that following chronic cannabinoid exposure, long-term adaptations of DA function within the PFC increase likelihood of impulsivity.

In contrast to CB1-dependent effects on impulsivity, cannabinergic effects on more direct measures of attention in the 5-CSRTT are relatively null. Accordingly, modest impairments in sustained attention have been found to be reversed after 2 weeks abstinence following chronic THC administration in rats, with more pronounced increases in impulsivity persisting after 5 weeks of abstinence (Irimia et al., 2015). The distinction between impulsivity- and sustained attention-related effects of DA signaling have been scrutinized more than those by eCB signaling though results are conflicting. DA-dependent effects on 5-CSRTT performance differ based on pharmacological modality of altered activity (chemogenetics vs. psychostimulants), individual differences in baseline task performance between subjects and region-specificity of manipulations made within the mesocorticolimbic system. These variables notwithstanding, evidence reveals DA signaling regulates sustained attention albeit to a lesser degree than impulsivity. In general, sustained attention is enhanced following local D1 receptor agonism in the medial PFC and NAc as well as after increased neuronal activation of the VTA via selective pharmacological manipulation of modified G_i-coupled muscarinic GPCRs (i.e., chemogenetics) (Granon et al., 2000; van Gaalen et al., 2006; Pattij et al., 2007b; Baarendse and Vanderschuren, 2012; Boekhoudt et al., 2017; Xue et al., 2018; Fitzpatrick et al., 2019). Yet, multiple pharmacological and biological variables must be accounted for when manipulating DA function for behavioral output in general, though perhaps more so with cognitive tasks susceptible to numerous factors. Further dissecting any dissociable effects of chemogenetics from other types of DAergic manipulations, as well differentiating region-specific effects, may prove useful to probe DA's attentional functions within the 5-CSRTT.

DA and eCB Signaling Effects on ASST Performance

The effects of both mesocorticolimbic DA and eCB signaling on ASST performance diverge by the task's separate components, with DA impacting both shifting attentional set and reversal learning and cannabinergic effects restricted to reversal learning. Converging lines of evidence suggest that D1 signaling within the medial PFC in rodents and the homologous DLPFC in primates is central to attentional set formation and shifting in the ASST (Dias et al., 1997; Ragozzino et al., 1999; Birrell and Brown, 2000; Stuss et al., 2000; Stefani et al., 2003; Tunbridge et al., 2004; Fletcher et al., 2005; Floresco et al., 2008; Nagano-Saito et al., 2008; Parsegian et al., 2011) while D2 and DAT signaling within the OFC and striatum support reversal learning, respectively (Cools et al., 2009; Izquierdo et al., 2010; Cheng and Li, 2013). Indeed, cognitive flexibility training has been shown to enhance measures of prelimbic DA and therapeutic cognitive benefits in rats (Chaby et al., 2019), while ADHD and schizophrenia patients, both strongly associated with dysregulated cortical DA function, display attentional dysfunctions particularly related to shifting attentional set similar to patients with frontal lobe damage (Pantelis et al., 1999; Luna-Rodriguez et al., 2018). In terms of OFC DA, low but not high doses of methylphenidate remediate the impairment of both attentional-set formation and reversal learning in the spontaneously hypertensive rat (SHR) model of ADHD (Cao et al., 2012), though this effect on reversal learning specifically is occluded by intra-OFC injections of the D2 antagonist haloperidol (Cheng and Li, 2013).

Insight into the cannabinergic effects on ASST, on the other hand, are far outnumbered by those of DA, though these limited studies suggest eCBs may be more important for reversal learning than shifting attentional set. Egerton et al. (2005) first demonstrated that acute THC in rats impairs reversal learning while sparing extradimensional set-shifting ability in the ASST (**Figure 7D**). This selective effect on reversal learning by THC has been corroborated in non-human primates in the CANTAB test (WrightJr., Vandewater et al., 2013). Reversal learning has also been shown to be impaired in rats by THC in an olfactory go/no-go discrimination task and by adolescent WIN exposure in the ASST (Sokolic et al., 2011; Gomes et al., 2015). Surprisingly, cannabinoid-induced deficits to ASST may violate notions of a PFC/OFC task-specific dichotomy as overexpression of CB1 specific to the medial PFC has been shown to selectively impair reversal learning in rats, a cognitive component of the ASST typically associated with OFC function (Klugmann et al., 2011). Also surprising is a recent finding that intra-PFC injection of cannabidiol (CBD), but not similarly administered THC, impairs shifting of attentional set in rats (Szkudlarek et al., 2019). While its pharmacodynamic profile is complicated, it is worth noting that CBD functions as a negative allosteric modulator at CB1 (Laprairie et al., 2015).

Finally, several studies using rats prenatally treated with methylazoxymethanol acetate (MAM) as a developmental model of schizophrenia suggest persistent psychotomimetic effects related to mesocortical neuroadaptations may result from aberrant eCB signaling during adolescence (Renard et al., 2017). Both MAM and pubertal WIN have been shown to impair reversal learning in the ASST, as well as enhance mobility effects of d-amphetamine administration and an increased number of spontaneously active VTA DA neurons (Gomes et al., 2015). Somewhat remarkably, cannabinoid effects on MAM treatment are suggested to be transgenerational as adolescent WIN exposure also increases VTA DA population activity, decreased burst firing and sensitization to d-amphetamine locomotor responses in **Figure 2** generation MAM-treated rats (Aguilar et al., 2018).

These results bring into question both the locus of reversal learning and the neural mechanisms underlining its impairment by cannabinoids. Also important are considerations of dissimilarities between DA manipulations and ASST models, since like most laboratory-controlled behaviors, performance variations may be attributed to different protocols. This is underlined by cannabinoid administration having been suggested to impact visual discrimination in general, which may broadly affect performance in operant chamber-based ASST paradigms (Arguello and Jentsch, 2004; Hill et al., 2006). Future research of reversal learning following cannabinoid exposure and manipulation should take such details into account.

Comparable Studies Targeting Separate Signaling Systems May Benefit Analysis of DA/eCB Interaction and Attentional Dysfunction

To assist in clarifying DA/eCB interactions and their effects on attention, a few effective approaches may be noted. Behavioral effects of DA/eCB interactions have been demonstrated combining targeted pharmacological manipulations with measures of negative affect, showing anxiolytic effects of CB1 activation in the amygdala is D1 and D2 dependent (Zarrindast et al., 2011). Additional work with conditional knock-out mice with CB1 expression constitutively removed from D1-expressing neurons revealed CB1/D1 interactions modify negative affect as well (Terzian et al., 2011). Another comparative pharmacology study reported acute cannabis decreases while cocaine increases reversal learning performance in human subjects (Spronk et al., 2016). Interestingly, recent data combining chemical lesions of the medial forebrain bundle and single-unit electrophysiological recordings suggests the hypoDAergic states associated with many neuropsychiatric disorders affecting attention may themselves cause impairments in CB1 functional modulation of both sensorimotor and executive networks (Antonazzo et al., 2020). This novel conception expands the operative role for DA as not only a modulator of glutamate and GABA transmission, but also as a newfound gatekeeper of CB1's own robust modulation of transmission in substrates important for attentional and behavioral control. Future studies in the causality of CB1 functional modification within DA-affected pathologies and related changes to attentional processes are certainly warranted.

Finally, DA/eCB interactions may also be investigated at the level of GABAergic interneurons. Many of the previously described alterations to mesocorticolimbic functional dynamics may involve FSI that are either directly targeted by eCBs or are ultimately subject to eCB-dependent adaptations, with attentional processing being impacted by both. In the PFC, parvalbumin expressing FSIs critically gate pyramidal activity and are sensitive to DA signaling. Furthermore, levels of dendritic D2 expression in PFC parvalbumin cells have been demonstrated to be regulated by CB1 signaling (Fitzgerald et al., 2012) (**Figure 7E**). CB1-expressing FSIs in the striatum are also associated with cognitive functions, strongly associated with impulsive behavior and most recently suggested to gate attention toward reward-predicting visual features (Caprioli et al., 2014; Wright et al., 2017; Pisansky et al., 2019; Boroujeni et al., 2020). As eCB-sensitive FSIs in both cortical and striatal regions hold strong potential as a nexus for DA/eCB overlap, studies investigating their role in cannabinoid-modulated attentional processes may clarify much detail lacking in the field.

CONCLUSION

Summary of Conclusion

In this review, we describe recent scientific studies suggesting cannabinoids modulate transient DA release events in a manner that may influence motivational and attentional processes alike. While we've acknowledged that DA/eCB interactions still need to be better investigated across multiple overlapping neural circuits, we would like to close by further considering the intertwined relationship between DA transients, motivation and attention. And finally, we offer some speculation into the clinical implications these findings may offer the treatment of neurobehavioral symptoms in psychiatric medicine.

Complex Interactions Complicate the Relationship Between DA and the Neural Circuitry of Motivation and Attention

Transient DA release events, the neural substrates of motivation, and the neural substrates of attention interact within a tangled thicket of intertwined circuits—the nodes of which likely influence each other and can be differentially modulated by eCBs at multiple levels. First, it is important to recognize that activation of either subcortical nodes of pre-attentive visual processing (e.g., the superior colliculus) or cortical nodes of attentional visual processing (e.g., V1) are sufficient to evoke transient DA release events in the striatum of the basal ganglia (Redgrave et al., 2008, 2016; Takakuwa et al., 2017, 2018). Thus, it becomes difficult to definitively know whether a striatal DA transient truly reflects the value of a desired outcome within a motivational context (as was the general assumption of this review), is the result of an animal responding to a pre-attentive visual stimulus or is the result of an animal giving a visual stimulus attentional consideration. Furthermore, it is becoming abundantly clear that DA transient release events are accompanied by the co-release of additional neurotransmitters (e.g., GABA, glutamate) from the same DA neuron, which may profoundly impact the post-synaptic effects of DA (Tritsch et al., 2016; Morales and Margolis, 2017). In addition, a wide array of discrete neural circuits converge on midbrain DA neurons and their striatal-terminals within the basal ganglia (Morales and Margolis, 2017). It is likely that these discrete circuits can be differentially modulated by eCBs to influence neural input onto DA neurons, the generation of action potentials within DA neurons, and/or the concentrations of neurotransmitter released from the terminals of DA neurons. Following this multifaceted level of modulation, the transient DA release events are then integrated with other neural signals encoding various functions of motivation and attention within nuclei of the basal ganglia to ultimately influence the generation of behavioral action (Den Ouden et al., 2012). Difficulty in dissociating such neural representations has been considered before and ascribed to confounding neural signals of reward expectancy and attentional allocation (Maunsell, 2004). Indeed, DA value signals and motivational states are commonly recognized variables that capably modulate shifts in attention (Engelmann and Pessoa, 2007; Mohanty et al., 2008; Sali et al., 2014; Bourgeois et al., 2016; Anderson, 2019). And, at the level of behavioral output, common measures of both motivation and attention are highly DA-sensitive. This makes dissociating DA's contribution to their different components somewhat difficult as most experimental assessments of attention are dependent on the subject's motivation. Take, for example, individual differences in DA function and their effect on cognitive

measures demanding attention in a clinical setting. Healthy individuals with relatively lower DA synthesis capacity have been found to exert relatively low cognitive effort, while increasing their DA levels with methylphenidate and the D2 antagonist sulpiride has been reported to enhance their reward perception and motivation for cognitive engagement (Westbrook et al., 2020). This observation of a low DA-low effort relationship may be applied to preclinical settings and aligns with a study in mice reporting that chemogenetic inhibition of VTA DA neurons decreased motivated responding in a 5-CSRTT but not measures of attentional processing *per se*—suggesting an apparent dissociation (Fitzpatrick et al., 2019). However, it has also been reported that increasing DA levels through chemogenetic excitation of the VTA, using selective activation of modified G_q-coupled muscarinic GPCRs, impairs sustained attention in the 5-CSRTT (Boekhoudt et al., 2017). While separate types of neural DA manipulations were utilized between these studies (cf. inhibitory G_i vs. excitatory G_q DREADDs), the seeming contradiction may indicate the difficulty in separating the components of motivation and attention by performing DA manipulations in operant tasks.

Clinical Implications and Considerations

The interrelated nature of motivation and attention may be an asset to research as much as a liability, and cannabinoid modulation of either may underline therapeutic targets for both constructs. In the clinical context, DA/eCB interactions may play a specialized role in ADHD patients to impact both motivation and attention. Impaired activity in both motivational and attentional networks typical in ADHD patients are stabilized by pharmacologically increasing brain DA concentration; furthermore, while cannabinoids generally negatively affect measures of impulsivity and attention, they uniquely enhance them in ADHD patients (Rubia et al., 2009; Cooper et al., 2017). Commonalities between motivation and attention in the preclinical setting may be found in the 5-CSRTT and impulsivity's translatability to compulsive behavior, as cannabinergic regulation of this particular trait may be applied to constructs other than attentional dysfunction. For example, modulation of impulsivity through CB1 antagonism has been correlated with decreases in both alcohol and nicotine intake in rats, offering potential for therapies targeting eCB tone in addiction-related disorders (De Bruin et al., 2011). DA's role in this broadly applicable trait is also noted. In humans, decreased D2/D3 binding and increased d-amphetamine-induced striatal DA release has been correlated with high levels of trait impulsivity and drug cravings (Buckholtz et al., 2010). Strongly modulated by both DA and eCBs, FSIs in the NAc are one candidate as a mediator of impulsive behavior through DA/eCB interaction, directly gating medium spiny neuron activity to regulate tracking of reward-predicting cues and inhibit premature responding (Caprioli et al., 2014; Wright et al., 2017; Pisansky et al., 2019; Boroujeni et al., 2020).

Generalizing this example of impulsivity to other behavioral components linked by motivation and attention, it is possible to conceive of various constructs impacted by both, and in turn, their susceptibility to eCB-modulated DA function. In this sense, interrogation of DA/eCB interactions within substrates known to modulate either motivation or attention might share explanatory potential across translatable constructs, particularly substance use disorders. Such studies may assess cannabinoid-induced changes to motivational and attentional processes through modifications of DA-mediated reward value signals, which have been shown to influence both types of measures. Yet, for clear dissociations between each measure, it will be important to characterize the separate neural representations contributing to their respective behavioral outputs. It is also important to note that while distinct measures of motivation and attention have been studied under conditions of cannabinoid exposure, there remains much to be learned about how these measures overlap within the context of DA/eCB interactions.

AUTHOR CONTRIBUTIONS

EO and DG conceptualized this review and equally shared in the majority of the writing. LH performed a secondary writing contribution that was lesser, but significant. All authors contributed to the article and approved the submitted version.

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Endocannabinoids Released in the Ventral Tegmental Area During Copulation to Satiety Modulate Changes in Glutamate Receptors Associated With Synaptic Plasticity Processes

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Endocannabinoids modulate mesolimbic (MSL) dopamine (DA) neurons firing at the ventral tegmental area (VTA). These neurons are activated by copulation, increasing DA release in nucleus accumbens (NAcc). Copulation to satiety in male rats implies repeated ejaculation within a short period (around 2.5 h), during which NAcc dopamine concentrations remain elevated, suggesting continuous neuronal activation. During the 72 h that follow copulation to satiety, males exhibit long-lasting changes suggestive of brain plasticity processes. Enhanced DA neuron activity triggers the synthesis and release of endocannabinoids (eCBs) in the VTA, which participate in several long-term synaptic plasticity processes. Blockade of cannabinoid type 1 receptors (CB1Rs) during copulation to satiety interferes with the appearance of the plastic changes. Glutamatergic inputs to the VTA express CB1Rs and contribute to DA neuron burst firing and synaptic plasticity. We hypothesized that eCBs, released during copulation to satiety, would activate VTA CB1Rs and modulate synaptic plasticity processes involving glutamatergic transmission. To test this hypothesis, we determined changes in VTA CB1R density, phosphorylation, and internalization in rats that copulated to satiety 24 h earlier as compared both to animals that ejaculated only once and to sexually experienced unmated males. Changes in glutamate AMPAR and NMDAR densities and subunit composition and in ERK1/2 activation were determined in the VTA of males that copulated to satiety in the presence or absence of AM251, a CB1R antagonist. The CB1R density decreased and the proportion of phosphorylated CB1Rs increased in the animals that copulated compared to control rats. The CB1R internalization was detected only in sexually satiated males. A decrease in α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor (AMPAR) density, blocked by AM251 pretreatment, and an increase in the proportion of GluA2-AMPARs occurred in sexually satiated rats. GluN2A- N-methyl-D-aspartate receptor (NMDAR) expression decreased, and GluN2B-NMDARs increased in these animals, both of which were prevented by AM251 pre-treatment.

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An increase in phosphorylated ERK1/2 emerged in males copulating to satiety in the presence of AM251. Results demonstrate that during copulation to satiety, eCBs activate CB1Rs in the VTA, producing changes in glutamate receptors compatible with a reduced neuronal activation. These changes could play a role in the induction of the long-lasting physiological changes that characterize sexually satiated rats.

Keywords: endocannabinoids, CB1 receptors, AMPA and NMDA glutamate receptors, mesolimbic circuit, ventral tegmental area, synaptic plasticity, sexual satiety, natural reward

INTRODUCTION

The mesolimbic (MSL) dopaminergic system is constituted by the dopaminergic neurons of the ventral tegmental area (VTA) that mainly project to the nucleus accumbens (NAcc) (Ikemoto, 2007). This circuit is activated by natural rewarding behaviors, such as sexual activity (Kelley and Berridge, 2002), increasing dopamine (DA) levels in the NAcc. Such DA increases occur after the exposure of male rats to estrous odors, to an inaccessible sexually receptive female, and in response to mating itself (Pfaus et al., 1990; Damsma et al., 1992; Mitchell and Gratton, 1994; Robinson et al., 2001), and are the result of the activation of VTA neurons. This has been demonstrated in studies showing that sex-related cues and copulation increase the expression of c-Fos, a marker of neuronal activity, in the DA neurons of the VTA (Balfour et al., 2006) and that the sexual behavior performance increases VTA neuronal firing (Hernandez-Gonzalez et al., 1997) and activates NAcc medium spiny neurons (MSNs) (Matsumoto et al., 2012). Conversely, the MSL system has been shown to participate in the regulation of sexual motivation and the reinforcing properties of copulatory behavior (Everitt, 1990; Melis and Argiolas, 1995; Pfaus, 2009).

Sexually experienced male rats that are allowed to copulate without restriction with a sexually receptive female will repeatedly ejaculate until becoming sexually satiated. Intense copulation is displayed along 2.5 h, time during which male rats are capable of executing 7 successive ejaculations, on an average, before becoming sexually inhibited. Interestingly, this sexual inhibition lasts 72 h, but the sexually satiated males require 15 days of sexual inactivity to fully recover their initial ejaculatory capacity (Rodríguez-Manzo and Fernández-Guasti, 1994; Rodríguez-Manzo et al., 2011). The duration of this sexual inhibitory state suggests the occurrence of neuroadaptations resulting from intense copulation within a short period. In support of this idea, the sexually satiated rats exhibit other physiological changes in addition to the sexual behavior inhibition, the most conspicuous of which is a generalized sensitization to drug actions, a phenomenon which also lasts 72 h (Rodríguez-Manzo et al., 2011).

It has been demonstrated that during the copulation to exhaustion process, the NAcc DA levels are continuously elevated (Fiorino et al., 1997), indicating the constant activation of MSL DA neurons. Enhanced midbrain DA neuron activity triggers the synthesis and release of endocannabinoids (eCBs) from their cell bodies in the VTA (Lupica and Riegel, 2005). These eCBs act retrogradely, activating presynaptic cannabinoid type 1 receptors (CB1Rs) located on GABAergic (Szabo et al., 2002) and glutamatergic axon terminals (Melis, 2004), where they regulate neurotransmitter release, thereby modulating the firing activity of midbrain DA neurons (Riegel and Lupica, 2004). This eCB-mediated regulation is exerted through the transient inhibition of neurotransmitter release by the following two short-term plasticity phenomena: depolarization-induced suppression of inhibition (DSI), when inhibiting GABA release (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), and depolarization-induced suppression of excitation (DSE), when inhibiting glutamate release (Kreitzer and Regehr, 2001; Melis, 2004). The eCBs are also involved in the induction of long-term forms of synaptic plasticity, namely long-term depression (eCB-LTD) at both, glutamatergic (Gerdeman et al., 2002) and GABAergic synapses (Chevaleyre and Castillo, 2003). Besides, glutamatergic transmission at the VTA has been implicated in the induction of DA neuron burst firing, responsible for massive synaptic DA release in the NAcc (Floresco et al., 2003), as well as in long-term synaptic plasticity phenomena at the VTA (Xin et al., 2016). VTA neurons that are activated by copulation receive glutamatergic inputs (Balfour et al., 2006). Plasticity phenomena of excitatory synaptic transmission involve changes in α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptor densities and subunit composition (Song and Huganir, 2002; Paoletti et al., 2013).

Previous work from our laboratory showed that the blockade of CB1Rs during copulation to satiety, interferes with the appearance of the long-lasting sexual behavior inhibition and drug hypersensitivity in the sexually satiated rats (Canseco-Alba and Rodríguez-Manzo, 2014; González-Morales and Rodríguez-Manzo, 2020), evidencing the involvement of eCBs in the induction of these phenomena. These effects were replicated by the specific blockade of CB1Rs at the VTA (Canseco-Alba and Rodríguez-Manzo, 2016). On these bases, we hypothesized that eCBs released at the VTA during copulation to satiety could induce synaptic plasticity processes through the activation of CB1Rs that might involve glutamatergic transmission. To test this hypothesis, in the present work, we first determined if there were changes in VTA CB1R density, phosphorylation, and internalization—all events indicative of CB1R activation—in sexually experienced unmated control males and in rats that ejaculated only once or copulated to satiety 24 h earlier. In addition, we established the occurrence of changes in glutamate AMPA receptor (AMPAR) and NMDA receptor (NMDAR) densities and subunit composition in the VTA of male rats that

copulated to satiety in the absence or presence of the CB1R antagonist, AM251. Finally, we explored if there were changes in the phosphorylation of the extracellular signal-regulated kinase (pERK 1/2), which has been associated with CB1R activation and to long-term synaptic plasticity processes.

MATERIALS AND METHODS

Animals

Sexually experienced, adult male (250–300 g b. wt.) and sexually receptive female (200 g b.wt.) Wistar rats from our own vivarium were used in this study. Animals were housed, eight per cage, under inverted light/dark cycle conditions (12 h light: 12 h dark, lights on at 22:00 h), at 22°C, and with free access to food and water. Males were rendered sexually experienced by subjecting them to five independent sexual behavior tests and those animals achieving ejaculation in <15 min, in at least three of these tests, were classified as sexually experienced and selected for the study. Female rats served as sexual stimuli; sexual receptivity was induced by the sequential s.c. injection of estradiol benzoate (13 μg/rat) followed 24 h later by progesterone (7 mg/rat). All experimental procedures (Protocol 0230-16) were approved by our Institutional Internal Committee for the Care and Use of Laboratory Animals (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio, CICUAL), which follows the regulations established in the Mexican Official Norm for the use and care of laboratory animals NOM-062-ZOO-1999.

Sexual Behavior and Sexual Satiety Tests

Sexual behavior tests were conducted 2 h after the beginning of the dark phase of the cycle, in a room under dim red light. Males were individually introduced into polycarbonate cylindrical arenas covered with fine sawdust. A 5-min adaptation period was permitted to the males before introducing a sexually receptive female. Sexual activity was allowed until the achievement of one ejaculation or the attainment of sexual satiety. In this last case, males had *ad libitum* copulation with a single sexually receptive female until a 90-min period elapsed since the last ejaculation, without accomplishment of another ejaculation, which is the criterion used to consider a male sexually satiated (Rodríguez-Manzo et al., 2011). After reaching the corresponding criteria, animals were returned to their home cages.

Drugs

All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). AM251 was dissolved in a mixture of saline solution (98%), Tween80 (1%), and dimethyl sulfoxide (DMSO, 1%) and was i.p. injected. Estradiol benzoate and progesterone were dissolved in sesame oil and s.c. injected.

Antibodies

The following primary antibodies were used: rabbit anti-CB1R, mouse anti-β-actin, goat anti-β-Arrestin2 (β-A2), mouse anti-GluN2B and mouse anti-GLUA2 (Santa Cruz Biotechnology, Dallas, TX, USA); rabbit anti-GluN2A, rabbit anti-GluN1and rabbit anti-GluA1 (Alomone Labs, Jerusalem, Israel); rabbit anti-CB1R phospho S316 (pCB1R) (Abcam, Cambridge, UK), rabbit anti-ERK 1/2 phospho Thr202/Tyr204 (pERK 1/2) (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-VGlut2 (Merck-Millipore, Burlington, MA, USA). Secondary Abs for immunoblotting include HRP-donkey anti-rabbit and HRP-goat anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA). For immunofluorescence, the secondary Abs used were donkey anti-goat Alexa Fluor 488, donkey anti-mouse Alexa Fluor 555, and donkey anti-rabbit Alexa Fluor 647, all from Invitrogen (Thermo Fisher Scientific, San Diego, CA, USA).

Western Blot

Twenty-four hours after the sexual behavior tests, the animals were anesthetized with sodium pentobarbital and thereafter sacrificed by decapitation, their brains extracted in <5 min and cooled in dry ice for 15 s. Using stereotaxic coordinates from a rat brain atlas (Paxinos and Watson, 2009) as a reference, 2 mm thick coronal slices from the region containing the VTA were obtained. Bilateral punches of the VTA (1 mm diameter) from each rat were obtained with the aid of sample punches (Fine Science Tools Inc., CA, USA). Samples of both hemispheres of each rat were pooled, put in a lysis buffer [Tris HCL (50 mM), NaCl (150 mM), Igepal (1%), Triton X-100 (1%)] containing protein phosphatase (Thermo Fisher, catalog # 78428, 100X) and protease (Thermo Fisher, catalog # 78430,100X) inhibitor cocktails, pH = 7.4 and stored at −80°C until further processing. Tissue samples were thawed, homogenized by sonication, and centrifuged at 14,000 rpm, for 30 min, at 4°C; the resulting pellet was removed and protein concentrations were quantified in the supernatant using the modified Lowry method (DC Protein assay instruction manual, BIORAD (Lowry et al., 1951; Peterson, 1979). Bovine serum albumin (BSA, Sigma catalog # 05470) was used to build protein reference standard curves.

From each tissue sample, 25 μg protein, diluted 1:1 in 2X loading buffer (Sigma, catalog # S3401) were boiled to 95°C for 5 min, loaded onto 8% SDS-polyacrylamide gels and separated by electrophoresis for 2 h, at 100 V. A protein molecular weight (MW) standard (Page ruler plus pre-stained protein ladder, Thermo Fisher, catalog # 26620) was added to each gel. Proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane for 1 h, at 100 V, 350 mA. PVDF membranes were blocked for 2.5 h, at room temperature, with 4% milk (Biorad, catalog # 1706404) in Tris-Buffered Saline (TBS) with 1% Tween20 (Sigma, catalog # P9416) (TBS-T) for CB1R and pERK 1/2 Abs, and with 2% milk for the rest of Abs used (GluN1, GluN2A, GluN2B, GluA1, GluA2, and pCB1R). Thereafter, the membranes were incubated with the primary antibody overnight, at 4°C. Membranes were then washed three times with TBS-T for 5–10 min, under agitation, and were incubated with the corresponding secondary antibody for 1.5 h at room temperature. After this, they were washed three times with TBS-T and once with TBS. A chemiluminescence kit (Millipore, catalog #WBKLS0500) was used to reveal the membranes using the BioradChemidoc MP Imaging system (Hercules, CA, USA). Optical density (OD) of the resulting bands was determined with the aid of the ImageJ program (NIH) and the values were normalized with those of β-actin, which served as

TABLE 1 | Summary of antibodies used for the Western blotting.

	Primary antibodies				Secondary antibodies		
Protein	**Source**	**Dilution**	**Catalog #**	**References**	**Source**	**Dilution**	**Catalog #**
Western blot							
CB1	Rabbit polyclonal	1:1200	sc-20754	Almada et al., 2016	Donkey anti-rabbit	1:10000	711-035-152
pCB1	Rabbit monoclonal	1:1000	ab186428				
GLUN1 (NMDA)	Rabbit polyclonal	1:700	AGC-001	Atkin et al., 2015			
GLUN2A (NMDA)	Rabbit polyclonal	1:700	AGC-002	Atkin et al., 2015			
GLUN2B (NMDA)	Mouse monoclonal	1:1000	sc-365597	Lai et al., 2019	Goat anti-mouse	1:10000	115-035-003
GluA1 (AMPA)	Rabbit polyclonal	1:600	AGC-004	Lin et al., 2011	Donkey anti-rabbit	1:10000	711-035-152
GluA2 (AMPA)	Mouse monoclonal	1:500	sc-517265	Scherma et al., 2020	Goat anti-mouse	1:10000	115-035-003
pERK1/2(Thr202/Tyr204)	Rabbit polyclonal	1:10000	9101	Travaglia et al., 2016	Donkey anti-rabbit	1:10000	711-035-152
β-Actin	Mouse monoclonal	1:10000	sc-47778	Xu et al., 2020	Goat anti-mouse	1:10000	115.035-003

a charge control. Details on the Abs used for Western blotting are summarized in **Table 1**.

Immunofluorescence for CB1R and β-A2

Double immunofluorescence and confocal microscopy were used to analyze CB1R co-localization with β-arrestin2 (βA2), as a tool to determine CB1 cannabinoid receptor internalization in the VTA of male rats that ejaculated once or copulated to satiety 24 h earlier.

Tissue Preparation

Twenty-four hours after the sexual behavior tests, animals were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and intracardially perfused with 250 ml 0.9% sodium chloride, followed by 400 ml paraformaldehyde (4%) in sodium phosphate solution (PBS) buffer 0.1 M supplemented with 0.9% sodium chloride, pH 7.3. At the end of this procedure, brains were extracted from the skull and immediately placed in a cryoprotectant solution (sucrose at 30% in PBS) and stored at 4°C until further use.

Sixty coronal sections (40 μm) of the brain region containing the VTA [coordinates from −5.2 to −6.3 mm anterior to bregma (Paxinos and Watson, 2009)] were obtained using a cryostat (Leica CM1100, Nussloch, Germany); 10 brain sections from each animal, representative of the whole VTA, were used for the immunofluorescence experiments.

Triple Immunofluorescence for CB1R, β-A2, and VGlut2

Tissue sections were washed with PBS, four times, for 10 min and incubated during 2.5 h with a blocking solution [2.0% BSA, (Sigma, catalog # 05470), 6% donkey serum (Sigma, catalog # D9663), 2% gelatin (Sigma, catalog # G7765), and 0.3% Triton X-100 (Sigma, catalog # T9284), all contained in PBS]. All incubations and washes were made under constant stirring. Thereafter, sections were incubated with the CB1R, β-A2, and VGlut2 primary antibodies, diluted in the blocking solution, first for 2 h at room temperature, then for 19 h at 4°C and finally, 2 h at room temperature. After incubations with the primary antibodies, the sections were washed four times, for 10 min each, with a solution containing 0.3% Triton X-100 and 2.0% gelatin mixed in PBS. Subsequently, they were incubated with the secondary antibodies and 1 μl DAPI (Sigma catalog # D8417), diluted in the blocking solution, for 2 h at room temperature. For immunohistochemical controls, the first antibodies were omitted, which resulted in the absence of immunoreactivity. After incubation, the slices were washed four times with 0.1% Triton X-100 in PBS, for 10 min each. Thereafter, the sections were mounted on gelatinized slides using antifade mounting medium (Prolong Diamond antifade kit, Molecular Probes catalog # P36962), and analyzed by confocal microscopy. Details on the Abs used for immunohistochemistry procedures are summarized in **Table 2**.

Confocal Analysis

Ten coronal brain sections, representative of the whole VTA, of three different rats from each group were used for CB1R and β-A2 immunodetection. The VTA region in these sections was localized with the 10X objective of a confocal microscope (Carl Zeiss, LSM 800 Airyscan). Once identified, the objective was changed to a 40x oil objective for image acquisition. The ZEN v10.0 black software was used for image acquisition and the ZEN 2.3 blue edition software for image processing (Carl Zeiss, Oberkochen, Germany). Images were acquired in the best signal mode with a 1 airy unit (AU) pinhole and the total optical area for the 40x objective was 24,336 μm^2. The maximum emission spectrum of each fluorophore was delimited with barrier filters to avoid crosstalk between spectral emission curves. The 450 nm laser was used to detect DAPI, the 488 nm laser to detect Alexa 488, the 555 nm laser to detect Alexa 555, and the 647 nm laser to detect Alexa 647. The final color of each fluorophore (secondary antibodies) in the images was selected from the image software: magenta for Alexa 647 (CB1R), red for Alexa 555 (VGlut2), and green for Alexa 488 (β-A2). The immunoreactivities (IRs) for CB1R and β-A2 and their co-localization were obtained from three different channels (channel 1: Alexa 488 for β-A2, channel 2: Alexa 647 for CB1R, and channel 3: CB1R-IR/β-A2-IR co-localization). The IR values were obtained in μm^2, which were provided by the ZEN blue software.

TABLE 2 | Summary of antibodies used for immunohistochemical procedures.

	Primary antibodies				Secondary antibodies		
Protein	**Source**	**Dilution**	**Catalog #**	**References**	**Source**	**Dilution**	**Catalog #**
Immunohistochemistry							
CB1	Rabbit polyclonal	1:75	sc-20754	Hayn et al., 2008	Donkey anti-rabbit, Alexa 647	1:175	A31573
β-Arrestin2	Goat polyclonal	1:75	sc-6387	Garduño-Gutiérrez et al., 2013	Donkey anti-goat, Alexa 488	1:250	A11055
VGlut2	Mouse monoclonal	1:330	MAB5504	Zhang et al., 2015	Donkey anti-mouse, Alexa 555	1:300	A31750

Quantitative Analysis

For the quantification of the CB1R and β-A2 densities, data were expressed as the ratio of each IR protein area/total analyzed area (24,336 μm^2), using the following formulae: CB1R-IR = [channel 1 + channel 3 (μm^2)]/24,336 μm^2; β-A2-IR = [channel 2 + channel 3 (μm^2)]/24,336 μm^2. CB1R-IR/β-A2-IR co-localization was considered indicative of receptor internalization (Garduño-Gutiérrez et al., 2013). The degree of CB1R internalization was determined dividing IR co-localization values by the total CB1R-IR using the following formula: [channel 3 (co-localization of CB1R-IR and β-A2-IR) (μm^2)]/[channel 1 (CB1R-IR area) + channel 3 (co-localization of CB1R-IR and β-A2-IR) (μm^2)]. Data of a total of 30 brain sections per group were used for statistical analysis.

Statistical Analyses

Data are expressed as mean ± standard error of the mean (SEM). Changes in protein expression (CB1R, pCB1R, GluA1, GluA2, GluN1, GluN2A, GluN2B, and pERK1/2) were conducted by means of a one-way ANOVA followed by Tukey's test. Paired comparisons were conducted with the unpaired *t*-test; when normality was not met, the Mann–Whitney U test was used. Immunohistochemistry data were not normally distributed and were therefore analyzed by means of the Kruskal–Wallis ANOVA followed by Dunn's test. The Sigma Plot program, version 12.0 was used for all statistical analyses. In all cases, differences with a $P \leq 0.05$ were considered significant.

Experimental Design

Three different groups of males were used to establish possible changes in CB1R and pCB1R densities ($n = 5$ each) as well as in CB1R internalization ($n = 3$): (1) sexually experienced males that had no sexual activity 1 week prior to sacrifice, which served as a control group; (2) sexually experienced males that ejaculated once and were sacrificed 24 h later, which were used as a reference for the effects of recent sexual activity; and (3) sexually experienced males that copulated to satiety and were sacrificed 24 h later, which constituted the group of interest.

For the determination of changes in the densities of AMPARs (GluA1 and GLUA2 subunits), NMDARs (GluN1, GluN2A, and GluN2B subunits) and the phosphorylated MAPK ERK1/2 (pERK1/2), a fourth group was included ($n = 5$). This group consisted of sexually experienced males that copulated to satiety in the presence of the CB1R antagonist, AM251 (0.3 mg/kg, i.p.), administered immediately before the copulation to satiety session, at a dose previously found to interfere with the appearance, 24 h later, of the long-lasting sexual behavior inhibition and drug hypersensitivity that characterize sexually satiated male rats (González-Morales and Rodríguez-Manzo, 2020). These animals were sacrificed 24 h later to find out if eCBs played a role in the putative changes in these proteins in the sexually satiated males. For the WB experiments, animals were anesthetized with sodium pentobarbital and sacrificed by decapitation to obtain the brain samples. For the immunohistochemical experiments, the animals were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.), and intracardially perfused.

Experiment 1. Evidence for CB1R Activation in the VTA by Copulation to Satiety

In each of the three groups of males, the density of CB1Rs, as well as changes in the density of phosphorylated CB1Rs, were determined by Western blot. In addition, immunohistochemistry and confocal microscopy were used to establish CB1R co-localization with β-A2, as an indicator of CB1R internalization (Garduño-Gutiérrez et al., 2013).

Experiment 2. Determination of Changes in AMPARs of the VTA Induced by Copulation to Satiety and the Possible Involvement of eCBs in Their Induction

The density of VTA AMPARs was established in each of the four groups of males, by determining the expression of the constitutive GluA1 subunit of these receptors.

The expression of AMPARs containing the GluA2 subunit was also established in each of these groups, establishing the density of the GluA2 subunit. A Western blot procedure was used for all determinations.

Experiment 3. Establishment of Changes in NMDARs of the VTA Induced by Copulation to Satiety and the Possible Involvement of eCBs in Their Induction

The density of VTA NMDARs was established in the same four groups of animals, by determining the expression of the obligatory, channel-forming GluN1 subunit of these receptors.

In addition, the densities of NMDARs containing the GluN2A subunit and of those containing the GluN2B subunit in all the four groups were established by determining the expression of the GluN2A or the GluN2B subunit. All determinations were carried on by Western blot.

Experiment 4. Establishment of Changes in VTA pERK1/2 Densities Induced by Copulation to Satiety and the Possible Involvement of eCBs in Their Induction

The expression of the phosphorylated form of the MAPK ERK1/2 (pERK1/2) was determined by Western blot in each of the four different groups of animals.

RESULTS

Evidence for CB1R Activation in the VTA by Copulation to Satiety

Changes in VTA CB1R density produced by sexual activity are shown in **Figure 1**. There was a significant effect of mating on CB1R expression [one-way ANOVA, $F_{(2,\ 12)} = 25.20$, $P < 0.001$]. *Post-hoc* analysis showed a significant decrease in the density of CB1Rs (**Figure 1A**) in both, the animals that executed one ejaculatory series (1 Ejac) and those that copulated to satiety (sexually satiated) the day before sacrifice, as compared to unmated sexually experienced control males (Tukey's test, $P < 0.001$ each). By contrast, as shown in **Figure 1B**, no significant differences in the expression of phosphorylated CB1Rs (pCB1R) were found among groups [one-way ANOVA, $F_{(2,\ 12)} = 2.33$, $P = 0.14$].

Immunohistochemical and confocal microscopy analyses of mating-induced CB1R internalization is shown in **Figure 2**. Sample photomicrographs of the VTA, taken at the level of −6.0 mm from bregma (**Figure 2A**), were included in **Figure 2**. The upper row of photomicrographs corresponds to control, sexually experienced unmated rats (**Figure 2B**), the middle row refers to males that ejaculated once (**Figure 2C**), and the lower row to sexually satiated animals (**Figure 2D**). Similar patterns of CB1R immunoreactivity (CB1R-IR, magenta) distribution can be observed in all groups, i.e., a substantial amount of CB1R-IR could be detected, which was mainly located intracellularly, uniformly distributed in the cytoplasm of cell bodies with a defined nucleus (DAPI, blue). In addition, CB1R-IR appeared as punctuate marks, outside these rounded structures, that could indicate their presence in fibers. Quantification of CB1R-IR (**Figure 2E**) did not show statistically significant differences among groups [Kruskal–Wallis ANOVA, $H_{(2,\ 30)} = 3.76$, $P = 0.15$].

Ventral tegmental area β-A2 immunofluorescence (β-A2-IR, green) can be detected in all groups. In the control group (**Figure 2B**), β-A2-IR appears mainly as punctuating marks, sometimes forming empty circles, and occasionally distributed in the cytoplasm of cell bodies with a defined nucleus (DAPI, blue color). In the samples of males that ejaculated once (**Figure 2C**), β-A2-IR appears sometimes forming empty circles, which might correspond to cell body membranes. Finally, in the sexually satiated males (**Figure 2D**), an increase in β-A2-IR, together with a clear redistribution of the immunoreactive mark can be appreciated. In this case, clusters of β-A2-IR can be observed predominantly in the cytoplasm of cell bodies with a defined nucleus. However, punctuate marks can also be seen independently of these cytoplasmic clusters. Quantification of β-A2-IR evidenced statistically significant differences in its expression among groups [Kruskal–Wallis ANOVA, $H_{(2,\ 30)} = 6.89$, $P = 0.03$]. *Post-hoc* analysis indicated a statistically significant increase in β-A2-IR in the VTA of the sexually satiated males when compared to animals that ejaculated only once (Dunn's test $P < 0.05$). The first two columns of the sample photomicrographs show the co-localization of CB1Rs with the β-A2 protein, which combined immunofluorescences produced a white color. Higher magnification images (2x) are included in the second column. Colocalization sample photomicrographs contain an additional immunoreactive mark for the glutamate vesicular transporter 2 (VGlut2-IR, red color), which presumably labels glutamatergic inputs to the VTA of subcortical origin, which are the most abundant (Omelchenko and Sesack, 2007). In the photomicrographs of control males, CB1R-IR/β-A2-IR colocalization is almost absent. In this sample image, the coincidence of both immunoreactive marks is only observed in the cytoplasm of one cell. VGlut2-IR is abundant and located mainly outside cell bodies; sometimes this mark coincides with the cell membrane, but the majority of VGlut2-IR can be seen in what appears to represent fibers. The sample photomicrograph of males that ejaculated once lacks CB1R-IR/β-A2-IR colocalization and the majority of VGlut2-IR is observed again in fibers. Finally, in the sample of the sexually satiated males CB1R-IR/β-A2-IR co-localization is evident in the cytoplasm of cell bodies with a defined nucleus, suggesting CB1R internalization. VGlut2-IR appears often in the vicinity of the cells showing CB1R-IR/β-A2-IR colocalization. Quantification of CB1R/β-A2 colocalization showed differences among groups [Kruskal–Wallis ANOVA, $H_{(2,30)} = 15.10$, $P < 0.001$]. A statistically significant increase in the colocalization of both marks was obtained in the sexually satiated males as compared to control unmated males and to animals ejaculating once (Dunn's test, $P < 0.05$ each).

Changes in AMPARs of the VTA Induced by Copulation to Satiety and Involvement of eCBs in Their Induction

The density of VTA AMPARs, determined by the expression of their constitutive GluA1 subunit, showed significant differences among groups [one-way ANOVA, $F_{(2,\ 12)} = 5.33$, $P = 0.02$] (**Figure 3A**). *Post-hoc* analysis showed that the density of the GluA1 subunit decreased significantly in the sexually satiated rats. This decrease was statistically significant as compared to males ejaculating once ($P < 0.05$), and very close to significance when compared to the control group ($P = 0.054$). Interestingly, in the animals that copulated to satiety in the presence of the CB1R antagonist AM251 (**Figure 3A**), a significant increase in the expression of the GluA1 subunit was found as compared to the sexually satiated rats without treatment (*t*-test, $P < 0.002$),

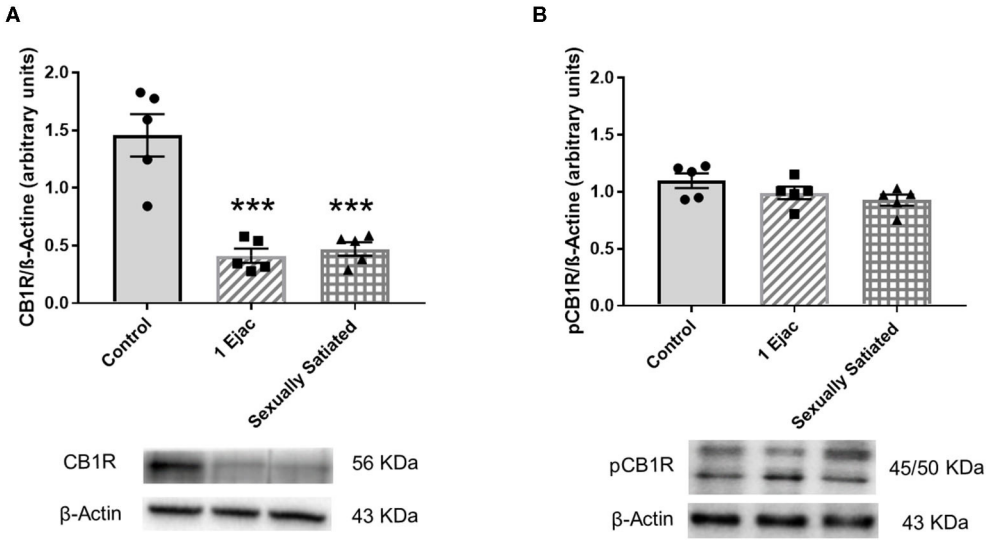

FIGURE 1 | Changes in the densities of CB1R and pCB1R in the VTA of male rats in response to copulation. Western blot analysis showing changes in CB1R **(A)** and phosphorylated CB1R (pCB1R) **(B)** densities in the VTA of males that ejaculated once (1 Ejac) and males that copulated to satiety (Sexually satiated) 24 h earlier when compared to control sexually experienced unmated rats (Control). Values are mean ± S.E.M of the protein/β-Actin optical density (O.D.) ratios of 5 rats per group. One-way ANOVA followed by Tukey test, $^{***}P < 0.001$ vs. control.

suggesting that the eCBs released during the sexual satiety development contributed to the decrease in AMPAR density.

There were changes also in GluA2 subunit expression among groups [one-way ANOVA, $F_{(2, 12)} = 22.71$, $P < 0.001$]. In this case, a significant increase in the expression of this subunit was found both in the animals ejaculating once ($P = 0.001$) and in the sexually satiated males ($P < 0.001$), when compared with control rats (**Figure 3B**). This increase was not modified in the males that copulated to satiety in the presence of the CB1R antagonist (**Figure 3B**), indicating that eCBs were not involved in this change.

Changes in NMDARs of the VTA Induced by Copulation to Satiety and Involvement of eCBs in Their Induction

Ventral tegmental area NMDAR density, determined through the measurement of its constitutive subunit GluN1, in the animals with different sexual activity showed differences among groups [one-way ANOVA, $F_{(2, 12)} = 6.05$, $P = 0.015$]. *Post-hoc* analysis indicated a statistically significant increase in GluN1 expression in the sexually satiated males only when compared to males ejaculating once ($P = 0.012$), but not in comparison with the control group (**Figure 4**). In the animals in which CB1Rs were blocked with AM251 during sexual satiety development, NMDAR expression was not significantly modified when compared to males copulating to satiety in the absence of treatment (**Figure 4**).

Establishment of changes in the expression of the GluN2A and the GluN2B NMDA subunits in the distinct groups is shown in **Figure 5**. As it can be seen, significant changes in GluN2A subunit expression were found among groups [one-way ANOVA, $F_{(2, 12)} = 14.3$, $P < 0.001$], which was statistically significantly reduced in the sexually satiated males as compared to both control rats ($P < 0.001$) and animals ejaculating once ($P = 0.01$) (**Figure 5A**). Measurement of the GluN2B subunit also showed a significant effect of mating on its expression [one-way ANOVA, $F_{(2,12)} = 14.5$, $P < 0.001$], increasing its density in both, the sexually satiated males ($P = 0.004$) and those ejaculating once ($P < 0.001$) as compared to control animals (**Figure 5B**). Pre-treatment with the CB1R antagonist in the animals copulating to satiety prevented the GluN2A subunit decrease (Mann–Whitney *U* test, $P = 0.03$) and the GluN2B subunit increase (*t*-test, $P = 0.005$) in the sexually satiated males (**Figures 5A,B**, respectively).

Changes in pERK1/2 in the VTA Induced by Copulation to Satiety and Involvement of eCBs in Their Induction

The expression of pERK1/2 was not modified by sexual activity (**Figure 6**) [one-way ANOVA, $F_{(2, 12)} = 3.12$, $P = 0.08$]. However, in the males that copulated to satiety in the presence of AM251, a statistically significant increase in pERK1/2 expression was found when compared to untreated sexually satiated animals (*t*-test, $P = 0.04$), suggesting that eCBs hinder the phosphorylation of this MAPK (**Figure 6**).

DISCUSSION

Main findings of the present series of experiments are as follows: (1) copulation to satiety promotes the release of eCBs in the VTA, which activate CB1Rs and produce their internalization and downregulation; (2) 24 h after the intense sexual activity that takes place during sexual satiety development, changes in

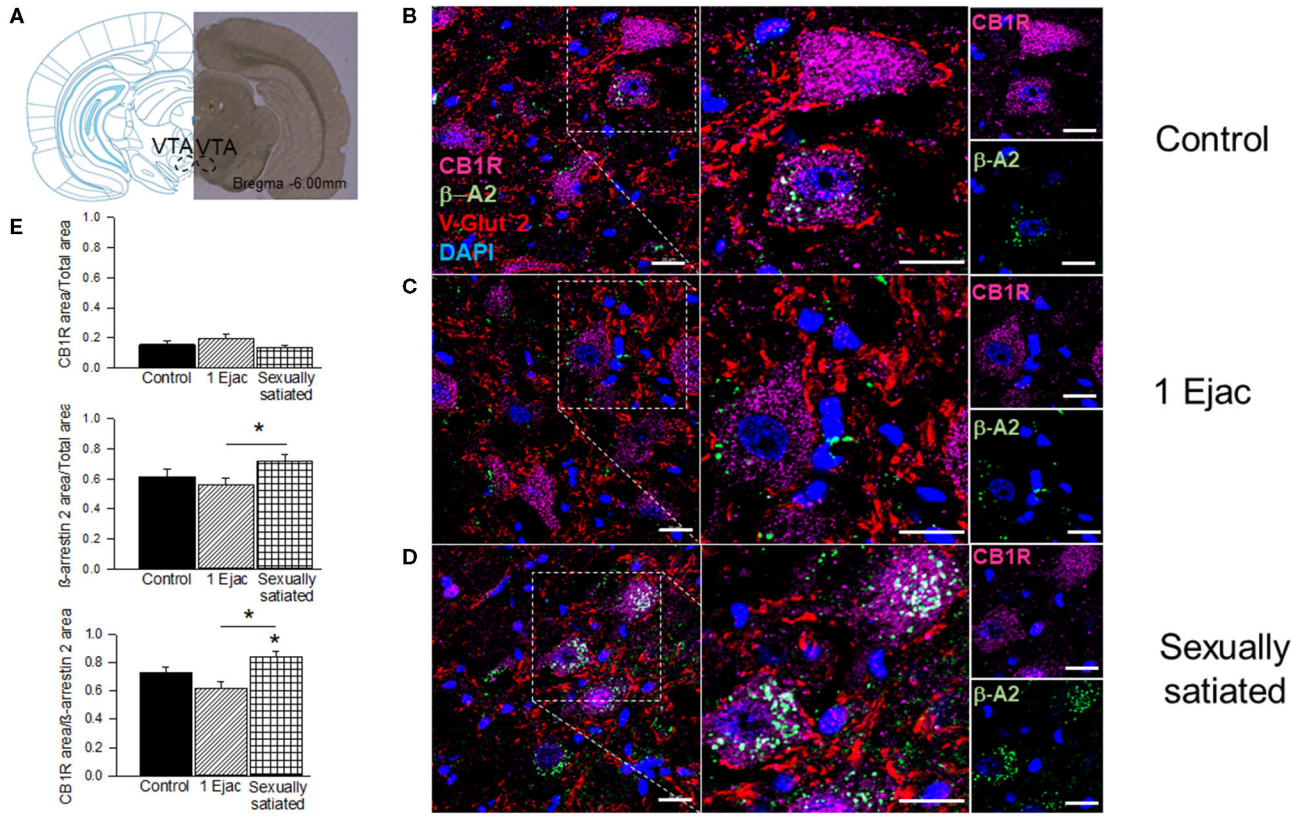

FIGURE 2 | CB1R internalization in the VTA of male rats that copulated to satiety. **(A)** Shows a coronal brain section, taken at −6.00 mm anterior to bregma, showing the VTA region from where the sample photomicrographs were obtained. **(B–D)** Show representative confocal photomicrographs from the VTA of unmated sexually experienced male rats (Control), males that ejaculated once (1 Ejac) and males that copulated to satiety (Sexually satiated) 24 h earlier, respectively. The first column shows 40X magnification images of CB1R (Alexa 647, magenta), β-A2 (Alexa 488, green) and VGlut2 (Alexa 555, red) immunoreactivity (IR); cell nuclei appear in blue (DAPI). The second column depicts a 2x enlargement of the selected region with the same IR marks. The third column shows individual CB1R and β-A2 IR-marks in the selected region. Co-localization of CB1R with β-A2- appears in white color. Scale bar 20 μm. **(E)** Shows the quantification of CB1R-IR, β-A2-IR as well as that of their co-localization. Kruskal-Wallis followed by Dunn's test, $^{*}P < 0.05$.

VTA glutamate AMPAR density and subunit composition and in NMDAR subunit composition are detected, coinciding with the period of sexual inhibition that characterizes sexually exhausted male rats; (3) the eCBs released during sexual activity are involved in the induction of changes in AMPAR and NMDAR, through the activation of CB1Rs, and prevent ERK1/2 phosphorylation in the sexually satiated rats.

Activation of CB1Rs at presynaptic inputs onto VTA DA neurons results from "on demand" postsynaptic eCB release during periods of increased DA neuronal activity (Lupica and Riegel, 2005). These eCBs exert control over MSL DA neuron activity by modulating GABAergic and glutamatergic inputs at the VTA (Riegel and Lupica, 2004). Sexual activity is a stimulus that enhances MSL DA neuron firing and, therefore, could promote eCB mobilization. The present study provides evidence showing that both, moderate (a single ejaculatory series) and intense copulation (until sexual satiety) activate CB1Rs at the VTA and induce their downregulation. Thus, 24 h after sexual activity, we found a significant reduction in VTA CB1R density in both copulating groups. Prolonged exposure to CB1R agonists is a condition found to produce CB1R downregulation, requiring new protein synthesis for receptor recovery (Hsieh et al., 1999). The majority of the available data on CB1R downregulation has been found following chronic tetrahydrocannabinol (THC) or synthetic cannabinoid agonist treatment (Sim-Selley, 2003), while only few studies report changes in the expression of CB1R in the brain induced by endogenous cannabinoids, and these are frequently associated with diseases involving pathological neuronal excitation. For example, a decrease in CB1R density has been documented in epileptic human hippocampal tissue (Ludányi et al., 2008), in rat hippocampus as a result of chronic stress (Hill et al., 2005; Hillard, 2014), chronic oxidative stress (Li et al., 2020), and in the ventral midbrain of patients with Parkinson's disease (Van Laere et al., 2012). The present study gives evidence for a reduction in VTA CB1R density under physiological conditions, produced by sexual activity. Little is known about the mechanisms involved in regulating the CB1R expression; again, these mechanisms have been related to disease states (Miller and Devi, 2011). Since the release of eCBs was produced by a physiological stimulus in our model, the mechanisms involved in CB1R density reduction are probably those acting in the natural receptor cycle, involving CB1R

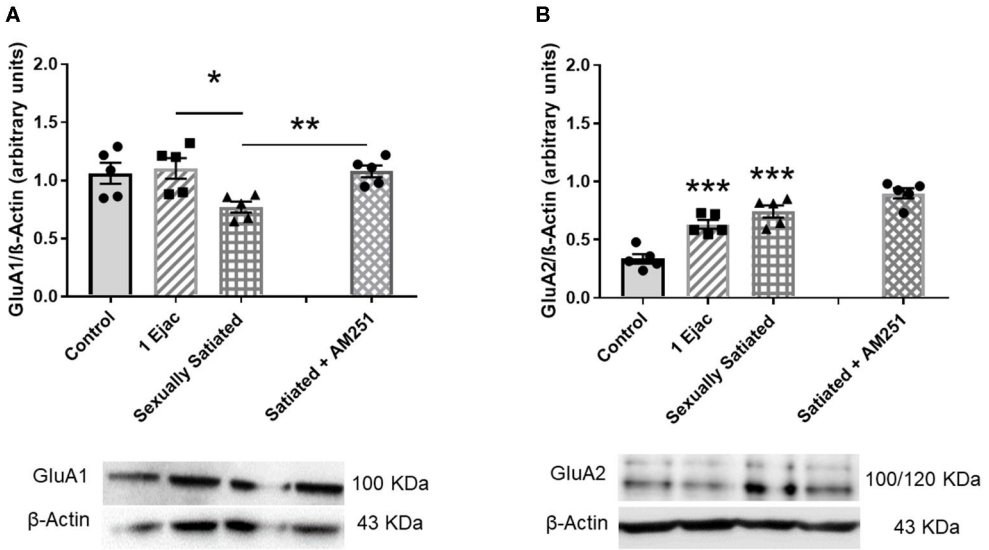

FIGURE 3 | Changes in AMPAR density and subunit composition in the VTA of sexually satiated rats and involvement of eCBs in their induction. Western blot analysis showing changes in GluA1 **(A)** and GluA2 AMPAR subunit **(B)** densities in the VTA of control sexually experienced unmated rats (Control), males that ejaculated once (1 Ejac) or copulated to satiety (Sexually satiated) 24 h earlier, and of males that copulated to satiety in the presence of the CB1R antagonist AM251 (Satiated + AM251). Differences among untreated groups with different sexual conditions were determined by means of a one-way ANOVA followed by Tukey test, $^{***}P < 0.001$; $^{*}P < 0.05$. A comparison between the rats that copulated to satiety in the absence or presence of AM251 was conducted with the unpaired *t*-test $^{**}P < 0.01$. Values are mean ± S.E.M of the protein/β-Actin optical density (O.D.) ratios of 5 rats per group.

internalization and subsequent receptor degradation (Hsieh et al., 1999). The decrease in CB1R expression here reported suggests that copulation might induce a prolonged increase in eCB levels in the VTA.

No changes in the expression of phosphorylated CB1Rs (pCB1Rs) were found in any of the copulating groups of rats as compared to unmated control males. However, the lack of change in pCB1R expression, combined with the decrease in CB1R density in the VTA of these animals, implies that a larger proportion of the expressed CB1Rs is phosphorylated 24 h after sexual activity. These data strongly suggest that the proportion of pCB1Rs increases in the VTA of male rats as a result of sexual activity, thereby evidencing CB1R activation. The G protein-coupled receptor (GPCR) phosphorylation followed by binding an arrestin is a common pathway for uncoupling GPCRs, like the CB1R, from G protein-dependent signaling (Moore et al., 2007). G protein receptor kinases (GRKs) are recruited to the plasma membrane and participate in phosphorylating the agonist-occupied receptor, increasing the affinity of β-arrestins for the receptor; β-arrestin binding prevents further receptor activation. Thus, ligand-induced CB1R phosphorylation results in receptor desensitization and β-arrestin recruitment (Nogueras-Ortiz and Yudowski, 2016). β-Arrestins also work as a scaffold for the endocytic machinery that internalizes GPCRs. The recruitment of β-arrestin2 (β-A2) has been associated with the internalization process of the CB1R (Gyombolai et al., 2013). In the present study, no difference in the magnitude of VTA pCB1Rs expression was found between animals that executed mild and intense copulation the day before, though an increase in CB1R/β-A2 co-localization, interpreted as indicative of CB1R internalization, was only found in the sexually satiated rats. It has been reported that, in contrast to synthetic cannabinoid agonists, which cause rapid CB1R internalization, an analog of the endogenous cannabinoid anandamide causes internalization only at high concentrations (Hsieh et al., 1999). Thus, it could be thought that after one ejaculation, the eCBs released by DA neurons activated CB1Rs inducing their phosphorylation but did not attain the concentrations needed to induce CB1R internalization, while in the animals that copulated to satiety, DA neurons were activated repeatedly, releasing eCBs for a longer period and reaching the concentrations needed to induce CB1R internalization. Another possible interpretation for this differential result is related to the timing of CB1R internalization, since short-term agonist exposure has been reported to induce rapid CB1R internalization and recycling (Hsieh et al., 1999). This rapid process could have occurred in the animals that experienced one ejaculation and therefore, CB1R/β-A2 colocalization was no longer observed 24 h later. In contrast, the repeated activation of DA neurons during copulation to satiety could have released a larger amount of eCBs over a longer lasting period, inducing several cycles of CB1R internalization, which were still detectable 24 h later. To the best of our knowledge, this is the first time that evidence is provided for *in vivo* CB1R internalization in VTA neurons, mediated by endogenous cannabinoids that were released by a natural stimulus, under physiological conditions. In this sense, this finding is of particular significance as it provides direct information on the functioning of the endogenous cannabinoid system in the MSL circuit, specifically in the VTA, contributing to our understanding of the role of the

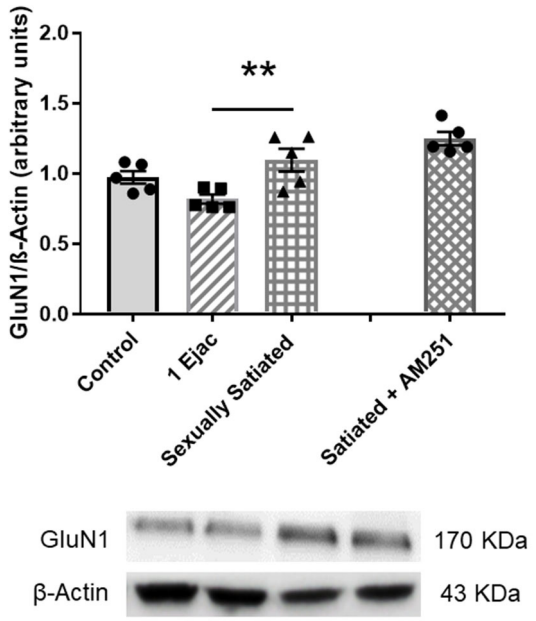

FIGURE 4 | VTA NMDAR density does not change in response to sexual activity in male rats. Western blot analysis showing changes in the expression of the NMDAR constitutive GluN1 subunit in the VTA of control sexually experienced unmated rats (Control), males that ejaculated once (1 Ejac) or copulated to satiety (Sexually satiated) 24 h earlier, and of males that copulated to satiety in the presence of the CB1R antagonist AM251 (Satiated + AM251). Differences among untreated groups with different sexual conditions were determined by means of a one-way ANOVA followed by Tukey test, $^{**}P < 0.01$. A comparison between the rats that copulated to satiety in the absence or presence of AM251 (Satiated + AM251) was conducted with the unpaired *t*-test, n.s. Values are mean ± S.E.M of the protein/β-Actin optical density (O.D.) ratios of 5 rats per group.

endogenous cannabinoid system in the function of the central nervous system.

The VTA neurons that are activated by copulation in male rats, identified by the mating-induced expression of Fos protein, receive glutamatergic afferents from the medial prefrontal cortex (mPFC) (Tzschentke, 2001; Balfour et al., 2006), the pedunculopontine nucleus (PPT), and the laterodorsal tegmental (LDT) area (Clements and Grant, 1990). In addition, local glutamatergic neurons were identified in the VTA (Yamaguchi et al., 2007; Morales and Root, 2014), which form functional synapses onto VTA dopaminergic neurons (Dobi et al., 2010; Wang et al., 2015). The sex-activated VTA neurons contain NMDARs, whose constitutive GluN1 subunit is phosphorylated in response to sexual behavior and sex-associated cues (Balfour et al., 2006). Thus, glutamatergic transmission at the VTA participates in the stimulation of the MSL system that takes place during sexual activity. Besides, it has been shown that the activation of NMDARs in this brain region is necessary to enable DA neurons to switch between tonic and phasic firing patterns (Sombers et al., 2009). Both, sexual cues and mating itself, trigger mesolimbic DA neuron phasic firing, resulting in an increase in DA release at the NAcc (Pfaus et al., 1990; Damsma et al., 1992; Wenkstern et al., 1993; Robinson et al., 2001). Together, these data show that glutamatergic synapses onto DA cell bodies in the VTA play an important role in the response of MSL DA neurons to sexual activity, a natural rewarding stimulus (Floresco et al., 2003).

In the present work, we provide evidence for changes in VTA glutamate receptor density and subunit composition that were detected in the sexually satiated rats 24 h after intense sexual activity. Thus, a decrease in the density of VTA AMPARs, concomitant to a significant rise in the expression of AMPARs containing the GluA2 subunit were found in the sexually satiated rats. Repeated activation of Ca^{2+}- permeable AMPARs has been reported to produce a long-lasting switch in their subunit composition, from GluA2-lacking to GluA2-containing receptors (Liu and Cull-Candy, 2000). Thus, the increased expression of the GluA2 subunit found in the sexually satiated males, suggests that the proportion of GluA2-containing AMPARs was increased as a result of the repeated activation of VTA neurons by glutamate during copulation to satiety. It is well-established that the presence of the GluA2 subunit in AMPARs renders these receptors impermeable to Ca^{2+} and Zn^{2+} and slows the channel kinetics (Liu and Zukin, 2007). Therefore, the predominance of GluA2-containing AMPARs results in a reduction of synaptic activity. On the other side, the decrease in the number of AMPAR may result from their removal from the cell surface by internalization and their subsequent lysosomal degradation. This phenomenon is associated with a specific form of long-term plasticity: long-term depression (LTD) (Chater and Goda, 2014). LTD of excitatory synapses can be produced by a switch in the composition of AMPARs from GluA2-lacking to GluA2-containing receptors, which show a lower single channel conductance (Mameli et al., 2007), and requires a loss of AMPARs from the synapse (Chater and Goda, 2014). The decrease in AMPAR expression and the increase in GluA2-containing receptor expression observed in this work in the sexually satiated rats are in line with those producing LTD.

As to the NMDAR, its density in the VTA was increased in the sexually satiated animals as compared to males that ejaculated only once but was not importantly modified with respect to control animals, in contrast to the evident changes found in the expression of NMDAR subunits. In this last case, a significant decrease in the expression of the GluN2A subunit, concomitant to a significant increase in that of the GluN2B subunit were found in the VTA of the sexually exhausted males, suggesting that the subunit composition of NMDARs is changed. Such changes in NMDAR composition would be relevant, as the GluN2A subunit confers these receptors a lower affinity for glutamate, faster kinetics, and greater channel open probability (Lau and Zukin, 2007). Synaptic activity regulates the molecular composition of NMDARs in such a manner that there is a relative abundance of GluN2A containing receptors in active synapses, which shifts to a reduction relative to the abundance of GluN2B containing NMDARs in inactive synapses (Ehlers, 2003). Therefore, a presumed increase in the proportion of GluN2B-NMDARs, with slower kinetics and reduced channel open probability, with regard to GluN2A-NMDARs, characterized by a high channel open probability and

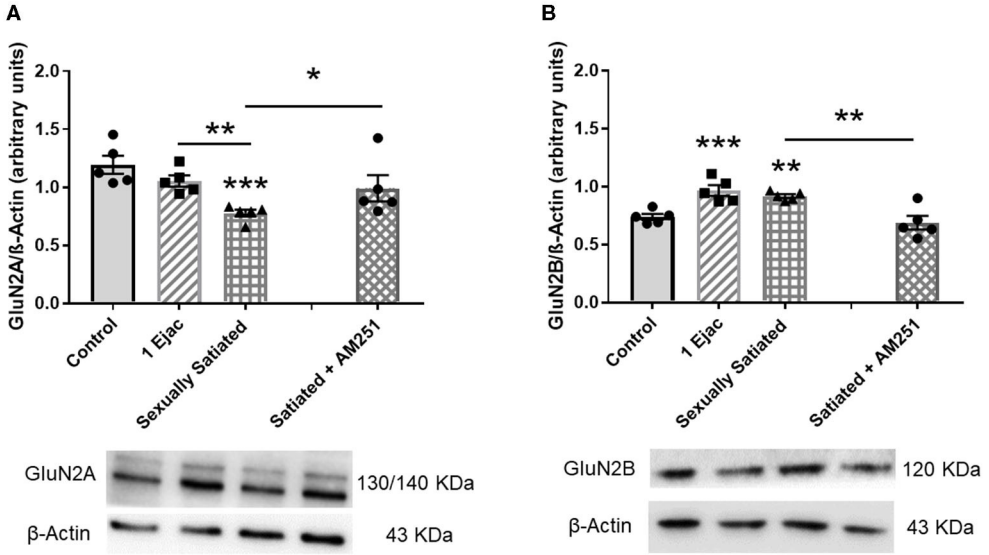

FIGURE 5 | Changes in VTA NMDAR subunit composition produced by copulation to satiety in male rats and involvement of eCBs in their induction. Western blot analysis showing changes in GluN2A **(A)** and GluN2B **(B)** NMDAR subunit expression in the VTA of control sexually experienced unmated rats (Control), males that ejaculated once (1 Ejac) or copulated to satiety (Sexually satiated) 24 h earlier, and of rats that copulated to satiety in the presence of the CB1R antagonist AM251 (Satiated + AM251). Differences among untreated groups with different sexual conditions were determined by means of a one-way ANOVA followed by Tukey test, $^{***}P < 0.001$, $^{**}P < 0.01$. A comparison between the rats that copulated to satiety in the absence or presence of AM251 (Satiated + AM251) was conducted with the Mann-Whitney *U*-test, $^{*}P < 0.05$ or the unpaired *t*-test $^{**}P < 0.01$. Values are mean ± S.E.M of the protein/β-Actin optical density (O.D.) ratios of 5 rats per group.

rapid deactivation (Paoletti et al., 2013), would result in a reduced activity of the neurons in which these changes occur. It could be speculated that the changes in expression of NMDAR subunits found in the VTA of the sexually satiated rats, might indicate a reduced activity of VTA glutamatergic synapses during the sexual inhibitory state of these animals. An increase in GluN2B subunit expression was also found in the males that ejaculated once, suggesting that glutamatergic stimulation provided by mild sexual activity is sufficient to initiate changes in NMDAR subunit composition.

We found only one study investigating changes in the expression of glutamate ionotropic receptors associated with male sexual activity in the MSL system. In that work, an increase in NMDAR density without changes in the expression of GluA1 and GluA2 AMPAR subunits was found in the NAcc of male rats, 24 h after the last of five consecutive daily mating sessions involving one ejaculation per day (Pitchers et al., 2012). Together with present findings, these data show that a natural rewarding behavior like sexual activity can induce changes in glutamate receptor expression in the MSL system.

Sexually satiated male rats do not respond to the presence of a sexually receptive female 24 h after copulation to satiety. This is an unexpected behavior since, on the one side, these animals are proofed sexually competent rats that rested around 20 h after intense sexual activity when presented to the new sexual partner and, on the other, copulation is an instinctive behavior that should be triggered by an adequate sign stimulus: in this case, the sexually receptive female. Considering that glutamatergic stimulation at the VTA participates in changing the activity pattern of DA neurons toward phasic firing, a phenomenon that occurs in response to rewarding stimuli, changes in the subunit composition of NMDARs, might reduce or cancel the ability of DA neurons to produce this phasic firing, thereby contributing to the lack of response of the sexually satiated rats to the presence of a sexually receptive female. Though the neuronal composition of the VTA is heterogeneous and, in addition to DA neurons (65%), there is an important population of GABA neurons (around 30%) and a small proportion of glutamate neurons (around 5%) (Dobi et al., 2010; Morales and Margolis, 2017). These GABA neurons exhibit NMDAR-mediated plasticity (Nelson et al., 2018) and can therefore be both, targets of glutamate and contribute indirectly to the induction of changes in DA neuron firing activity (Bouarab et al., 2019).

One of the goals of the present study was to determine the possible participation of the eCBs released during copulation to satiety in the changes in glutamatergic transmission resulting from intense sexual activity. Our results show that the blockade of the actions of eCBs at CB1R during copulation to satiety, by the pretreatment with the CB1R antagonist AM251, interfered with the decrease in AMPAR density and blocked the changes in the expression of NMDAR subunits, but did not modify the changes in the subunit composition of AMPARs. The question emerges as to the mechanisms involved in these eCB-induced changes in glutamatergic receptors.

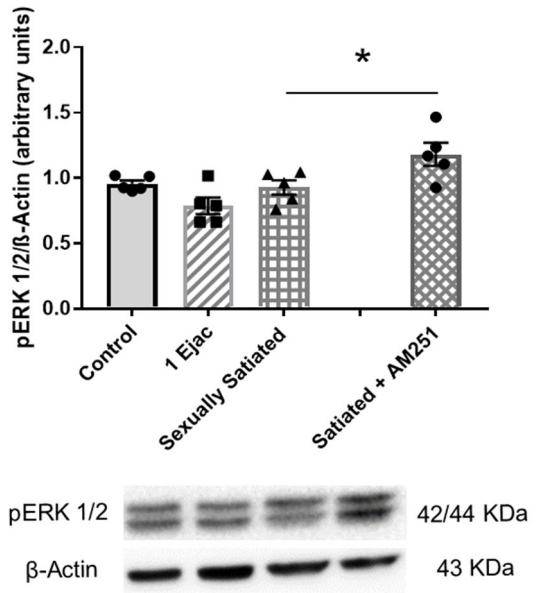

FIGURE 6 | Involvement of eCBs in the changes in pERK 1/2 expression in the VTA of sexually satiated rats. Western blot analysis showing changes in pERK1/2 expression in the VTA of control sexually experienced unmated rats (Control), males that ejaculated once (1 Ejac) or copulated to satiety (Sexually satiated) 24 h earlier, and of rats that copulated to satiety in the presence of the CB1R antagonist AM251 (Satiated + AM251). Differences among untreated groups with different sexual conditions were determined by means of a one-way ANOVA, n.s. A comparison between the rats that copulated to satiety in the absence or presence of AM251 (Satiated + AM251) was conducted with the unpaired *t*-test, $^{*}P < 0.05$. Values are mean ± S.E.M of the protein/β-Actin optical density (O.D.) ratios of 5 rats per group.

It has been shown that prolonged DA neuron depolarization within the VTA induces the release of eCBs, which activate CB1Rs at glutamatergic presynaptic terminals suppressing glutamatergic transmission onto DA neurons (Melis, 2004), through the short-term plasticity phenomenon called DSE (Kreitzer and Regehr, 2001). It has been considered that DSE plays a neuroprotective role against glutamate-induced excitotoxicity (Diana and Marty, 2004). The changes in VTA glutamatergic receptors reported here were detected particularly in the sexually satiated rats and are therefore consistent with a longer or repeated glutamatergic receptor stimulation. The eCBs have been found to produce also long-term plasticity phenomena, like long-term depression (eCB-LTD) of glutamatergic transmission (Robbe et al., 2002), requiring either long or repeated CB1R activation to be induced (Diana and Marty, 2004). This eCB-LTD has been observed in VTA DA neurons and is produced by a decrease in glutamate release, presumably exerted by 2-AG through the activation of CB1Rs (Haj-Dahmane and Shen, 2010). This form of plasticity is mechanistically different from the eCB-mediated mGluR-LTD, also described in the VTA (Bellone and Lüscher, 2005), in which the postsynaptic group I mGluR activation, triggers the synthesis and release of 2-AG, presumed to mediate a switch in AMPAR subunit composition to induce LTD. The excitatory synapses onto DA neurons of the VTA contain GluA2-lacking AMPARs and mGluR-LTD induces an increase in the intracellular calcium of DA neurons (Morikawa et al., 2003) leading to eCB release (Wilson and Nicoll, 2002) that depend on the switch from GluA2-lacking to GluA2-containing AMPARs. These two forms of LTD of glutamatergic synapses on DA neurons of the VTA coexist and might be part of the mechanisms contributing to the induction of the long-lasting physiological changes observed in sexually satiated rats. We have demonstrated that interfering with CB1R activation during sexual satiety development hinders the appearance of the long-lasting sexual behavior inhibition and drug hypersensitivity in rats that copulate to satiety (González-Morales and Rodríguez-Manzo, 2020). Blockade of CB1Rs during intense copulation could have interfered with eCB-LTD but not with mGluR-LTD, since the released glutamate would have activated mGluRs. Present data showing that CB1R blockade during copulation to satiety did not cancel the increase in GluA2-containing AMPARs support this notion.

Although eCB-mediated LTD plasticity phenomena may play a role in the induction of the long-lasting plastic changes that characterize sexually exhausted rats, their dependence on CB1R activation makes eCB-LTD the most likely candidate. Electrophysiological studies should be conducted to evaluate this possibility.

Extracellular signal-regulated receptor kinase (ERK) participates in the induction of some forms of LTD (Gallagher et al., 2004; Grueter et al., 2006; Kellogg et al., 2009) and cannabinoid receptors are known to activate ERK (Derkinderen et al., 2003); therefore, we decided to look for changes in ERK phosphorylation in the VTA of sexually satiated rats that might be related to CB1R activation. Our data failed to detect changes in pERK1/2 expression in the VTA of the copulating males; however, blockade of eCB actions during intense copulation resulted in an increase in VTA pERK1/2 expression in the sexually satiated males, suggesting that the released eCBs hinder the increase in the phosphorylation of this MAP kinase. Contrary to present results, CB1R-mediated ERK1/2 phosphorylation has been shown to occur and to participate in eCB-dependent LTD at inhibitory synapses (I-LTD) onto VTA DA neurons, as a result of repeated cocaine exposure (Pan et al., 2011). We could not find any data on CB1R-mediated ERK1/2 phosphorylation at excitatory synapses; however, it has been reported that sustained CB1R stimulation activates ERK1/2 only transiently and that the duration of ERK1/2 activation is regulated by CB1R desensitization (Daigle et al., 2008). However, in our study the observed increase in pERK1/2 expression in the AM251 pre-treated males did not involve CB1R activation but must have been the result of stimulating other type of receptors, for instance, NMDA glutamate receptors (Krapivinsky et al., 2003).

In sum, the results of the present work show that eCBs are released in the VTA in response to both mild and intense sexual activity; however, only in the rats that copulated to satiety did eCBs induce excitatory synaptic plasticity. These data suggest that the magnitude of eCB release was

different in these two groups. Copulation to satiety appears to have released a larger amount of eCBs in the VTA, which impacted glutamatergic transmission presumably onto DA neurons, although VTA GABA neurons expressing glutamate receptors could also be a target. The nature of the glutamate receptor changes promoted by eCBs is consistent with a reduction in the excitatory effects of glutamatergic input, which might result in a reduced VTA DA and maybe also GABA neuron activity. The present work provides evidence for a physiological role of eCB release from VTA DA neurons *in vivo*, that might represent a protective mechanism against excessive activation produced by heightened natural rewarding stimulation.

CONCLUSION

Evidence implicates eCBs in the induction of the long-lasting physiological changes observed in sexually satiated rats: the sexual inhibition and generalized sensitization to drug actions. The MSL system, specifically the VTA, has been found to play a significant role in the control of these two phenomena (Canseco-Alba and Rodríguez-Manzo, 2014; González-Morales and Rodríguez-Manzo, 2020). CB1Rs on axon terminals synapsing onto DA neurons in this brain region are the target of these eCB-mediated actions and glutamatergic transmission appears to be a principal player. The results of the present series of experiments demonstrate that eCBs are released in the VTA during copulation to satiety and activate CB1Rs producing changes in glutamate receptors that are consistent with a reduction of glutamate excitatory effects onto DA and perhaps GABA neurons.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL) at CINVESTAV.

AUTHOR CONTRIBUTIONS

GR-M conceived and designed the study and wrote the manuscript. EG-M and RG-G performed the experiments and participated in the drafting of the manuscript. EG-M prepared the figures. All authors analyzed the data, discussed the results, and commented on the manuscript.

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frontiers in Synaptic Neuroscience

REVIEW
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Endocannabinoid Modulation of Nucleus Accumbens Microcircuitry and Terminal Dopamine Release

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The nucleus accumbens (NAc) is located in the ventromedial portion of the striatum and is vital to valence-based predictions and motivated action. The neural architecture of the NAc allows for complex interactions between various cell types that filter incoming and outgoing information. Dopamine (DA) input serves a crucial role in modulating NAc function, but the mechanisms that control terminal DA release and its effect on NAc neurons continues to be elucidated. The endocannabinoid (eCB) system has emerged as an important filter of neural circuitry within the NAc that locally shapes terminal DA release through various cell type- and site-specific actions. Here, we will discuss how eCB signaling modulates terminal DA release by shaping the activity patterns of NAc neurons and their afferent inputs. We then discuss recent technological advancements that are capable of dissecting how distinct cell types, their afferent projections, and local neuromodulators influence valence-based actions.

Keywords: dopamine, nucleus accumbens, endocannabinoids, circuitry, optogenetics

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INTRODUCTION

Accurate valence-based predictions and appropriate action sequences are necessary for survival, and disruptions in this process underlie numerous neuropsychiatric disorders. The nucleus accumbens (NAc) is a brain region that is central to valence-based predictions and goal-directed actions, and neuropsychiatric treatments often target neural signaling in the NAc (e.g., Deep Brain Stimulation (DBS), pharmacotherapies). State-of-the-art neuroscience techniques capable of interrogating cell type- and anatomically specific neural elements are continuously clarifying how NAc function controls stimulus encoding and goal-directed behavior. Here, we will discuss ongoing efforts aimed at elucidating NAc control of behavior and propose critical, outstanding questions.

The NAc is a relatively small region of the ventral striatum that relays input from cortical and subcortical "limbic" brain regions onto basal ganglia motor circuits (Groenewegen et al., 1987; Klawonn and Malenka, 2018), allowing emotion to influence action (Mogenson et al., 1980; Floresco, 2015). Dysfunction in this region is also involved in numerous neuropsychiatric disorders including addiction, depression, and chronic stress (Russo and Nestler, 2013; Rudebeck et al., 2019). Seminal work aimed at understanding the function and dysfunction of the NAc has identified separate cell populations based on molecular profile, electrophysiological properties, or output target that differentially influence valence-based behaviors (Gerfen and Surmeier, 2011; Lobo and Nestler, 2011). This classification system of striatal neurons has massively influenced our understanding of the neurobiology of motivated behavior, disease, and its treatment (Alexander et al., 1986; Albin et al., 1989). However, this overall framework implies separate homogenous cell

populations that do not exist in the NAc. Rather than distinct cell populations defining specific circuits that serve separate functions, the NAc controls behavior through coordinated interactions between complex microcircuits, consisting of diverse cell types with different afferent inputs and output targets. For the purposes of this review, we will focus on how neuromodulatory systems interact with local NAc microcircuitry and associated networks to influence valence processing and associated actions.

Another common misconception is that the NAc functions as a "reward center." Support for this idea is based on evidence that disruptions in NAc function through experimental manipulation or disease diminish goal-directed action and predictably reduce reward seeking (Ungerstedt, 1976; Zhou and Palmiter, 1995). Nevertheless, targeted inactivation of the NAc using lesions or pharmacology does not disrupt hedonic reactions (Berridge, 2007) or appetitive responding when the reinforcement contingency is simple or reward cost is low (Parkinson et al., 2002; Corbit and Balleine, 2011). Moreover, the NAc is required for overcoming physical or cognitive costs in pursuit of reward (Salamone and Correa, 2002) or to avoid aversive stimuli (Pezze and Feldon, 2004; Klawonn and Malenka, 2018). Thus, rather than a "reward-region," the NAc appears to more generally allow stimuli associated with motivationally salient events to invigorate the initiation and continuation of approach or avoidance behavior. The neural mechanisms that mediate this rather broad and still somewhat poorly defined role remain difficult to pinpoint due to the highly integrative architecture of the NAc, receiving input from cortical and subcortical regions that is filtered by complex interactions within the NAc.

In this review, we will focus on two important filters of NAc function: dopamine (DA) and endocannabinoid (eCB) neuromodulators. DA release in the NAc controls motivated action (Salamone and Correa, 2002) and DA dysfunction in this region contributes to numerous neurological and neuropsychiatric disorder that are characterized by aberrant forms of goal-directed behavior (Russo and Nestler, 2013). Additionally, alterations in NAc eCB signaling are indicated in a variety of disorders (Araque et al., 2017; Patel et al., 2017). As reviewed previously (Oleson et al., 2012; Covey et al., 2014, 2015, 2017), eCB signaling modulates midbrain DA neuron activity to shape downstream DA concentration changes in the NAc during goal-directed action. Here, we will discuss ongoing work investigating how eCB signaling influences neural circuitry within the NAc to locally shape terminal DA release. Specifically, we will cover the complex neural architecture of the NAc (section "Neural Architecture of the NAc") and how eCB signaling modulates interactions between various NAc cell types (section "Endocannabinoid Control of NAc Microcircuits") to locally influence DA release (section "DA Signaling in the NAc"). We conclude that an understanding of how the NAc controls action cannot be accomplished by isolating individual components of NAc function (e.g., cell firing, receptor binding), but by dissecting how various cell types, their afferent projections, and local modulators interact to control complex behaviors. We end by discussing recent technological advancements that will facilitate this endeavor (section "Conclusions, Caveats, and Future Directions").

NEURAL ARCHITECTURE OF THE NAc

The NAc is bound medially by the septum, ventrally by the olfactory tubercle, and extends rostrally from the bed nucleus of the stria terminalis to the rostral pole (Zahm and Brog, 1992). The NAc is generally subdivided into the more dorsal core (NAcC) and ventral shell (NAcSh) subregions (Zaborszky et al., 1985) that can be immunohistochemically distinguished based on expression of several proteins, including calcium-binding protein calbindin D28k (core > shell), acetylcholinesterase (shell > core), and substance P (shell > core) (Zahm and Brog, 1992). The cellular architecture of the NAc consists primarily of medium spiny neurons (MSNs), which constitute ∼95% of the cell types in the NAc and 100% of the projection neurons (Meredith et al., 1993; Meredith, 1999). Although small in number relative to MSNs, separate classes of NAc interneurons with distinct molecular profiles and electrophysiological properties exert a profound influence on NAc cell excitability and terminal neurotransmitter release. We will provide a brief overview of each neuron population below but refer readers to excellent reviews for a more detailed discussion (Tepper et al., 2018; Castro and Bruchas, 2019; Robinson and Thiele, 2020; Schall et al., 2021).

Medium Spiny Neurons (MSNs)

Medium spiny neurons–or spiny projection neurons (SPNs)–are named as such based on their medium size (∼15 um diameter) and dendritic arbors that are covered in small membrane protrusions [i.e., spines; (Kawaguchi, 1993; Meredith, 1999)]. MSNs exhibit low baseline firing rates (∼1 Hz) *in vivo* (O'Donnell et al., 1999) and are resistant to excitation due to intrinsic properties combined with afferent control. NAc MSNs possess an inward rectifying K^+ conductance and hyperpolarized resting membrane potential (−70 to −85 mV) and are subject to substantial tonic inhibition from GABAergic interneurons. Thus, strong excitatory input, is required to reach spike threshold and drive MSN output. This cellular architecture is conceptualized as a filter that allows transmission of only the most salient information onto downstream motor nuclei of the basal ganglia.

Medium spiny neurons are segregated into two populations according to whether they express D1- or D2-type DA receptors (Gerfen and Surmeier, 2011; Lobo and Nestler, 2011). D1- and D2 type receptors are G-protein couple receptors (GPCRs) that differ in their downstream signaling cascades. D1-type receptors (including D1 and D5) are "excitatory" in that they positively couple to $G\alpha_s$ and stimulate adenylyl cyclase (AC) signaling cascades. Conversely, D2-type receptors (including D2, D3, and D4) are "inhibitory" in that they positively couple to $G\alpha_{i/o}$ to inhibit AC production and reduce cell excitability (Lachowicz and Sibley, 1997). It should be noted that DA receptor binding has no direct effect on cell firing, but modulates how synaptic transmission alters cell function by positively or negatively influencing voltage-dependent conductance. Both MSN populations are distributed equally throughout most of

the NAc, although parts of the medial NAcSh express relatively low-levels of D2-MSNs (Gangarossa et al., 2013). Few striatal MSNs express both receptors, but D1/D2 co-expression increases along a ventromedial gradient from 7.3% in NAcC to ∼14.6% in NAcSh (Surmeier et al., 1996; Gagnon et al., 2017). As we discuss below (see section "Non-canonical Neuromodulator Control of DA Release"), the two MSN populations can also be distinguished based on neuropeptide signaling mechanisms.

In addition to DA receptor expression, MSNs are also differentiated by their projection target. D1-MSNs preferentially project to the ventral mesencephalon, including dense synapses onto GABA and DA neurons in the ventral tegmental area (VTA). In contrast, D2-MSNs preferentially target GABA neurons in the ventral pallidum (VP), which creates an indirect pathway to the VTA (Lobo and Nestler, 2011). These dichotomous pathways are akin to the "direct" and "indirect" pathways in the dorsal striatum that define the basal ganglia motor loops (Gerfen and Surmeier, 2011; Kravitz et al., 2012). Molecular and anatomical differences are often proposed to allow D1- and D2-MSNs in the NAc to exert opposing control over behavior. In support of this model, optogenetic activation of NAc D1-MSNs facilitates a cocaine conditioned place preference (CPP) (Lobo et al., 2010; Koo et al., 2014), whereas chemogenetic inhibition of D1-MSNs (Calipari et al., 2016) or D2-MSN activation suppresses cocaine CPP (Lobo et al., 2010; Koo et al., 2014). Moreover, D2-MSN signaling is required for avoidance learning and D1-MSNs support reward learning (Hikida et al., 2010, 2013).

The functional distinction between D1- and D2-MSN populations–D1 projections to the midbrain promote approach while D2 projections to the VP facilitate avoidance–is supported by numerous studies. However, this approach/avoidance segregation between MSN populations is over-simplified. First, while D1-MSNs projecting from the NAcC to the VTA express only the D1 receptor, ∼50% of D1-MSNs also project to the VP along with D2-MSNs (Lu et al., 1998; Kupchik et al., 2015; Soares-Cunha et al., 2020). Activity between the two populations is also interconnected through local GABAergic collaterals: D1-MSNs project onto other D1-MSNs but D2-MSNs project onto both D1- and D2-MSNs (Taverna et al., 2007). Additionally, D1 and D2-MSN activity–based on fiber photometry measurements of Ca^{2+} signaling–are dramatically different in the medial NAcC during a cocaine CPP task (Calipari et al., 2016), while Ca^{2+} signaling in the lateral NAc during an operant lever pressing task is strikingly similar between D1- and D2-MSNs (Natsubori et al., 2017). Thus, D1- and D2-MSN activity levels exhibits regional and context-dependent similarities and differences, rather than a dichotomous profile/phenotype.

Moreover, optogenetic manipulation studies are inconsistent (**Table 1**). Notably, both D1- and D2-MSNs (Soares-Cunha et al., 2016) and their projections to the VP (Soares-Cunha et al., 2018), facilitate the motivation to work for sucrose reinforcement (Soares-Cunha et al., 2016). Moreover, both populations reinforce behavior, as measured with optogenetic intracranial self-stimulation (ICSS), although reinforcement rate is much greater in D1 versus D2 MSNs (Cole et al., 2018). Alternatively, optogenetic activation of D1-MSNs supports a CPP, while D2-MSN activation has no effect (Cole et al., 2018) or is aversive (Soares-Cunha et al., 2020). However, elegant work by Soares-Cunha et al. (2020) demonstrates that optogenetic activation of both D1- and D2-MSNs supports a place preference or aversion, depending on stimulation parameters. Overall, rather than exerting diametric control over goal-directed behavior by driving approach or avoidance, both MSN populations support a more nuanced and overlapping role in various aspects of motivated actions that is unlikely to be recapitulated by bulk measures or manipulations of population-level signaling dynamics during rudimentary behavioral assays (e.g., place preference, fear conditioning).

Excitatory Inputs

As noted above, action potential generation in NAc MSNs relies on excitatory input. Glutamatergic projections arise from numerous regions including, but not limited to, anterior cortical areas, amygdala, hippocampus, and thalamus (Goto and Grace, 2005; Sesack and Grace, 2010; Russo and Nestler, 2013). For the purposes of this review, we will focus our discussion on the more widely studied excitatory NAc projections arising from the prefrontal cortex (PFC), basolateral amygdala (BLA), and ventral hippocampus (vHPC). Anatomically distinct glutamatergic inputs to the NAc are proposed to relay distinct forms of environmental information; the vHPC encodes contextual information, the BLA relays emotionally salient events, and the PFC signals value (Everitt and Wolf, 2002; Kelley, 2004; Pennartz et al., 2011; Russo and Nestler, 2013). Inputs are also anatomically distributed across the NAc; cortical areas preferentially innervate the NAcC, while the vHPC preferentially projects to the NAcSh, and the BLA projects equally to both (Britt et al., 2012; Li Z. et al., 2018; Deroche et al., 2020). However, there is striking overlap in the density and behavioral influence of these glutamatergic inputs. First, all regions similarly innervate D1- and D2-MSNs (Barrientos et al., 2018; Li Z. et al., 2018). Moreover, optogenetic activation of NAc inputs from either PFC, vHPC, or BLA is reinforcing, as measured by ICSS (Britt et al., 2012; Mateo et al., 2017). The activity patterns of these separate NAc inputs (as measured by fluorescent Ca^{2+} sensors) are also strikingly similar during reward-seeking tasks, and their optogenetic activation similarly reduces food consumption (Reed et al., 2018). Accordingly, and for the sake of simplicity, we depict each region as a single glutamate source in our NAc functional connectivity map (**Figure 1**) and receptor interaction diagram (**Figure 2**).

Despite the general functional overlap among glutamatergic inputs to the NAc, heterogeneity emerges in the input-output connections of separate projections. Deroche et al. (2020) found that excitatory synaptic strength–as measured by the amplitude of optogenetically evoked excitatory post-synaptic currents (oEPSCs) or the probability of post-synaptic action potential generation–varied according to the afferent input and target subpopulation. Excitatory synaptic strength from the BLA was greatest onto D1-MSNs, while the PFC and vHPC exhibited stronger inputs to D2-MSNs (Deroche et al., 2020). However, separate work using similar approaches found substantially stronger inputs from the vHPC onto D1-MSNs (MacAskill et al., 2012; Scudder et al., 2018) due to more

TABLE 1 | Optogenetic manipulations of NAc neurons.

Target	Opsin	Site	Freq (Hz)	Pulse (ms)	Power (mW)	Pattern	Task	Effect	References
D1-MSN	ChR2	Shell	10	NR	2–4	3 min ON/5 min OFF	Coc CPP	⬆	Lobo et al., 2010
	ChR2	Shell	10	100	16–28	3 min ON/5 min OFF	Morph CPP	⬆	Koo et al., 2014
	ChR2	Shell	4	5	10	Continuous	RTPP	⬆	Lafferty et al., 2020
	ChR2	Core	40	12.5	10	Cue-paired (1 s)	PR	⬆	Soares-Cunha et al., 2016
	ChR2	Core	Constant	NR	1	1 s /Lever press	ICSS	⬆	Cole et al., 2018
	ChR2	Core	Constant	NR	1	1 s /Movement	RTPP	⬆	Cole et al., 2018
	ChR2	Core	40	12.5	5	1 s ON/59 s OFF	CPP	⬆	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 min ON/1 min OFF	CPP	⬇	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 s ON/59 s OFF	Coc CPP	⬆	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 min ON/1 min OFF	Coc CPP	⟷	Soares-Cunha et al., 2020
	eArcht3.0	Shell	Constant	NR	10	8 min ON	Feeding	⬆	Lafferty et al., 2020
D2-MSN	ChR2	Shell	10	NR	2–4	3 min ON/5 min OFF	Coc CPP	⬇	Lobo et al., 2010
	ChR2	Shell	10	100	16–28	3 min ON/5 min OFF	Morph CPP	⬇	Koo et al., 2014
	ChR2	Shell	4	5	10	Continuous	RTPP	⬇	Lafferty et al., 2020
	ChR2	Core	40	12.5	10	Cue-paired (1 s)	PR	⬆	Soares-Cunha et al., 2016
	ChR2	Core	Constant	NR	1	1 s /Lever press	ICSS	⬆	Cole et al., 2018
	ChR2	Core	Constant	NR	1	1 s /Movement	RTPP	⟷	Cole et al., 2018
	ChR2	Core	40	12.5	5	1 s ON/59 s OFF	CPP	⬆	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 min ON/1 min OFF	CPP	⬇	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 s ON/59 s OFF	Coc CPP	⬆	Soares-Cunha et al., 2020
	ChR2	Core	40	12.5	5	1 min ON/1 min OFF	Coc CPP	⬇	Soares-Cunha et al., 2020
	eNpHR3.0	Core	Constant	NR	15	Cue-paired (10 s)	PR	⬇	Soares-Cunha et al., 2016
	eArcht3.0	Shell	Constant	NR	10	8 min ON	Feeding	⬆	Lafferty et al., 2020
FSIs	ChR2	Shell	20	10	5	Continuous	RTPP	⬇	Qi et al., 2016
	ChR2	Core	20	15	10	0.5 s ON/9.5 s OFF	CPP	⬆	Chen et al., 2019
	ChR2	Core	20	15	10	0.5 s ON/9.5 s OFF	LiCl CPA	⬇	Chen et al., 2019
	eNpHR3.0	core	Constant	NR	3	ITI (2.5–20 s)	5-CSRTT	⬆	Pisansky et al., 2019
CINs	ChR2	Shell	15	5	9–10	2 s ON/2 s OFF	Coc CPP	⬇	Lee et al., 2016
	ChR2	Shell	15	5	9–10	Continuous in one	RTPP	⟷	Lee et al., 2016
	ChR2	Shell	15	5	9–10	2 s /Nose poke	ICSS	⟷	Lee et al., 2016
	ChR2	Core	10	5	140–200	Continuous	CPP	⟷	Witten et al., 2010
	ChR2	Core	10	5	140–200	Continuous	CPP	⟷	Witten et al., 2010
	ChR2	Core	10	5	10	Cue-paired (120 s)	PIT	⬇	Collins et al., 2019
	eNpHR3.0	Core	Constant	NR	70–140	Continuous	Coc CPP	⬇	Witten et al., 2010
	eNpHR3.0	Core	Constant	NR	70–140	Continuous	CPP	⟷	Witten et al., 2010
	eNpHR3.0	Shell	Constant	NR	2–3	Continuous	RTPP	⟷	Lee et al., 2016
	eNpHR3.0	Shell	Constant	NR	2–3	Continuous	Coc CPP	⬆	Lee et al., 2016
LTSIs	ChR2	Core	20	4	NR	0.5 s ON/9.5 s OFF	Coc CPP	⬆	Ribeiro et al., 2019
	ChR2	Core	20	49	NR	Continuous	Coc CPP	⬇	Ribeiro et al., 2019
	ChR2	Core	20	4	NR	0.5 s ON/9.5 s OFF	CPP	⟷	Ribeiro et al., 2019
	ChR2	Core	20	49	NR	Continuous	CPP	⟷	Ribeiro et al., 2019

Shown are the targeted neuron populations (Target), Opsin, NAc subregion (Site), optogenetic experimental parameters (Opsin), Frequency (Freq), Pulse width (Pulse), Light power (Power), and Stimulation (Pattern), behavioral task (Task), and behavioral effect (Effect; Increase: ⬆, Decrease ⬇:, No Effect: ⟷).
Reported values are from each reference (Ref), unless values were not reported (NR).
Behavioral tasks include drug conditioned place preference (CPP), real-time place preference (RTPP), and intracranial self-stimulation (ICSS) to assess hedonic processing or positive reinforcement; progressive ratio (PR) operant schedule to assess motivation; lithium-chloride conditioned place aversion (LiCl CPA) to assess avoidance; 5-choice serial reaction time task (5-CSRTT) to assess impulsivity; and Pavlovian-to-instrumental transfer (PIT) to assess conditioned motivation.

proximal synaptic connections at D1- versus D2-MSN dendrites (MacAskill et al., 2012). Because optogenetic excitation often relies on viral transduction, contrasting findings may arise due to slight differences in injection site or viral infectivity. Moreover, genetically- and functionally-distinct subpopulations of glutamatergic inputs arising from the same projection region differentially affect behavior. Optogenetic activation of BLA excitatory projections to the NAc following expression of ChR2 under the regulatory elements of the CaMKIIα promoter is reinforcing (Stuber et al., 2011). However, Shen et al. (2019) recently identified a separate, non-overlapping population of BLA to NAc glutamatergic projections that express the

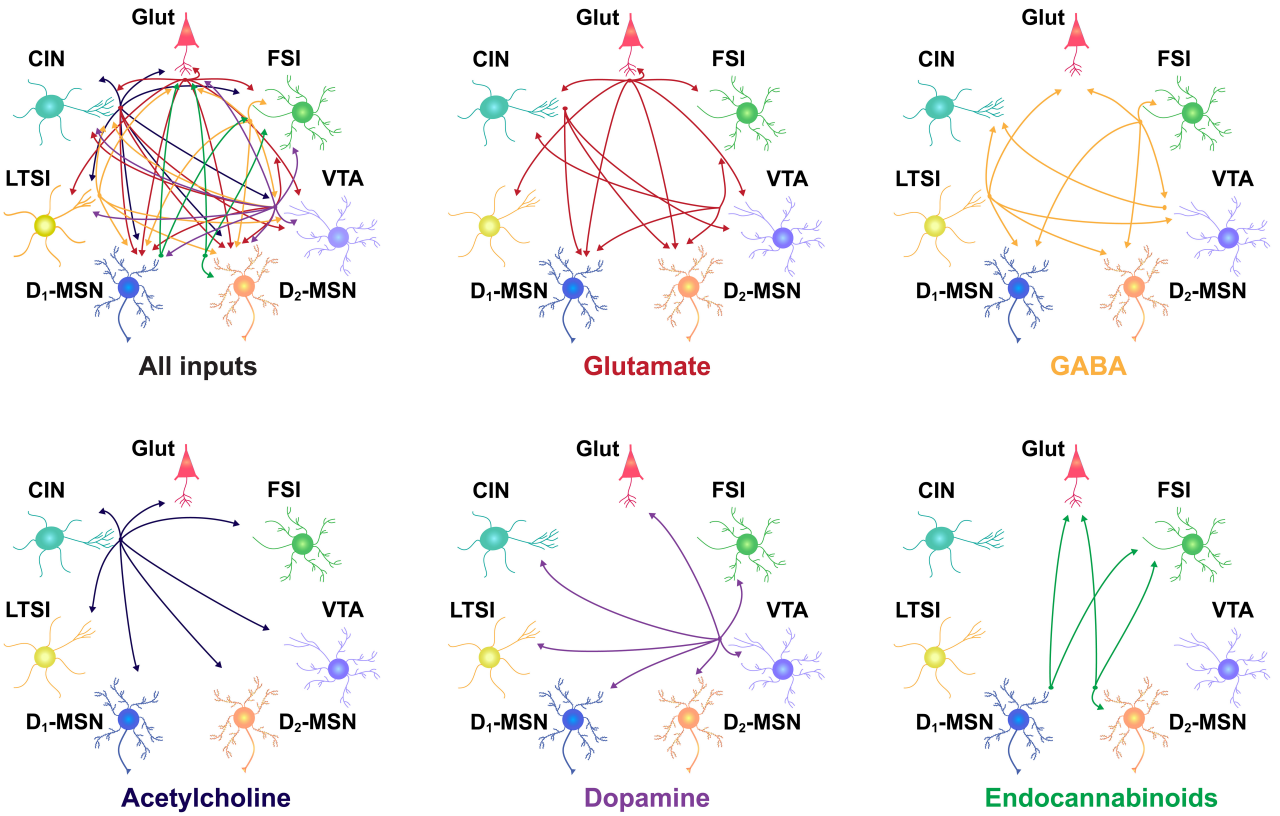

FIGURE 1 | Neurochemical communication in the NAc. Neurotransmitter release from separate cellular populations in the NAc, including glutamatergic (Glut) neurons from throughout the brain, fast-spiking interneurons (FSIs), the ventral tegmental area (VTA), D2- and D1-medium spiny neurons (MSNs), low-threshold spiking interneurons (LTSIs), and cholinergic interneurons (CINs). Input-output relationship is depicted by the direction of the arrow and separated according to neurotransmitter molecule, listed below each diagram.

neuropeptide cholecystokinin (CCK) (Shen et al., 2019). These CCK-expressing projections from the BLA preferentially innervate D2-MSNs in the NAc and optogenetic activation of this circuit is aversive, highlighting the critical importance of dissecting the anatomical and molecular properties of separate NAc circuits. As will be discussed below (section "Endocannabinoid Control of NAc Microcircuits"), the strength of input- and output-specific NAc projections are also distinguished based on their regulation by eCB signaling.

GABAergic Interneurons

One GABAergic interneuron population is often distinguished based on the expression of the calcium binding protein parvalbumin (PV) and are therefore referred to as PV interneurons. We will use the term fast-spiking interneurons (FSIs) here because a population of GABAergic NAc interneurons display electrophysiological properties characteristic of FSIs (see section "Endocannabinoid Control of NAc Microcircuits") and release GABA, but do not express PV (Winters et al., 2012; Schall et al., 2021). Similar to MSNs, NAc FSIs are "medium" sized (Kawaguchi, 1993), lack spontaneous activity *in vitro*, and rely on excitatory input from similar brain regions for action potential generation, including the PFC (Yu et al., 2017), BLA (Yu et al., 2017), vHPC (Yu et al., 2017; Scudder et al., 2018; Trouche et al., 2019), and VTA (Qi et al., 2016). However, excitatory synaptic strength is much greater onto FSIs compared with neighboring MSNs (Wright et al., 2017; Yu et al., 2017; Scudder et al., 2018), resulting in high maximal firing rates, upward of ∼150 Hz (Taverna et al., 2007), and sustained feedforward inhibition of target MSNs *in vivo*. While lateral inhibition from MSN collaterals is prominent in the dorsal striatum (Chuhma et al., 2011), FSIs provide the major source of inhibitory control in the NAc (Qi et al., 2016; Wright et al., 2017; Yu et al., 2017; Schall et al., 2021). FSIs also form electrical and chemical FSI-to-FSI connections (Winters et al., 2012), allowing synchronized and widespread inhibitory control over both D1- and D2-MSNs (Scudder et al., 2018). Notably, FSIs do not make synaptic contacts with low threshold spiking interneurons (LTSIs) or cholinergic interneurons (CINs) (Straub et al., 2016). Recent work shows

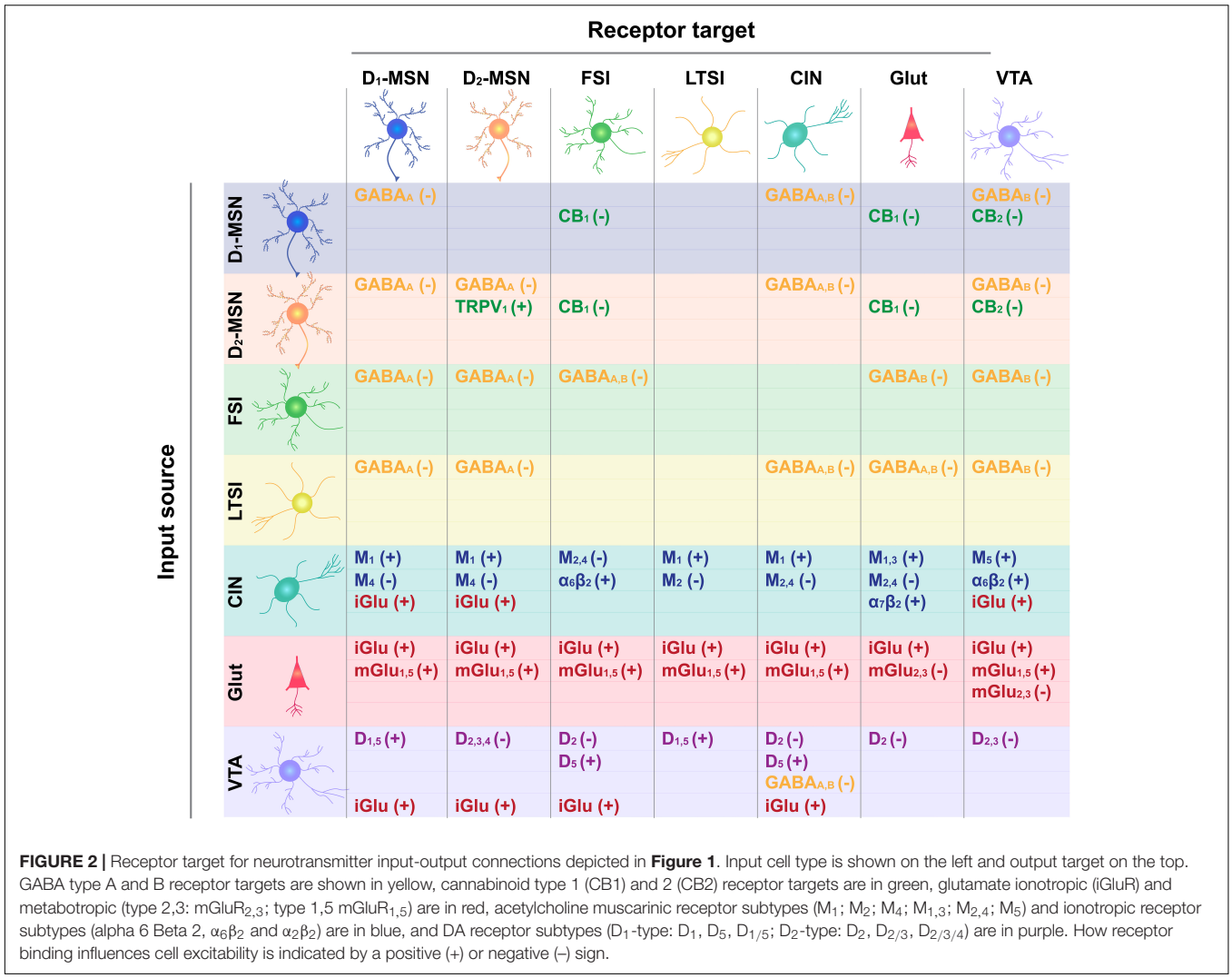

FIGURE 2 | Receptor target for neurotransmitter input-output connections depicted in **Figure 1**. Input cell type is shown on the left and output target on the top. GABA type A and B receptor targets are shown in yellow, cannabinoid type 1 (CB1) and 2 (CB2) receptor targets are in green, glutamate ionotropic (iGluR) and metabotropic (type 2,3: $mGluR_{2,3}$; type 1,5 $mGluR_{1,5}$) are in red, acetylcholine muscarinic receptor subtypes (M_1; M_2; M_4; $M_{1,3}$; $M_{2,4}$; M_5) and ionotropic receptor subtypes (alpha 6 Beta 2, $\alpha_6\beta_2$ and $\alpha_2\beta_2$) are in blue, and DA receptor subtypes (D_1-type: D_1, D_5, $D_{1/5}$; D_2-type: D_2, $D_{2/3}$, $D_{2/3/4}$) are in purple. How receptor binding influences cell excitability is indicated by a positive (+) or negative (–) sign.

that FSIs also gate NAc function via $GABA_B$ receptors located on glutamatergic inputs that preferentially synapse onto D1-MSNs (Manz et al., 2019). Whether this $GABA_B$-mediated inhibition differs across glutamatergic projections or post-synaptic interneuron populations is not clear. As will be discussed below (section "Endocannabinoid Control of NAc Microcircuits"), FSIs can be further distinguished as the only NAc neuronal population that expresses the cannabinoid type 1 (CB1) receptor within the NAc (Winters et al., 2012).

While FSIs provide robust inhibitory control over NAc neuronal activity, establishing how FSIs influence behavior has been difficult. Qi et al. (2016) found that direct optogenetic activation of NAc PV-expressing FSIs or their glutamate inputs, promotes place aversion. In contrast, Chen et al. (2019) found optogenetic activation of this neuronal population elicits a place preference and their inhibition is aversive. Discrepancies between these two studies may be ascribed to different optogenetic stimulation protocols (**Table 1**). Altogether, differences across studies highlight that behaviorally relevant patterns of FSI activity–which are highly dynamic and uncoordinated during reward-seeking tasks (Berke, 2008)–may be difficult to recapitulate using synchronous, population-wide activation or inhibition.

A second GABAergic interneuron population are the LTSIs. Similar to FSIs, LTSIs are medium-sized (9–24 um) neurons that lack dendritic spines (i.e., "aspiny"), receive afferent input from similar regions as MSNs (Ribeiro et al., 2019), and control information flow into and out of the NAc via local GABAergic modulation. LTSIs are named as such based on their low-threshold Ca^{2+} spike that, in conjunction with their relatively depolarized membrane potential (∼−50 mV) and spontaneous activity patterns, supports a highly excitable neuronal population (Scudder et al., 2018; Tepper et al., 2018; Robinson and Thiele, 2020). Striatal LTSIs send long axonal projections (up to 1 mm) that synapse onto distal dendrites of D1- and D2-MSNs and CINs (Straub et al., 2016). Similar to FSIs, LTSIs do not show biased input onto D1- versus D2-MSNs (Scudder et al., 2018). This anatomical arrangement allows LTSIs to influence NAc output over large distances, but their effect on MSN spiking activity is weaker compared to the more proximal synapses formed

by FSIs. In addition to GABA, LTSIs are also highly enriched in the neuropeptides somatostatin (SOM), the neuropeptide Y (NPY) receptor, and nitric oxide synthase (NOS), and are often categorized as the SOM/NPY/NOS+ interneurons (Kawaguchi, 1993; Tepper et al., 2018).

Targeted manipulations of LTSI subpopulations indicate an important role in motivated behavior. Optogenetic activation of SOM-expressing NAc neurons enhances a cocaine CPP, which is suppressed by optogenetic inhibition (Ribeiro et al., 2019). Chemogenetic stimulation of NOS-expressing interneurons in the NAc facilitates the acquisition rate of cocaine self-administration (Smith et al., 2017) and sucrose reinforcement (Bobadilla et al., 2017). Finally, NPY infusions into the NAc produce a CPP (Brown et al., 2000), elevate extracellular dopamine levels measured with microdialysis (Sorensen et al., 2009), and increase motivation to seek and consume sucrose (van den Heuvel et al., 2015). As noted above, SOM, NOS, and NPY are typically expressed in the same LTSI cells along with GABA (Tepper et al., 2018). Thus, manipulations that use any one of these molecular markers to target one subpopulation of LTSIs will likely alter multiple modes of neuromodulatory signaling. Moreover, common optogenetic approaches are optimized for manipulating target populations on fast timescales that may not mimic endogenous neuropeptide or gaseous transmitter signaling.

Cholinergic Interneurons (CINs)

Cholinergic interneurons can be distinguished from other NAc neuron populations by their much larger cell bodies (∼20–30 um diameter), long (up to 1 mm) axonal branches, and expression of the acetylcholine (ACh) synthesizing enzyme choline acetyltransferase. Striatal CINs rest at a relatively depolarized potential (∼−60 mV) (Bennett et al., 2000; Gonzales and Smith, 2015) and exhibit tonic firing patterns (∼2–10 Hz) (Zhou et al., 2002). Accordingly, CINs are also referred to as "Tonically Active Neurons" (TANs). Afferent input to CINs arises from similar extra-striatal regions as MSNs and local LTSIs, but not from FSIs or other CINs (Guo et al., 2015). Notably, the majority of synaptic connections onto striatal NAc CINs are GABAergic (Gonzales and Smith, 2015), including extrinsic GABAergic input from the VTA (Brown et al., 2012).

Cholinergic interneurons exert complex actions on NAc microcircuitry through ACh release onto various cholinergic receptor subtypes, including ionotropic nicotinic receptors (nAChRs) and metabotropic muscarinic receptors (mAChRs) that are located on axon terminals and somatodendritic compartments (Gonzales and Smith, 2015). nAChRs form pentameric ion (Na^+, K^+, and Ca^{2+}) channels consisting of a combination of α (α2–α10) and β (β2–β4) subunits, with the α6/β2 variety forming the predominant type in the NAc (Zhou et al., 2002; Exley and Cragg, 2008). Analogous to D1- and D2-MSNs, mAChRs can be separated into two categories; $G_{q/11}$-coupled M1-like receptors (M1, M3, and M5) that enhance internal calcium release through stimulation of phospholipases, and the $G_{i/o}$-coupled M2-like receptors (M2 and M4) that block calcium channel activity by reducing cyclic AMP formation through the inhibition of adenylyl cyclase (Gonzales and Smith, 2015). Moreover, the vast majority of CINs express the vesicular glutamate transporter-3 (vGlut3) and can release glutamate onto both ionotropic and metabotropic glutamate receptors in the NAc (Nelson et al., 2014; Mateo et al., 2017).

The variety of receptor subtypes and binding sites allows CINs to exert direct or indirect excitatory and inhibitory effects on NAc circuitry via pre- or post-synaptic targets. For example, CINs can excite output targets via nAChRs on glutamatergic, GABAergic, or dopaminergic neurons (Nelson et al., 2014; Mateo et al., 2017) or through M1 mAChRs expressed on MSNs and GABAergic interneurons (Abudukeyoumu et al., 2019). Accordingly, feedforward excitation or inhibition is possible through potentiation of glutamate or GABA signaling, respectively. Witten et al. (2010) found optogenetic excitation of NAc CINs inhibited MSN firing via nAChR-dependent activation of GABA release, although the source of GABAergic input is unclear. Alternatively, Mateo et al. (2017) found that CIN activation elicited a nAChR-dependent activation of PFC glutamatergic input to MSNs. In contrast, binding the inhibitory M2/M4 receptors on axon terminals can suppress pre-synaptic neurotransmitter release from glutamatergic, GABAergic, or dopaminergic terminals, and from CINs themselves (Threlfell et al., 2012; Shin et al., 2017), leading to an inhibitory or dis-inhibitory effect. Moreover, by acting as a diffuse, volume transmitter that coordinates activity over large distances, CIN-evoked acetylcholine release can simultaneously affect numerous receptor systems in the NAc (Gonzales and Smith, 2015).

Neural manipulation of NAc CINs support a crucial role in reward-related behaviors, although the direction of this effect is unclear (**Table 1**). Optogenetic activation of NAc CINs elicits DA release (Mateo et al., 2017), which generally promotes reinforcement. Yet, optogenetic excitation or inhibition of NAc CINs does not support a place preference, *per se*, but can alter a cocaine CPP. Optogenetic inhibition both suppresses (Witten et al., 2010) and facilitates (Lee et al., 2016) a cocaine CPP, while optogenetic excitation augments a cocaine CPP (Lee et al., 2016) or has no effect (Witten et al., 2010). Suppression of CIN activity using a membrane-tethered toxin against voltage-gated calcium channels leads to anhedonic behaviors, defined as a decrease in sucrose preference and immobility (Warner-Schmidt et al., 2012) and chemogenetic inhibition of NAc CINs suppresses the ability of rewards (food, social interaction, and cocaine) to increase NAc DA release measured by microdialysis (Hanada et al., 2018). As we discuss further in section "Endocannabinoid Control of NAc Microcircuits," the relationship between CIN activity, NAc DA release, and valence-driven behavior remains unclear, but is an intense area of investigation.

ENDOCANNABINOID CONTROL OF NAc MICROCIRCUITS

The eCB system–consisting of fatty acid signaling molecules, their synthetic and degradative enzymes, and constituent

receptors–is a vast signaling network that controls synaptic transmission throughout the brain and periphery (Katona and Freund, 2012; Covey et al., 2017). The primary site of eCB action in the brain is the CB1 receptor, which functions to suppresses pre-synaptic neurotransmitter release via inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), inhibition of adenylyl cyclase (AC), and activation of inwardly rectifying K^{+} channels. CB1 receptor expression exhibits a decreasing dorsolateral to ventromedial gradient in the striatum, with relatively sparse expression in the NAc (Herkenham, 1992; Pickel et al., 2006). Nevertheless, NAc CB1 receptors are critical to short- and long-term forms of synaptic plasticity (Robbe et al., 2002; Grueter et al., 2010) and tightly regulate NAc neurotransmission (Caille et al., 2007) and appetitive behavior (Covey et al., 2017; Mateo et al., 2017). CB1 receptors bind the endogenous fatty acid signaling molecules 2-arachidonoylglycerol (2-AG) and arachidonoyl ethanolamide (AEA, also known as anandamide) (Katona and Freund, 2012; Covey et al., 2017). Both 2-AG and AEA signaling requires their enzymatic synthesis, which is initiated by membrane depolarization, activation of metabotropic receptors coupled to PLCβ (e.g., $G_{q/11}$-coupled group 1 metabotropic glutamate receptor-mGluR1/5, muscarinic acetylcholine-mACh-types M1/M3), and increased intracellular [Ca^{2+}]. However, important differences in 2-AG and AEA signaling allow dissociable functions.

2-Arachidonoylglycerol is more abundant than AEA in most brain regions, is a full agonist for the CB1 receptor, and is the primary eCB involved in CB1-mediated inhibition of pre-synaptic neurotransmitter release (Ohno-Shosaku and Kano, 2014). This canonical mode of CB1 receptor signaling depends on the *de novo* synthesis and retrograde mobilization of 2-AG from post-synaptic sites onto pre-synaptic CB1-expressing terminals. 2-AG mobilization requires the biosynthetic enzyme, *sn*-1-diacylglycerol lipase-alpha (DGLα), which is expressed in the plasma membrane at dendritic spines of NAc MSNs post-synaptic to CB1 receptor-expressing terminals (Matyas et al., 2007). Cessation of 2-AG signaling occurs primarily through pre-synaptic enzymatic degradation via monoacylglycerol lipase (MAGL), which is localized to the pre-synaptic membrane (Long et al., 2009). Thus, 2-AG levels are determined by the balance between post-synaptic production by DAGLα and pre-synaptic degradation by MAGL.

Arachidonoyl ethanolamide synthesis and degradation mechanisms are less understood. While post-synaptic depolarization and intracellular Ca^{2+} influx support AEA synthesis, the precise mechanisms are unclear (Di Marzo and De Petrocellis, 2012). AEA is synthesized by N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase-D (NAPE-PLD), but alternative synthetic pathways exist (Okamoto et al., 2007). AEA signaling is terminated at post-synaptic sites by the membrane-bound fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) and potentially through membrane transport via a lipophilic carrier protein (Ronesi et al., 2004). Moreover, AEA is a partial agonist at both the CB1 receptor and Transient Receptor Potential Vanilloid type 1 (TRPV1) channels, non-selective cation channels expressed by D2 MSNs in the NAc (Deroche et al., 2020). Differences in binding affinity may allow AEA to engage CB1 receptors versus TRPV1 channels in a concentration-dependent manner, such that low AEA levels preferentially bind CB1 receptors while higher concentrations affect TRPV1 channels (Di Marzo and De Petrocellis, 2012).

In the NAc, CB1 receptors are expressed on pre-synaptic glutamatergic terminals from the PFC, BLA, and vHPC (Robbe et al., 2002; Grueter et al., 2010; Winters et al., 2012; Wright et al., 2017; Deroche et al., 2020), but not on thalamic inputs (Wu et al., 2015). CB1 receptors are also on inhibitory GABAergic terminals of FSIs (Winters et al., 2012; Wright et al., 2017), but not LTSIs, MSNs, or CINs (Winters et al., 2012; Mateo et al., 2017). This contrasts with the dorsal striatum where CB1 receptors are expressed by both FSI and MSN collaterals (Freiman et al., 2006), and at lower levels on CINs and LTSIs (Fusco et al., 2004). Moreover, while CB1 receptors in the NAc are exclusively expressed by GABAergic neurons with FSI-like electrophysiological properties, ∼50% of these neurons do not express the characteristic PV marker (Winters et al., 2012), which could arguably define another interneuron subpopulation. Collectively, CB1 receptors act within the NAc to suppress excitatory input from throughout the brain and inhibitory input from FSIs.

Cannabinoid type 1-mediated inhibition of pre-synaptic input occurs across a range of time scales. A short-term depression (STD) (lasting tens of seconds) of glutamate input onto MSNs or FSIs and GABAergic input from FSIs onto MSNs occurs following brief (∼5–10 s) post-synaptic depolarization (Winters et al., 2012; Wright et al., 2017; Yu et al., 2017). This eCB-mediated STD (eCB-STD)–termed depolarization-induced suppression of inhibition (DSI) or excitation (DSE)–arises from retrograde eCB mobilization onto pre-synaptic CB1 receptors and allows fine-tuned regulation of ongoing pre-synaptic input. CB1 receptor signaling in the NAc also supports long-term depression (LTD) of CB1-expressing glutamatergic terminals from the PFC, BLA, and vHPC (Robbe et al., 2002; Grueter et al., 2010; Deroche et al., 2020) and GABAergic terminals from CB1-exprressing FSIs (Wright et al., 2017). eCB-LTD at both excitatory and inhibitory synapses depends on post-synaptic Ca^{2+} signaling, activation of the Gq-coupled mGluR5, post-synaptic TRPV1 channel activation, and pre-synaptic CB1 receptor signaling. The functional relevance of receptor-mediated eCB-LTD in the NAc is indicated by studies demonstrating that mGluR5-dependent eCB-LTD is eliminated following exposure to drugs of abuse, presumably due to occlusion (Mato et al., 2005; McCutcheon et al., 2011). Moreover, blocking mGluR5 signaling in the NAc suppresses drug-seeking in a CB1 receptor-dependent manner (Li X. et al., 2018). Accordingly, mGluR5 receptors may represent a promising target for treating substance-abuse and addiction.

Recent work demonstrates that both forms of eCB-mediated plasticity exhibit dramatic heterogeneity according to the projection site and post-synaptic target. Projection-specific ChR2 expression in the PFC, BLA, and vHPC demonstrated greater eCB-STD at BLA inputs on D1-MSNs and PFC to D2-MSNs inputs, while vHPC inputs on D1- and D2-MSNs were equivalent (Deroche et al., 2020). TRPV1 agonism similarly

led to inhibition of PFC-evoked oEPSCs in D1- and D2-MSNs, vHPC inputs to D1- but not D2-MSNs, and BLA to D2-MSN inputs while augmenting BLA to D1-MSN inputs (Deroche et al., 2020). Further complexity arises from differential CB1 receptor expression on subpopulations of excitatory inputs arising from the same brain region. The CCK-expressing BLA to NAc projections identified by Shen et al. (2019) (see section "Excitatory Inputs") also express CB1 receptors (∼90% overlap). These CCK/CB1-expressing projections preferentially synapse onto D2- but not D1-MSNs, and CB1-mediated inhibition of synaptic activity in this pathway supports resilience to the effects of chronic social defeat stress (Shen et al., 2019), a function long-attributed to the NAc (Fox and Lobo, 2019). Continued technological development will be instrumental in further dissecting the immense synapse-specific heterogeneity in the NAc (section "Conclusions, Caveats, and Future Directions").

DA SIGNALING IN THE NAc

Dopamine neurotransmission in the NAc occurs at *en passant* release sites from dopaminergic axons projecting primarily from the VTA. DA receptors are located on somatic dendrites or axon terminals of all neuron classes in the NAc. The timing and concentration of DA released at target sites arises from a dynamic balance between vesicular release, DA transporter (DAT)-mediated reuptake, and diffusion (Rice and Cragg, 2008). Both release and reuptake are controlled by interactions between membrane excitability, ion channels, G-protein-coupled receptors, and downstream effector molecules, all of which are tightly regulated by local circuit interactions at DA axon terminals (Sulzer et al., 2016; Covey et al., 2017). Recent work argues that NAc DA signaling exerts dissociable effects on behavior depending on the source of modulation, such that cell body-independent mechanisms determine dopaminergic control of motivation, while cell body spiking support DA's canonical role in reward learning (Mohebi et al., 2019). While there remains little evidence to support such an anatomically dissociable role of DA in controlling behavior, the relationship between terminal DA release and cell body firing has long been recognized as non-linear (Rice and Cragg, 2008), and terminal modulation is a primary factor that dictates how DA neurons ultimately influence NAc function. Yet, how DA terminal modulation controls DA concentration dynamics that associate with or drive discrete behavioral events is poorly understood. Below, we will discuss how prominent afferent projections to the NAc and local microcircuitry interact with eCB signaling to modulate DA release.

DA Neuron Autoregulation

Dopamine neurons control their own activity through feedback mechanisms at axon terminals via D2 DA autoreceptor signaling and DAT-mediated reuptake. As discussed above (section "Medium Spiny Neurons (MSNs)"), D2 receptors are coupled to inhibitory $G_{i/o}$-coupled signaling, thus DA autoreceptors function similarly to pre-synaptic CB1 receptors and suppress pre-synaptic neurotransmitter release, in addition to inhibiting DA synthesis and suppressing vesicular packaging (Ford, 2014). D2 autoreceptors also control DAT function through protein-protein interactions that can increase DAT membrane expression and the rate of DAT-mediated DA reuptake (Lee et al., 2007). Below, we will discuss how these autoregulatory functions interact with a variety of incoming signals that act on receptors expressed by DA terminals to alter DA release via modulation of membrane excitability, DAT function, and DA synthesis.

Glutamatergic Control of NAc Dopamine Release

As discussed above (section "Excitatory Inputs"), the NAc receives extensive glutamatergic input from throughout the brain. Glutamate acts on both ionotropic (AMPA, NMDA, and kainate) and metabotropic receptors (mGluRs) that are expressed at somatodendritic and axonal compartments throughout the NAc. Eight types of mGluRs are classified into three groups (Niswender and Conn, 2010) that are either $G_{q/11}$-coupled (Group 1: mGluR1/5) and stimulate AC and phospholipase C (PLC) signaling cascades or Group 2 (mGluR2/3) and Group 3 (mGluR4/6/7/8) that inhibit AC signaling, Ca^{2+} channels, and activate K^+ channels through $G\alpha_{i/o}$ signaling. Both mGluR1/5 and mGluR2/3 are densely expressed at both pre- and post-synaptic sites throughout the NAc (Manzoni et al., 1997). Glutamate signaling alters striatal DA release through direct and indirect actions involving ionotropic and mGluRs. Thus, precisely how glutamate input to the NAc alters terminal DA release is unclear.

For example, local application of ionotropic glutamate receptor agonists (kainate, AMPA, and NMDA) inhibits DA release in the NAc (Yavas and Young, 2017) while AMPA receptor antagonists increase DA released in striatal slices, specifically following high intensity stimulation (10 Hz, 3 s) (Avshalumov et al., 2008). This suppression of DA release arises from AMPA receptor-dependent H_2O_2 generation in striatal MSNs, which acts as a retrograde messenger to inhibit DA release through activation of K^+ channels on DA axons (see section "eCB Control of NAc DA Release" below). Synaptic overflow of glutamate during high intensity stimulation can also inhibit DA release via activation of group 1 (Zhang and Sulzer, 2004) or group 2/3 mGluRs (Yavas and Young, 2017) on DA terminals. An inhibitory effect of glutamate receptor activation could also arise through feedforward inhibition via excitation of GABA neurons (see section "GABAergic Control of NAc Dopamine Release").

In contrast to the inhibitory effect of glutamate, Mateo et al. (2017) recently found that, unlike the dorsal striatum (Chen et al., 1998), DA axons in the NAc express AMPARs and their activation, via local pressure application of AMPA or optogenetic activation of PFC glutamatergic inputs, elicits DA release. However, PFC-evoked DA release remains partly sensitive to nAChR inhibition (Mateo et al., 2017), indicating a contribution from AMPAR-mediated feedforward excitation of NAc CINs (see section "Cholinergic Control of NAc DA Release"). An additional source of glutamatergic modulation arises from VTA projections that co-release DA and glutamate or are exclusively glutamatergic (Zhang et al., 2015). Recent

work demonstrates that, similar to other glutamate inputs, VTA glutamatergic projections reinforce behavior independently of DA co-release (Zell et al., 2020).

GABAergic Control of NAc Dopamine Release

Local regulation of NAc DA release by GABAergic signaling can arise via metabotropic $GABA_B$ receptors located on DA terminals. However, $GABA_B$ antagonists do not alter evoked (single- or pulse train) DA release in NAc slices suggesting a lack of tonic $GABA_B$-mediated inhibition of terminal DA release, although GABAergic tone may be artificially suppressed in slice preparations (Pitman et al., 2014). GABA release from FSIs onto $GABA_B$ receptor-expressing glutamatergic terminals (Manz et al., 2019) also inhibit DA release. Because ionotropic $GABA_A$ receptors are not expressed by DA axons (Sulzer et al., 2016), elevated dialysis DA levels following local $GABA_A$ receptor antagonism (Adermark et al., 2011), or inhibition of DA release in striatal slices by a $GABA_A$ agonist (Brodnik et al., 2019) arise through an indirect mechanism. Indeed, $GABA_A$-mediated inhibition of DA release in NAc slices is blocked by a $GABA_B$ antagonist (Brodnik et al., 2019), indicating that $GABA_A$ receptors can act upstream to modulate GABAergic output onto $GABA_B$ receptors on DA axons. This putative circuit remains to be elucidated. Notably, while GABAergic projections from the VTA preferentially synapse onto NAc CINs (Brown et al., 2012), GABA-mediated inhibition of DA release does not rely on striatal CINs (Pitman et al., 2014). Future work is required for elucidating the circuit- and receptor-specific mechanisms by which GABA transmission modulates NAc DA release.

Cholinergic Control of NAc DA Release

Cholinergic interneurons exert powerful control over dopaminergic transmission in the NAc through extensive axonal branches (section "Cholinergic Interneurons (CINs)") that are closely intermingled with DA varicosities. ACh release from CINs binds ionotropic nAChRs on DA terminals, which increases intracellular Ca^{2+} flux (Zhou et al., 2002; Exley and Cragg, 2008). Accordingly, NAc CINs are capable of directly eliciting DA release *in vivo*, independently of midbrain neuronal activity (Mateo et al., 2017). Ca^{2+} entry through nAChRs may further potentiate DA release through mobilization of readily releasable pools of vesicular DA (Turner, 2004). However, numerous *in vitro* studies indicate that nAChRs control DA release in a complex manner according to ongoing patterns of DA release, increasing DA release evoked by low-frequency stimulation of DA neurons, and inhibiting DA release during high frequency stimulation (Zhou et al., 2002; Zhang and Sulzer, 2004). Thus, when nAChRs are antagonized or become desensitized by nicotine, DA release evoked by high frequency burst firing patterns is potentiated. In support of this *in vitro* work, NAc infusions of a nAChR antagonist increase cue-evoked DA release and invigorate reward-seeking (Collins et al., 2016), while optogenetic stimulation of NAc CINs during cue presentation suppresses cue-evoked reward seeking. Collectively, the *in vivo* measures are largely in agreement with *in vitro* work; nAChRs on DA terminals facilitate DA release when the DA neuronal firing rate is low, such as in anesthetized animals (Mateo et al., 2017), but suppress DA release during periods of increased DA neuron activity, as occurs during specific epochs of reward-seeking sequences (Collins et al., 2016, 2019). An improved understanding of the relationship between ACh transmission and terminal DA release will likely be afforded by recent advancements in optical imaging approaches that permit rapid ACh and DA detection during behavior (Sabatini and Tian, 2020).

Cholinergic interneurons also target mAChRs, which are of the $G_{q/11}$-coupled M5 subtype on DA terminals. Activation of M5 mAChRs in dorsal striatum (Foster et al., 2014) or NAc (Shin et al., 2017) brain slices inhibits DA released by electrical stimulation (Foster et al., 2014), but potentiates DA released by optogenetic activation of DA neurons (Shin et al., 2017). Thus, M5 mAChRs increase DA release through direct actions on DA terminals but can inhibit DA release via a polysynaptic route that is revealed by electrical stimulation. ACh release onto pre-synaptic $G_{i/o}$-coupled M4 mACh autoreceptors in the NAc provides another source of regulation by decreasing CIN output onto nAChRs and potentiating DA release evoked by high frequency firing patterns (Threlfell et al., 2012; Shin et al., 2017). mAChR agonists may also potentiate DA overflow by slowing DAT-mediated DA uptake through an unidentified mechanism (Shin et al., 2017). DA release is also modulated by ACh interactions with glutamate signaling, such that NAc DA release is facilitated by ACh acting at nAChRs on glutamate terminals arising from the PFC (Mateo et al., 2017) or VTA (Shin et al., 2017). Finally, a subpopulation of CINs also express the vesicular glutamate tranporter-3 (vGlut-3) and are thus capable of increasing DA release through glutamate co-transmission (Mateo et al., 2017).

eCB Control of NAc DA Release

Despite the important role of eCB signaling in controlling NAc microcircuitry (section "Endocannabinoid Control of NAc Microcircuits") and modulating DA input to the NAc (Oleson et al., 2012; Covey et al., 2015, 2017), our understanding of how eCBs influence terminal DA release is limited. Because DA neurons do not express CB1 receptors (Julian et al., 2003), the ability of CB1 receptor manipulations to control NAc DA release and motivated action (Oleson et al., 2012; Wang et al., 2015; Covey et al., 2018) has generally been ascribed to CB1-mediated changes in pre-synaptic input onto midbrain DA neurons projecting to the NAc. However, mounting evidence demonstrates that CB1 receptors also control DA release at the level of NAc terminals. For example, voltammetry recordings in striatal brain slices found that CB1 agonists inhibit DA release following pulse-train stimulation (10 Hz, 3 s) of striatal DA terminals (Sidlo et al., 2008). Thus, CB1 receptors can exert a similar effect as nAChRs and H_2O_2 to suppress DA release evoked by more intense stimulations. The CB1-mediated inhibition of DA release depends on H_2O_2-mediated activation of K_{ATP} channels in DA terminals (Sidlo et al., 2008). However, the precise mechanisms by which CB1 receptors control DA release,

including the site of eCB production and receptor binding, remains unclear.

Recent work identified a complex circuit by which eCBs locally control NAc DA release (Mateo et al., 2017). In this study, optogenetic stimulation of CINs elicited NAc DA release recorded with FSCV in anesthetized mice and in brain slices. Moreover, a CB1 agonist was found to inhibit CIN-evoked DA release, although the site of CB1 receptor action is not readily apparently because, as mentioned above, neither CINs nor DA neurons express CB1 receptors. Rather, it was found that CIN stimulation facilitates glutamatergic transmission via pre-synaptic α7-expressing nAChRs located on PFC terminals. Increased glutamate release, in turn, drives DA release through at least two mechanisms: (1) directly via glutamate release onto AMPA receptors located on DA terminals or (2) indirectly through excitation of CINs and activation of nAChRs on DA terminals. In the NAc, CIN-evoked eCB production also occurs through facilitation of glutamate release onto NAc MSNs, which drives eCB mobilization onto CB1 receptor-expressing PFC terminals. Behavioral relevance for CB1 regulation of this pathway was obtained by assessing ICSS for optogenetic stimulation of PFC to NAc inputs, which has previously been shown to support high rates of reinforcement (Britt et al., 2012). ICSS rates were suppressed by inhibiting degradation of the endogenous CB1 receptor ligand 2-AG with an MAGL inhibitor. Notably, an opposite effect on reinforcement rates is observed following MAGL inhibition when behavior is maintained by stimulation of the midbrain (Oleson et al., 2012) or sucrose reinforcement (Covey et al., 2018), highlighting site-specific control of reinforcement by eCB signaling. Moreover, ICSS rates were augmented by pathway-specific CB1 receptor deletion, presumably due to loss of inhibitory feedback onto PFC terminals. Collectively, this work demonstrates that NAc CIN activation evokes DA release, in part, through excitation of CB1 receptor-expressing PFC glutamate terminals.

While the work by Mateo et al. (2017) elegantly dissects complex mechanisms by which eCBs and NAc microcircuitry interact to shape NAc DA release, the behavioral relevance of this circuit remains to be fully elucidated. NAc nAChR signaling suppresses DA release during periods of heightened DA neuron activity (section "Cholinergic Control of NAc DA Release") and in response to motivationally salient stimuli during reward seeking (Collins et al., 2016, 2019). Moreover, CB1 receptor agonists enhance NAc DA release when administered systemically or in the VTA (Oleson et al., 2012; Covey et al., 2018), but suppresses DA release evoked by local stimulation in the NAc (Mateo et al., 2017). Thus, how CIN-evoked DA release and its suppression by CB1 receptors on PFC terminals associates with or drives distinct behaviors remains elusive. Additional questions remain regarding CB1 receptor control of terminal DA release. CB1 receptors are expressed on axon terminals of glutamatergic inputs from the PFC, BLA, and vHPC (Grueter et al., 2010; Deroche et al., 2020), as well as GABAergic FSIs (Wright et al., 2017), but how these inputs, or their modulation by eCBs, controls terminal DA release is not known.

Emerging evidence also indicates that CB2 receptor signaling locally modulates NAc DA release. Unlike CB1Rs, CB2Rs do not alter Ca^{2+} or inwardly rectifying K^{+} channels (Felder et al., 1995). While CB2 receptors have historically been thought to reside primarily in the periphery, with high expression levels in the spleen and immune cells, more recent work has identified CB2 receptors throughout the brain, including the midbrain and striatum (Jordan and Xi, 2019). In contrast to CB1 receptors, CB2 receptors are expressed by DA neurons and their activation inhibits DA neuronal firing (Zhang et al., 2014). Moreover, both systemic and local administration of the CB2 agonist JWH133 into the NAc dose-dependently reduces extracellular DA levels measured with microdialysis (Xi et al., 2011; Zhang et al., 2017). CB2R activation also inhibits DA release in dorsal striatal slices through an mAChR M4-dependent mobilization of 2-AG from D1-MSNs onto CB2 receptors, which are presumably located on DA terminals (Foster et al., 2016). The ability to negatively regulate NAc DA transmission supports the CB2 receptor as a potential target for drug addiction therapy (Jordan and Xi, 2019).

Non-canonical Neuromodulator Control of DA Release

In addition to "classic" neurotransmitters (e.g., glutamate, GABA, Ach), several neuropeptides control DA neurotransmission via alterations in vesicular DA release, transporter-mediated uptake, or degradation. Although MSNs are typically categorized by DA receptor expression (i.e., D1- versus D2-type), MSNs are also differentiated by which endogenous opioid they produce (Castro and Bruchas, 2019). D1-MSNs produce dynorphin (DYN) that functions as the endogenous ligand for the κ-opioid receptors (KORs) while D2-MSNs produce enkephalin (ENK) that binds both Δ-opioid receptors (DORs) and μ-opioid receptors (MORs). Opioid receptors are inhibitory GPCRs (i.e., $G_{i/o}$–coupled) that suppress neuronal function and are expressed throughout the NAc. KORs are expressed on DA terminals (Svingos et al., 2001) and reduce DA transmission by suppressing vesicular release probability and DA synthesis, and increasing the rate of DAT-mediated DA uptake (Thompson et al., 2000; Britt and McGehee, 2008). In contrast, DOR activation generally increases DA release in the NAc through circuit interactions potentially involving inhibition of KOR signaling (Svingos et al., 1999). MORs are the majority of cell types in the NAc and their activation can increase or decrease NAc DA release through a variety of direct and indirect actions (Britt and McGehee, 2008; Gomez et al., 2019).

Low threshold spiking interneurons also control DA release via neuropeptide signaling through the release of SOM and expression of NPY receptors. Infusions of SOM into the NAc potently increases dialysate DA levels, possibly by potentiating NAc glutamate release (Pallis et al., 2001). Similar to SOM, NPY infusions into the NAc elevate extracellular DA levels (Sorensen et al., 2009), produce a place preference (Brown et al., 2000), and increase motivation to seek out and consume sucrose (van den Heuvel et al., 2015). While NPY and SOM influence NAc DA dynamics and behavior, the direct mechanism remains elusive.

Insulin provides another form of neuropeptide control of DA release. Insulin receptors (IRs) are receptor tyrosine kinases that are densely expressed on the majority of CINs (96%) in

the NAc, with limited expression on DA axons (Stouffer et al., 2015). IR binding on CINs increases CIN firing and potentiates DA release via a nAChR binding on DA axons (Stouffer et al., 2015). Somewhat paradoxically, IR binding also increases DA uptake rate, which diminishes the potentiation in vesicular DA release (Stouffer et al., 2015). Notably, insulin regulation of NAc DA release depends on diet, such that IR signaling increases DA release during food restriction, but loses efficacy following a high fat diet (Stouffer et al., 2015).

CONCLUSIONS, CAVEATS, AND FUTURE DIRECTIONS

In this review, we described how varied cell types in the NAc are modulated by numerous afferent projections and local neuromodulators. Yet, how these complex neural circuit mechanisms ultimately influence behavior remains unclear. "Traditional" neural manipulation techniques (e.g., lesions or pharmacology) demonstrate a crucial role for the NAc in goal-directed action, but these approaches are generally unable to isolate specific cell types and circuits. More recent optogenetic developments that permit manipulations or measurements of genetically defined cell types represent an important advancement in differentiating the heterogenous signaling modalities in the NAc. However, these approaches generally still lack the requisite resolution for identifying how individual cells, their afferent inputs, and local neuromodulators interact in a spatiotemporally specific manner to control output targets and influence behavior. First, synchronized neuronal manipulations may identify that a target population *can* affect behavior, but this likely does not reflect how separate neurons with unique firing patterns and distinct synapse-specific connections (i.e., neuronal "ensembles" expanded upon in section "Characterization and Manipulation of Defined Neuronal Ensembles") normally control behavior. This is clearly highlighted by work showing that optogenetic activation of D1- or D2-MSNs (Hikida et al., 2010, 2013; Lobo et al., 2010; Koo et al., 2014; Soares-Cunha et al., 2020) or FSIs (Chen et al., 2019) in the NAc can promote reward or aversion depending on the stimulation pattern (see **Table 1**). Moreover, any neuronal population is unlikely to be deterministic in isolation, and direct activation or inhibition of targeted cell types does not reflect normal modes of receptor-mediated synaptic transmission, which occurs across varied spatial and temporal scales on a variety of receptor systems. Finally, genetic profile is not deterministic. Spatially intermixed neurons may display a similar molecular profile, but possess distinct input-output connectivity and perform functionally heterogeneous roles (see section "Characterization and Manipulation of Defined Neuronal Ensembles"). Below, we will briefly discuss recent advancements that are building upon existing technology to better understand neural circuit control of behavior.

Fluorescent Detection and Modulation of Neuronal Excitation

The degree to which neural manipulation techniques inform how the brain controls behavior relies on the fidelity by which these approaches mimic endogenous neural mechanisms. A number of genetically encoded fluorescent sensor techniques have been developed that can inform neural manipulation approaches by permitting cell type- and circuit-specific monitoring of numerous neural signaling mechanisms. We refer the reader to excellent reviews that offer a more in-depth discussion on this topic (Wang et al., 2018; Sabatini and Tian, 2020). These techniques rely on genetically modified fluorescent proteins that emit light following a conformational change in response to a specific cell signal, such as a change in membrane potential, ion flux, intracellular signal transduction, or receptor binding. Fluorescent detection of electrical signaling in the brain is accomplished using genetically encoded voltage indicators (GEVIs) (Knopfel and Song, 2019). GEVIs are able to provide information-rich readouts of continuous hyperpolarizing and subthreshold depolarizing signals with subcellular resolution. However, a number of technical hurdles related to adequate signal-to-noise ratios and stable expression in the plasma membrane of live brain tissue currently limits their widespread use in behaving animals. Alternatively, genetically encoded Ca^{2+} indicators (GECIs) such as GCaMPs are widely used *in vivo* to measure intracellular Ca^{2+} flux, which is often interpreted as a proxy of cell firing or neurotransmitter release (Pal and Tian, 2020). Similar intracellular signaling sensors have been developed for monitoring second messenger signals, such as kinases or GTPases (Rost et al., 2017). In recent years, a large number of receptor-based sensors that convert ligand binding into fluorescence emission have been developed to study neurochemical signaling (Sabatini and Tian, 2020). These tools dramatically expand the scope of neurochemical sensing technology, offering rapid detection of numerous ligands. Continued development and implementation of fluorescent sensors with improved signal-to-noise ratios, faster kinetics, and expanded spectral range will permit sensitive and simultaneous detection of various neural signaling mechanisms during behavior.

While improved neural monitoring technology can theoretically better inform cell manipulation approaches, it is important to consider the differences between what is being measured and manipulated. For example, measures of Ca^{2+} flux are not a direct readout of action potential generation, which is often what is targeted with common optogenetic manipulations (e.g., ChR2). Another major issue with interpreting direct manipulations of neuronal function (e.g., opto- or chemogenetic approaches) is that these methods bypass normal modes of synaptic neurotransmission. This is particularly important because most neurons–including all classes of NAc neurons–release multiple neuromodulators that act on various receptor subtypes, making it difficult to identify the neural effector (e.g., SOM/NPY/NO+ LTSIs). Recent developments allow spatially- and temporally-resolved control of cell type-specific receptor signaling mechanisms, permitting investigation into the interplay between intracellular signaling and synaptic function (Spangler and Bruchas, 2017). For example, optogenetically activated chimeric G-protein coupled receptors (Opto-XRs) express a modified, light-sensitive extracellular receptor binding domain but a conserved intracellular loop, allowing anatomically- and temporally-resolved control of endogenous signaling

mechanisms using light (Airan et al., 2009; Siuda et al., 2015). The ability to manipulate a specific receptor in a defined cell type using the opto-XR approach will likely prove particularly useful for elucidating eCB control of neural circuit function.

Another technique involving modified receptor-based technology includes the inducible Tango (iTango) approach that links light- and ligand-dependent receptor activity with inducible signaling pathways to drive gene expression (Kim et al., 2017; Lee et al., 2017). With this approach, coincident light delivery and endogenous ligand binding lead to the release of an intracellular transcription factor that is designed to drive protein expression in cells that bind the ligand of interest. This can be used, for example, to express fluorescent proteins in cells that bind a specific ligand during a particular event, creating a snapshot of neural signaling during that period. Alternatively, the engineered transcription factor can drive expression of an excitable opsin or a caspase to then identify how neurons activated during the event of interest influence behavior. A number of additional optogenetic-based methods have been developed to directly target intracellular processes, even within specific organelles (Rost et al., 2017).

Characterization and Manipulation of Defined Neuronal Ensembles

Cell type-specific genetic techniques are powerful tools for probing spatially intermixed cell populations. However, the use of widespread molecular markers to target an entire region or cell type within a region (e.g., glutamate- or GABA-expressing neurons), regardless of their activity state during the behavior of interest, likely masks the underlying neural ensembles–consisting of a small percentage of the target population–that allow animals to discern the complexity of their environment. The idea that distinct neuronal subpopulations control specific neural functions and behaviors stems from work on "engrams," defined as enduring physical changes elicited by learning that underlie memory formation (DeNardo and Luo, 2017). This work typically uses immediately early genes (IEGs) such as *Fos* and *Arc* that are expressed within minutes following neuronal activation, but only in a small population within a particular brain region (∼2–12%) (Cruz et al., 2013). Activated IEG promoters can be targeted using pharmacology (Koya et al., 2009) or viral-genetic approaches (Zhou et al., 2019) to drive protein expression for labeling or manipulating neurons activated by a particular event (e.g., drug exposure, fear conditioning). In the NAc, this approach has demonstrated that a small population of accumbal neurons controls the hyperlocomotor or reinforcing effects of cocaine (Koya et al., 2009; Zhou et al., 2019). Viral strategies that use synthetic, activity-dependent promoters to drive IEG expression in genetically defined cell types allow improved isolation of active subpopulations (Bobadilla et al., 2020). Activity-dependent labeling of DA neurons may elucidate physiological factors that control neuronal and behavioral responses to certain events (e.g., drug exposure, stress) and promote pathological states.

From Cells to Circuits to Treatments

While a cell type- and circuit-specific understanding of NAc function is a noble endeavor, how such an understanding would influence neuropsychiatric therapy is unclear. It is currently not possible to treat patients with manipulations that alter brain function with cellular resolution. However, understanding how precise neural signaling systems in specific brain regions control valence-based actions may improve treatment protocols. For example, deep brain stimulation (DBS) is a treatment that involves targeted delivery of electricity to a select brain region using an implanted electrode. The NAc is a target for DBS treatment in multiple conditions including obsessive compulsive disorder (OCD) (Koya et al., 2009), treatment-resistant bulimic anorexia nervosa (Fernandes Arroteia et al., 2020), and substance use disorders (Hassan et al., 2020). While the precise mechanism by which DBS relieves symptoms remains elusive, an improved understanding of how NAc circuitry controls of behavior may inform more targeted manipulation of specific neural elements or circuits at distinct time points in conjunction with pharmacotherapies. For example, eCB manipulations along with NAc DBS may improve outcomes by filtering a subpopulation of synaptic connections that are affected by stimulation. Moreover, cell- or ensemble-specific experiments may identify cellular markers that promote pathophysiology (e.g., psychiatric symptoms, neurodegeneration), potentially leading to more targeted therapeutics.

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Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Discordant Effects of Cannabinoid 2 Receptor Antagonism/Inverse Agonism During Adolescence on Pavlovian and Instrumental Reward Learning in Adult Male Rats

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The endocannabinoid system is responsible for regulating a spectrum of physiological activities and plays a critical role in the developing brain. During adolescence, the endocannabinoid system is particularly sensitive to external insults that may change the brain's developmental trajectory. Cannabinoid receptor type 2 (CB2R) was initially thought to predominantly function in the peripheral nervous system, but more recent studies have implicated its role in the mesolimbic pathway, a network largely attributed to reward circuitry and reward motivated behavior, which undergoes extensive changes during adolescence. It is therefore important to understand how CB2R modulation during adolescence can impact reward-related behaviors in adulthood. In this study, adolescent male rats (postnatal days 28–41) were exposed to a low or high dose of the CB2R antagonist/inverse agonist SR144528 and Pavlovian autoshaping and instrumental conditional behavioral outcomes were measured in adulthood. SR144528-treated rats had significantly slower acquisition of the autoshaping task, seen by less lever pressing behavior over time [$F_{(2,\ 19)} = 5.964$, $p = 0.010$]. Conversely, there was no effect of adolescent SR144528 exposure on instrumental conditioning. These results suggest that modulation of the CB2R in adolescence differentially impacts reward-learning behaviors in adulthood.

Keywords: endocannabinoid, appetitive, autoshaping, conditioning, adolescent

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Ellner D, Hallam B, Frie JA, Thorpe HHA, Shoaib M, Kayir H, Jenkins BW and Khokhar JY (2021) Discordant Effects of Cannabinoid 2 Receptor Antagonism/Inverse Agonism During Adolescence on Pavlovian and Instrumental Reward Learning in Adult Male Rats. Front. Synaptic Neurosci. 13:732402. doi: 10.3389/fnsyn.2021.732402

INTRODUCTION

The endocannabinoid system (ECS) is responsible for the regulation of many biological systems through the activities of endogenous cannabinoids (eCB) such as anandamide and 2-arachidonoylglycerol, eCB synthesizing and degrading enzymes, and the endogenous receptors cannabinoid receptor 1 (CB1R) and CB2R. The CB2R had traditionally been labeled a peripheral cannabinoid receptor but its more recent discovery in the brain (Atwood and Mackie, 2010) has

Abbreviations: CB-R, Cannabinoid type—receptor; CS, Conditioned stimulus; DA, Dopamine; ECS, Endocannabinoid system; eCB, Endogenous cannabinoids; NAc, Nucleus accumbens; PCA, Pavlovian conditioning approach; PFC, Prefrontal cortex; PND, Postnatal day; VTA, Ventral tegmental area.

garnered interest regarding its role in the CNS. Studies have localized CB2R expression to regions important to the mesocorticolimbic signaling pathway, namely the prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA) (Aracil-Fernandez et al., 2012; Zhang et al., 2014). In particular, CB2R are expressed on the dendrites of dopamine (DA) neurons in the VTA, which project to the NAc and are integral to reward response (Zhang et al., 2017). Activation of the CB2R inhibit DAergic neurons firing, and ultimately decreases DA release into the NAc (Zhang et al., 2014; Han et al., 2017; Galaj and Xi, 2019).

Despite their identification in reward-relevant brain regions, only a handful of studies have explored the relationship between CB2R activation and modulation on related behavioral processes. CB2R activity appears to modulate reward and drug seeking behavior, in line with their expression in the mesocorticolimbic pathway (Zhang et al., 2014; Ghosal et al., 2019; Martin-Sanchez et al., 2019). Inhibition of DAergic neurons projecting from the VTA to the NAc by CB2R activation mitigates cocaine self-administration in adult mice (Zhang et al., 2014). Additionally, CB2R antagonist/inverse agonist exposure during the acquisition phase of conditioned place preference testing decreased in the rewarding effects of alcohol (Martin-Sanchez et al., 2019). These studies suggest that the CB2R plays a direct role in adulthood drug and reward seeking behavior. Importantly, however, it is unclear if CB2R modulation during critical periods of mesocorticolimbic development has any lasting impact on future reward response.

Adolescence is characterized by various developmental changes that occur in the period between childhood and adulthood (Spear, 2000). During this time, there is extensive reorganization of cortical and limbic neurocircuitry, which contributes to natural cognitive, emotional, and reward development (Paus et al., 2008). Many neurotransmitter systems fluctuate significantly during this period of neurodevelopment (Meyer et al., 2018; Thorpe et al., 2020), and make the brain highly susceptible to social, nutritional and environmental influences, as well as insults by drugs of abuse (Thorpe et al., 2020). The ECS has been shown to play a crucial role in adolescent neuronal development, including modulating the ratio of excitatory and inhibitory signaling in the PFC (Meyer et al., 2018), and CB2R expression specifically increases in the PFC, NAc, and hippocampus during the rat equivalent of adolescence (Amancio-Belmont et al., 2017). The existence of adolescent-specific stressors such as family, academic, and peer pressures make this a unique period for environmental modulation of ECS activity, which has been extensively implicated in stress response (Tottenham and Galvan, 2016). In addition, adolescents are especially vulnerable to drug use. Cannabis is one of the most commonly used drugs among this age group, so understanding the impact of adolescent cannabinoid exposure is of utmost importance (Hamidullah et al., 2020, 2021). Previous human and animal studies suggest that adolescent exposure to cannabinoids dysregulates ECS activity, which may have long-term behavioral implications that can persist into adulthood (Hamidullah et al., 2020; Thorpe et al., 2020). For instance, treatment of adolescent rats with the CB1R/CB2R agonist WIN55,212-2 (Schoch et al., 2018) or the CB1R/CB2R partial agonist and the primary psychoactive constituent of cannabis Δ9-tetrahydrocannabidiol (THC) (Kruse et al., 2019) have been shown to modulate the response to food-predictive cues in adulthood.

Since CB2R have been implicated in the rewarding effects of a variety of drugs (e.g., nicotine, alcohol) (Ishiguro et al., 2007; Navarrete et al., 2013), our interest was assessing the persisting impact of adolescent CB2R modulation on future reward learning. Furthermore, since pharmacological modulation of CB1R and CB2R has been shown to impact reward learning in adulthood, we focused on the impact of CB2R modulation on two forms of reward learning behaviors: Pavlovian conditioning and instrumental conditioning. Autoshaping is a form of Pavlovian conditioning in which an animal develops a response to a neutral stimulus (i.e., a lever) that predictive of an outcome, such as the delivery of a palatable food reward, that is not contingent on the animal's response. Behaviors exhibited during this task are reflective of either sign-tracking or goal-tracking, which are the attribution of incentive salience to the stimulus or the place of reward delivery (i.e., food dispenser), respectively (Flagel et al., 2010). Instrumental learning is a form of operant conditioning, in which the delivery of a reinforcer is contingent on an animal's behavior, and thus exhibiting said behavior is considered goal-directed. While there is some overlap between the circuitry mediating Pavlovian vs. instrumental conditioning, the exact mechanisms behind these learning behaviors may be unique (Cain and LeDoux, 2008; Guo et al., 2016; Bouton et al., 2021; Doñamayor et al., 2021). Pavlovian and instrumental learning studies both demonstrate ventral striatum activation, with Pavlovian tasks showing higher recruitment of the left putamen of the dorsal striatum than instrumental tasks, which preferentially activate the caudate (Chase et al., 2015).

While existing studies have examined behavioral outcomes linked with short-term modulation of the CB2R (Aracil-Fernandez et al., 2012; Zhang et al., 2014), the long-term effects of CB2R modulation during adolescence remain largely unknown. Thus, the objective of our study was to understand the role of CB2R in adolescent development as it relates to reward learning in adulthood and processing by pharmacologically inhibiting CB2R during adolescence in a rodent model. We hypothesized that adolescent CB2R inhibition would hamper acquisition of reward-paired lever pressing in both the autoshaping and instrumental reward-learning tasks in a dose-dependent manner.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (n = 7–8/experiment/group) were obtained from Charles River Laboratories (Saint Constant, Quebec) at postnatal day (PND) 21. Males were used as previous studies report inconsistent effects of SR144528, the antagonist/inverse agonist used in this study, in females (Craft et al., 2012). All rats were weaned at PND23 and pair-housed on a 12:12 light:dark cycle. Rats were food restricted to 85–90% of their baseline body weights beginning on PND56

to encourage food-motivated behavior and exploration during behavioral testing. Animal care, behavioral testing, anesthesia, and euthanasia procedures were performed in accordance with the Animal Use Protocol approved by the University of Guelph Animal Care Committee.

Drug Administration

SR144528 is a potent and highly selective CB2R antagonist/inverse agonist (Rinaldi-Carmona et al., 1998; Portier et al., 1999) and was obtained from the National Institute of Mental Health Drug Repository Program. SR144528 was dissolved in a 1:1:18 vehicle solution of Cremophor: 95% ethanol:0.9% saline. Rats were given intraperitoneal injections once daily from PND28 to PND41. Rats received either 0, 3.2, or 6.4 mg/kg of SR144528 at a volume of 1–2 ml/kg. The initial dose of 3.2 mg/kg was selected based on prior behavioral investigations in male rats that suggest this dose does not antagonize CB1R (Craft et al., 2012). To determine if our observed outcomes were dose-dependent, a cohort administered 6.4 mg/kg of SR144528 was also included in our investigations.

Testing Apparatus

Testing was conducted using eight HABITEST® Operant Cages (24 × 30.5 × 29 cm; Coulbourn Instruments) placed in HABITEST® Isolation Cubicles (Coulbourn Instruments, model: H10-24). The chambers were composed of two front and back aluminum walls, two clear acrylic side walls, a clear acrylic roof, and floor of stainless-steel rods (5 mm diameter) that were 1.5 cm apart from each other. Each isolation cubicle was equipped with an exhaust fan to provide background noise and ventilation. One of the aluminum sidewalls was outfitted with a food dispenser bordered by two retractable levers. Lever presses were automatically measured and entries into the food dispenser were recorded via a photocell using Graphic State software. The reinforcing stimulus was 45 mg banana flavored sucrose pellets.

Autoshaping

The autoshaping protocol used in this study was modified from Khokhar and Todd (2018). Magazine training occurred on the first of 13 testing days. On the first day, rats were habituated to the apparatus. During this session, both levers were retracted, and one sucrose pellet was released approximately once every 30 s (± 30 s) for a 30 min session. No data was collected on this day.

Days 2–13 involved the rats learning to associate one of the two levers with reward delivery. Throughout the 60 min session, the conditioned stimulus lever (CS+) was presented 25 times, followed each time by the non-contingent delivery of a sucrose pellet, whereas the unconditioned stimulus lever (CS−) was presented 25 times without sucrose pellet delivery. Assignment of the left and right levers as the CS+ or CS− was counterbalanced in each group. The length of the inter-trial interval was randomized (60 ± 15 s). Lever presentation was pseudorandomized, with the same lever presented no more than two times in a row. Each lever was inserted into the chamber for 30 s. Food cup entries were only counted during CS+ lever presentations. Lever and food cup entry probabilities were calculated as the ratio of CS+ lever presentations with a lever press or food cup entry, respectively, divided by the total number of CS+ presentations in the session.

Instrumental Learning

The 14-day instrumental learning protocol was adapted from Bouton et al. (2011). Two retractable levers were positioned in the chamber: one eliciting the presentation of a reward when pressed (CS+) and the other not paired with a reward delivery when pressed (CS−). The assignment of each lever as CS+ or CS− levers was pseudorandomized within each chamber such that the CS+ was assigned to the right lever and CS− assigned to the left lever in half of the chambers. All groups were counterbalanced for CS+ position throughout behavioral testing.

Rats underwent magazine training as described for the autoshaping protocol during days 1–2. During days 3–14, rats completed 32 min sessions of lever press training occurring on a variable interval 30 s reinforcement schedule. Both levers were inserted 2 min after session initiation and remained presented for the duration of the session. Approximately once every 30 s (± 30 s), the CS+ lever would enter a "working state" in which pressing the lever resulted in delivery of a sucrose pellet at the food dispenser. The lever would stay in this "working state" until pressed, after which the 30 s variable interval was reset.

Data Collection and Statistical Analysis

The total number of CS− lever presses, CS+ lever presses and food dispenser entries were collected across experimental days. Results obtained from behavioral testing were evaluated with repeated-measures analyses of variance (RMANOVA) followed by Fisher's *post hoc* testing when appropriate. Data were assessed using Greenhouse-Geisser correction when sphericity was violated followed by Fisher's Least Significant Difference *post hoc* test where main effects were significant. Data analysis was performed using IBM SPSS Statistics 26. Results with a p-value < 0.05 were considered statistically significant.

RESULTS

Adolescent SR144528 Treatment Alters Sign- and Goal-Tracking Behaviors in Adulthood

There was a significant main effect of adolescent SR144528 treatment [$F_{(2,\ 19)} = 6.466$, $p = 0.0072$], session [$F_{(11,\ 209)} = 16.10$, $p < 0.0001$], and a treatment by session interaction [$F_{(22,\ 209)} = 1.598$, $p = 0.0489$] on the number of CS+ lever pressing (**Figure 1A**). *Post hoc* analysis revealed that CS+ lever pressing was significantly greater in vehicle treated rats compared to rats treated with 3.2 mg/kg of SR144528 on sessions 4, 5, and 11 ($p < 0.05$). Similarly, the vehicle treated group pressed the CS+ lever more than the 6.4 mg/kg treated group on sessions 5, 6, and 9–12.

There was a significant main effect of session [$F_{(11,\ 209)} = 3.749$, $p = 0.0180$] and treatment [$F_{(2,\ 19)} = 6.398$,

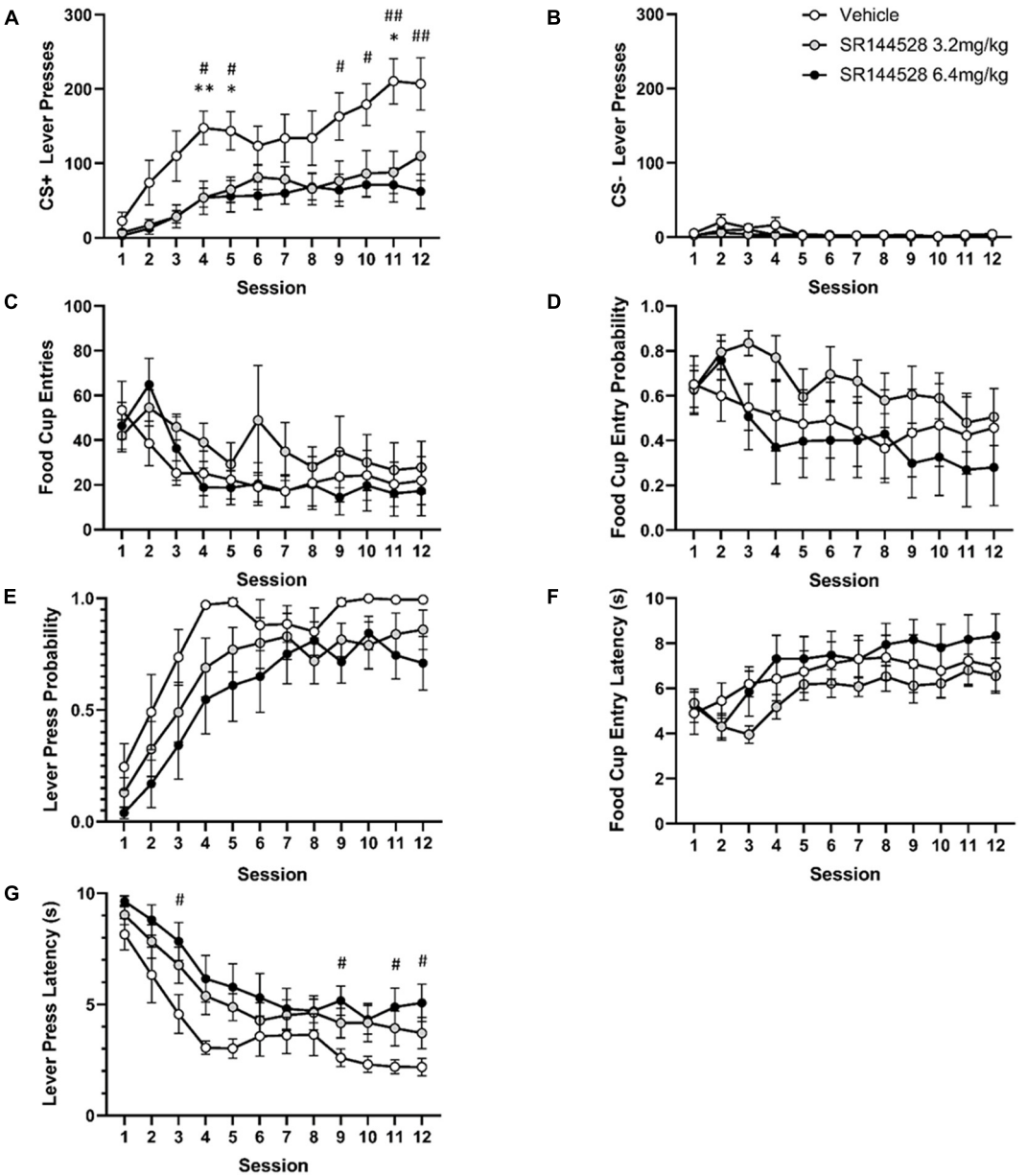

FIGURE 1 | Antagonism/inverse agonism of CB2R during adolescence modulates adulthood autoshaping task performance. **(A)** Mean lever presses on the CS+ lever. **(B)** Mean lever presses on the CS– lever. **(C)** Probability of a lever press at least once during its presentation. **(D)** Probability of entering the food cup during CS+ lever presentation. **(E)** Mean latency to press the CS+ lever. **(F)** Mean latency to food cup entry. **(G)** Mean cumulative food cup entries during CS+ lever presentation. $^{*}p < 0.05$, $^{**}p < 0.005$ vehicle versus 3.2 mg/kg SR144528. $^{\#}p < 0.05$, $^{\#\#}p < 0.005$ vehicle vs 6.4 mg/kg SR144528. Data presented as mean ± SEM.

$p = 0.0075$] on CS− lever pressing, but no *post hoc* comparisons were significant (**Figure 1B**).

Food cup entries [$F_{(11,\ 209)} = 4.714$, $p = 0.0036$; **Figure 1G**] and food cup entry probability [$F_{(11,\ 209)} = 2.990$, $p = 0.0313$; **Figure 1D**] decreased across sessions. Lever press probability [$F_{(11,\ 209)} = 28.66$, $p < 0.0001$; **Figure 1C**] and food cup entry latency [$F_{(11,\ 209)} = 6.949$, $p = 0.0002$; **Figure 1F**] increased across sessions. There was no main effect of treatment, however, on food cup entries [$F_{(2,19)} = 0.6816$, $p = 0.5177$; **Figure 1G**], probability [$F_{(2,19)} = 1.264$, $p = 0.3053$; **Figure 1D**], or latency [$F_{(2,19)} = 1.175$, $p = 0.3302$; **Figure 1F**], nor was there an interaction between session and treatment on food cup entries [$F_{(22,209)} = 0.7570$, $p = 0.7754$; **Figure 1G**], probability [$F_{(22,209)} = 0.5403$, $p = 0.9551$; **Figure 1D**], or latency [$F_{(22,209)} = 0.6401$, $p = 0.8916$; **Figure 1F**].

There was a significant effect of session [$F_{(11,\ 209)} = 36.24$, $p < 0.0001$] and treatment [$F_{(2,\ 19)} = 3.713$, $p = 0.0435$] on latency

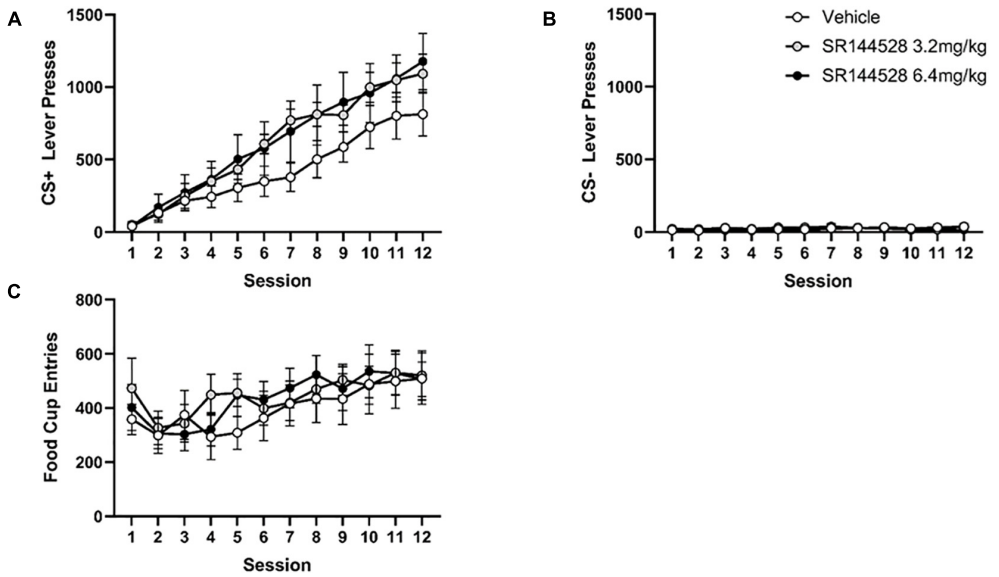

FIGURE 2 | Antagonism/inverse agonism of CB2R during adolescence does not impact instrumental learning in adulthood. **(A)** Mean CS+ lever presses. **(B)** Mean CS– lever presses. **(C)** Mean cumulative food cup entries. Data presented as mean ± SEM.

to press the CS+ lever (**Figure 1E**). *Post hoc* multiple comparisons showed significantly lower latency to press in the vehicle group compared to the 6.4 mg/kg dose of SR144528 on sessions 3, 9, 11, and 12 ($p < 0.05$).

Adolescent Exposure to SR144528 Does Not Affect Instrumental Learning in Adulthood

There was overall increase in CS+ presses during acquisition with a main effect of session [$F_{(2, 21)} = 1.204$, $p < 0.0001$], but no effect of treatment on CS+ responding (**Figure 2A**).

Pressing of the CS− lever during learning acquisition decreased across sessions in all treatment groups (**Figure 2B**), though there was no significant main effects of session or treatment, nor any interactions.

Food dispenser entries during acquisition did not significantly differ across treatment groups (**Figure 2C**). There was a main effect of day [$F_{(4, 75)} = 5.912$, $p = 0.01$] such that the number of food cup entries increased over sessions.

DISCUSSION

We found that adolescent treatment with the CB2R antagonist/inverse agonist SR144528 reduced sign-tracking behavior in adulthood. Our results suggest that suppression of CB2R activity during adolescence impairs adulthood sign-tracking as evidenced by an attenuation of CS+ lever pressing and a shift toward the goal-tracking phenotype during autoshaping acquisition but did not impact instrumental learning. While there were no significant differences between the high and low dose treatment groups in this task, CS+ lever pressing behavior differed significantly between the high dose compared to the vehicle treated group on more days than the low dose group and was the only group to have significantly higher latency to press the CS+ lever compared to controls. We suspect that a potential ceiling effect on lever presses/probability, or floor effect on lever press latency may have contributed to our inability to see a dose-dependent relationship.

Localization of CB2R along the mesocorticolimbic pathway supports its role in reward circuitry and drug seeking behavior (Zhang et al., 2017). Acute CB2R activation has been shown to inhibit VTA DAergic neuronal firing and cocaine self-administration (Zhang et al., 2014), possibly through decreased DA released into the NAc (Zhang et al., 2014; Chase et al., 2015; Galaj and Xi, 2019). Similarly, it has recently been shown that CB2R-null mice also show elevated DA levels in the NAc in response to THC, further implicated CB2R as important mediators of mesolimbic DA signaling (Li et al., 2021). The decreased sign-tracking behavior seen following CB2R antagonism/inverse agonism in our study may, therefore, be due to a compensatory increase in NAc-VTA receptor sensitivity and/or expression following prolonged inhibition during adolescence (Zhang et al., 2014). It is well established that the NAc is integral to sign-tracking behavior (Chang et al., 2012), and lower accumbal DA is correlated with impaired cue-directed performance (Fitzpatrick and Morrow, 2016) whereas elevated DA neuron firing is apparent in rats that exhibit stronger reward-cue associations (Flagel et al., 2010; Chang et al., 2012; Chase et al., 2015). The discordant effects of adolescent CB2R pharmacological manipulation on Pavlovian and instrumental reward learning further support the modulation of DAergic signaling; flupenthixol (a DA receptor D1/D2 antagonist) blocks Pavlovian goal approach without impacting instrumental incentive learning (Wassum et al., 2011). However, studies also implicate CB2R activation may inhibit (Foster et al., 2016;

Lopez-Ramirez et al., 2020) or stimulate (Lopez-Ramirez et al., 2020) DA release in the dorsal striatum that is contingent on non-cannabinoid receptors. As this region is relevant to both Pavlovian and instrumental learning through unique network recruitment (Chase et al., 2015), characterization of CB2R function across striatal networks and neuronal populations may reveal differential mechanisms within the dorsal striatum that are specific to sign- or goal-tracking behavior development.

Although we posit that long-term disruption of CB2R in mesolimbic circuits are underlying the observed alterations to sign-tracking behavior, other CB2R-related mechanisms may also be relevant to these findings. For instance, recent findings show that CB2R, but not CB1R, regulates physiological stress response to predator cues in rats such that their activity attenuates anxiety-like behavior following stress exposure (Ivy et al., 2020). Likewise, prolonged stress shifts rats from sign- to goal-directed behavior (Fitzpatrick et al., 2019). Prolonged inhibition of CB2R during adolescent development may therefore impair stress resilience in SR144528-treated rats, thus contributing to the shift from sign- to goal-directed reward learning mechanisms. This hypothesis is not mutually exclusive with the proposed involvement of CB2R expression in the VTA-NAc pathway; Ivy et al. (2020) detected increased *Cnr2* mRNA encoding CB2R mRNA in the PFC following predator stress. While they did not examine mRNA levels in the NAc or VTA, these increases were specific to the PFC and further implicate the mesocorticolimbic circuitry in CB2R's role in cue-directed strategies.

While previous studies have assessed the impacts of adolescent exposure to agonists of both CB1 and CB2R on Pavlovian reward learning, our study adds specificity for CB2R in this process as well as explores of the impact of antagonism/inverse agonism. Adolescent exposure to the full CB1R/CB2R agonist WIN-55,212 increased goal-tracking in rats that also exhibited sign-tracking, producing an "intermediate" phenotype. Adolescent consumption of an edible form of THC, a partial agonist at both CB1R and CB2R, in male rats increased sign-tracking behavior (especially early in acquisition), while reducing goal-tracking behaviors in adulthood; these effects were not seen in female rats (Kruse et al., 2019). While the findings here cannot be directly compared due to methodological differences (e.g., length of conditioning, and different ages at exposure), our findings oppose the findings from these studies, consistent with the effects of SR144528. Our findings are also consistent with the effects of acute treatment with the CB1R antagonist rimonabant, where reductions in sign-tracking were observed (Bacharach et al., 2018). Based on consistent evidence between our study with another that used a non-specific cannabinoid receptor agonist THC (Kruse et al., 2019), alongside the observations of endogenous CB2R activity that may be related to learning (Fitzpatrick et al., 2019; Ivy et al., 2020), we suspect that adolescent treatment with a CB2R-specific agonist would shift Pavlovian reward learning toward sign-tracking and away from a goal-tracking phenotype.

Limitations of this study include the exclusive use of male rats (especially in light of the findings with edible THC highlighted above) and CB2R modulation only during early adolescence. In addition, SR144528 is both an antagonist and inverse agonist at CB2R, and has low affinity for CB1R (Rinaldi-Carmona et al., 1998; Portier et al., 1999). As the results of our study are consistent with the properties of SR144528 as an antagonist and an inverse agonist, we cannot conclude the pharmacological mechanism responsible for its influence on CB2R-mediated reward learning in adulthood. While a previous study suggests that SR144528 does not affect CB1R-mediated behaviors in male rats, its effects on these behaviors were inconsistent in females (Craft et al., 2012), and as such its use in females should be further validated before investigating sex-specific differences related to reward learning.

We report that inhibition of the CB2R during adolescence decreases sign-tracking behavior but does not affect goal-tracking behavior in adulthood. The present paper extends current literature on the impact of endocannabinoid system disturbances during adolescent development and adds to the expanding literature investigating cannabinoid receptors in the context of reward-related behaviors. Our paper provides new insights into adolescent receptor modulation of CB2R and provides evidence that CB2R may have a pivotal role in cue associative learning and reward motivation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Guelph Animal Care Committee.

AUTHOR CONTRIBUTIONS

HT, JF, and JK contributed to conception and design of the study. JF programmed the experiments. DE, BH, JF, HT, HK, and BJ performed the experiments. DE, BH, and JF performed statistical analysis. DE, BH, HT, and JF wrote sections of the manuscript. JK secured funding and supervised all students. All authors contributed to manuscript revision, read, and approved the submitted version.

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Activity-Dependent Modulation of Tonic GABA Currents by Endocannabinoids in *Hirudo verbana*

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Endocannabinoids are lipid neuromodulators that are synthesized on demand and primarily signal in a retrograde manner to elicit depression of excitatory and inhibitory synapses. Despite the considerable interest in their potential analgesic effects, there is evidence that endocannabinoids can have both pro-nociceptive and anti-nociceptive effects. The mechanisms contributing to the opposing effects of endocannabinoids in nociception need to be better understood before cannabinoid-based therapies can be effectively utilized to treat pain. Using the medicinal leech, *Hirudo verbana*, this work investigates whether endocannabinoids modulate tonic inhibition onto non-nociceptive afferents. In voltage clamp recordings, we analyzed changes in the tonic inhibition in pressure-sensitive (P) cells following pre-treatment with endocannabinoids, 2-arachidonoylglycerol (2-AG) or anandamide (AEA). We also tested whether high frequency stimulation (HFS) of nociceptive (N) cells could also modulate tonic inhibition. Both endocannabinoid application and N cell HFS depressed tonic inhibition in the P cell. Depression of tonic inhibition by N cell HFS was blocked by SB 366791 (a TRPV1 inhibitor). SB 366791 also prevented 2-AG-and AEA-induced depression of tonic inhibition. HFS-induced depression was not blocked by tetrahydrolipstatin (THL), which prevents 2-AG synthesis, nor AM 251 (a CB1 receptor inverse agonist). These results illustrate a novel activity-dependent modulation of tonic GABA currents that is mediated by endocannabinoid signaling and is likely to play an important role in sensitization of non-nociceptive afferent pathways.

Keywords: endocannabinoids, 2-arachidonoylglycerol (2-AG), anandamide (AEA), TRPV, tonic inhibition, *Hirudo verbana*, synaptic depression

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INTRODUCTION

Endocannabinoids are lipid neuromodulators synthesized in an activity-dependent manner (Castillo et al., 2012). The main endocannabinoid ligands are 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamide (AEA) and these can act on metabotropic cannabinoid receptors (CB1 and CB2) or transient receptor potential (TRP) channels, e.g., TRPV (Edwards, 2014; Muller et al., 2019; Ye et al., 2019). Synthesis takes place primarily in the postsynaptic cell, which allows them to function as retrograde messengers and modulate diverse forms of synaptic plasticity (Castillo et al., 2012).

Endocannabinoids can modulate excitatory or inhibitory synapses over both short- and long-term time scales, often via presynaptic depression of neurotransmitter release (Castillo et al., 2012). Furthermore, these effects can be homosynaptic or heterosynaptic (Piette et al., 2020). In situations where inhibitory synapses are depressed, this can lead to disinhibition of excitatory synapses (Zhu and Lovinger, 2007; Kim et al., 2019). This disinhibition effect can lead to endocannabinoids eliciting long-term potentiation (LTP) in excitatory, glutamatergic synapses (Carlson et al., 2002; Brown et al., 2013; Cui et al., 2015; Silva-Cruz et al., 2017). Endocannabinoid-mediated disinhibition and its effects on LTP are likely to play an important role in modulating nociceptive synaptic circuits. In rodents, while endocannabinoid-mediated depression of glutamatergic synapses in the spinal cord represents a likely anti-nociceptive effect (Kato et al., 2012), endocannabinoid depression of inhibitory synapses has also been observed and leads to disinhibition of nociceptive circuitry in the spinal cord that generates a pro-nociceptive effect (Pernia-Andrade et al., 2009).

There is interest in using comparative approaches to understand the basic biology of pain (Walters and Williams, 2019). Applying this approach to endocannabinoid modulation of nociception makes sense given the interest in cannabinoid-based approaches in treating pain and that the endocannabinoid system is well-conserved across the animal kingdom (Elphick, 2012; Paulsen and Burrell, 2019). The central nervous system (CNS) of the medicinal leech, *Hirudo verbana*, is an especially useful organism in which to carry out these studies. The *Hirudo* CNS possesses mechanosensory neurons that are functionally similar to mammals, including rapidly adapting touch (T), slow adapting pressure sensitive (P), and both mechanical and polymodal nociceptive (N) neurons (Nicholls and Baylor, 1968; Blackshaw et al., 1982; Pastor et al., 1996; Lewin and Moshourab, 2004; Abraira and Ginty, 2013; Burrell, 2017). Furthermore, recent studies have found that genes associated with mechanosensation in mammals are also expressed in the *Hirudo* mechanosensory neurons, e.g., piezo, DeG/ENaC, and trp encoding genes (Heath-Heckman et al., 2021). As in mammals, *Hirudo* possesses the endocannabinoids 2-AG and AEA, with the former being more abundant (Matias et al., 2001). *Hirudo* possesses genes encoding the proteins required for 2-AG synthesis, [diacylglycerol lipase (DAGL) α and β (accession #s KU500007 & MT610103), 2-AG metabolism (monoacylglycerol lipase (MAGL) (KY971276)], AEA metabolism [fatty acid amide hydrolase (FAAH) (pending)], and TRPV (Heath-Heckman et al., 2021). *Hirudo* MAGL (hirMAGL) has been studied in some detail and exhibits considerable structural and functional conservation at the protein's catalytic active site (Kabeiseman et al., 2020). Using pharmacological approaches, multiple drugs blocking DAGL (RHC-80267, OMDM-188, and tetrahydrolipstatin/Orlisat) and activating (capsaicin, resiniferatoxin) or inhibiting (capsazepine, SB-366791) TRPV channels have been effective in studying putative endocannabinoid-mediated modulation of synapses and behavior in *Hirudo* (Yuan and Burrell, 2010, 2012, 2013b; Wang and Burrell, 2016, 2018).

The pro- and anti-nociceptive effects of endocannabinoids reported in rodent models (Pernia-Andrade et al., 2009; Kato et al., 2012; Sdrulla et al., 2015) are also observed in *Hirudo* (Yuan and Burrell, 2013b; Summers et al., 2017; Wang and Burrell, 2018). 2-AG activation of TRPV-like channels depresses nociceptive (N) cell synapses and decreases behavioral responses elicited by N cell activation (Yuan and Burrell, 2010, 2012, 2013b). On the other hand, endocannabinoids potentiate non-nociceptive pressure (P) cell synapses and increase the behavioral responses elicited by P cells (Higgins et al., 2013; Wang and Burrell, 2016, 2018). It is this latter effect that we will focus on.

Potentiation of P cell synapses by endocannabinoids is long-lasting (30–60 min) and proposed to be a disinhibition-mediated process, based on previous experiments in which inhibition of either GABA receptors or chloride importers prevents endocannabinoid-mediated potentiation (Higgins et al., 2013; Wang and Burrell, 2016). Disinhibition of P cell synapses is presynaptic in nature, supporting the idea that GABAergic input is modulating the amount of neurotransmitter (glutamate) released at the presynaptic terminal (Wang et al., 2015). In additional, high frequency stimulation (HFS) of the N cells also produces a similarly persistent potentiation of P cell synapses in *Hirudo* in a 2-AG-dependent manner that requires disinhibition (Wang and Burrell, 2016). Potentiation of P synapses by N cell HFS or exogenously applied endocannabinoids is TRPV-dependent, but P cells lack these TRPV-like channels (Higgins et al., 2013; Summers et al., 2014; Wang and Burrell, 2016). We hypothesize, as shown in **Figure 1A**, that endocannabinoids act on *Hirudo* TRPV-channels located on GABAergic neurons that modulate the P cell. The GABAergic neurons undergo endocannabinoid/TRPV-mediated long-term synaptic depression, which leads to disinhibition of the P cell synapses.

In this study we examined whether endocannabinoids and N cell HFS actually depressed GABAergic input onto the P cells at the same time points that we observed potentiation of P synapses. Other studies regarding endocannabinoid-mediated disinhibition have focused on GABAergic IPSPs. Since no spontaneous IPSPs are observed in P cell recordings, we examined whether endocannabinoids mediated tonic GABAergic inhibition instead. Tonic GABAergic inhibition was observed in the P cell and endocannabinoids and N cell HFS elicited long-term depression of this tonic GABAergic input.

MATERIALS AND METHODS

Ganglion Preparation

Medicinal leeches of the species *Hirudo verbana* (2–3g) were acquired from commercial suppliers (Leeches United States Ltd., Westbury, NY and Leech.com, Perris, California) and housed in artificial pond water (0.052% w/v Instant Ocean Sea Salt, Aquarium Systems) kept at 15°C with an alternating 12-h light/dark cycle. A single *Hirudo* would be chilled for 30 min at 4°C in pond water to lightly anesthetize the animals. Dissections of individual ganglia were conducted in ice-cold normal *Hirudo* saline solution (110 mM NaCl, 5 mM NaOH, 4 mM KCl, 1.8 mM

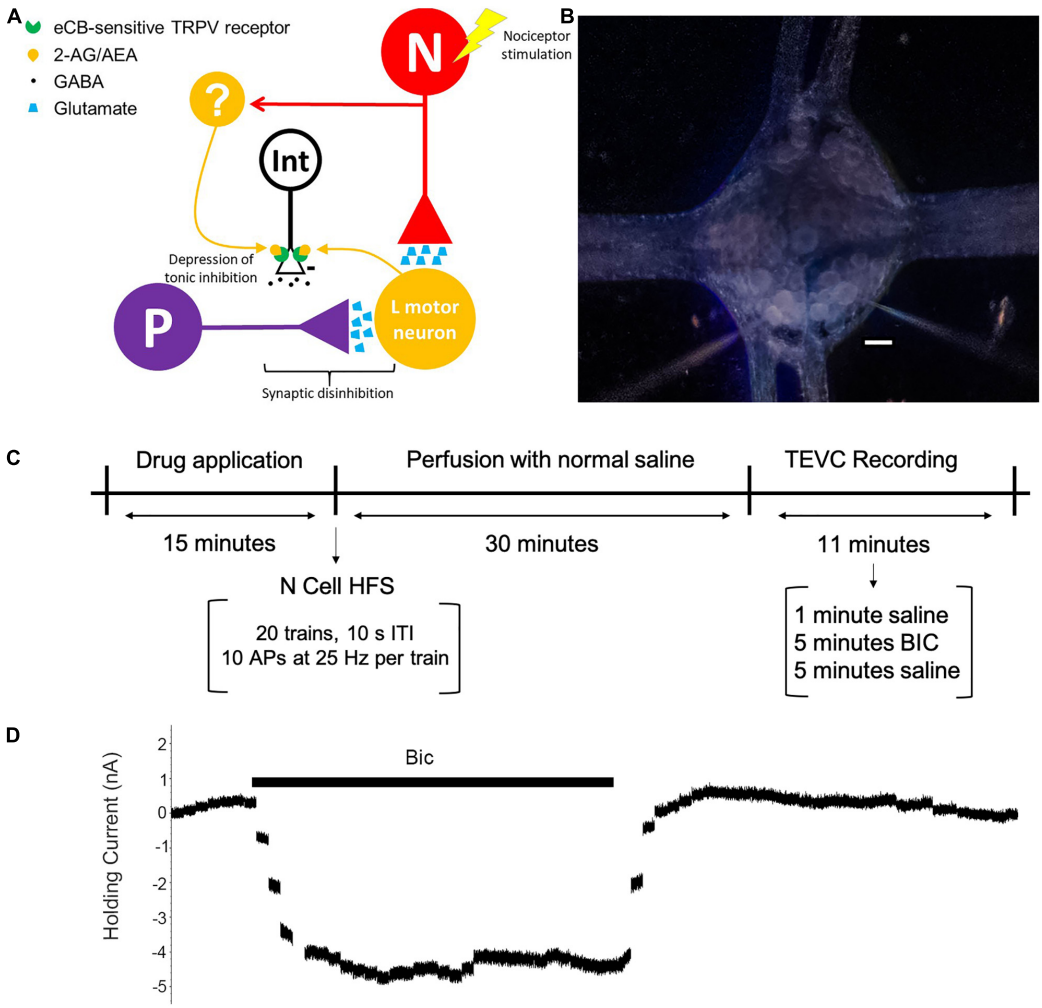

FIGURE 1 | (A) Proposed circuit for N cell-initiated disinhibition of P cell synapses through activity-dependent endocannabinoid modulation. The N and P afferents have converging inputs onto multiple postsynaptic targets, including the longitudinal (L) motor neuron. An unidentified GABAergic interneuron (Int) has tonic inhibitory input onto the P cell [indicated by the minus (–) sign]. It is hypothesized that HFS of an N cell elicits endocannabinoid (2-AG or AEA) production that depresses synaptic transmission by the neuron that is the source of tonic inhibition on to the P cell. The source of endocannabinoid signaling may be the L motor neuron, which is known to produce 2-AG following low frequency afferent input (Yuan and Burrell, 2010). However, it is possible that other, unknown neurons (labeled as "?") that also receive N cell input (indicated by the red arrow) are the source of 2-AG and/or AEA. **(B)** Acutely isolated ganglion with two microelectrodes. Scale bar = 50 μm. **(C)** Experimental timeline. Ganglia received a bath application of drug for 15 min followed by a 30 min wash-out period with normal saline. TEVC recordings (see section "Materials and Methods") were next performed in the P cell by first perfusing with saline for 1 min to establish a baseline, followed by perfusion with BIC for 5 min, and 5 final minutes in normal saline in order to allow the cell to return to its baseline holding current. Control experiments without BIC were perfused with normal saline throughout the entire TEVC experiment. Experiments with N cell HFS received stimulation after a drug pretreatment (if applied) and before the washout period. **(D)** Example of BIC effect on holding current. Plot consists of a series of concatenated recordings of P cell holding current prior to, during, and after application of BIC (1 sec TEVC recording with 10 sec intervals between each recording over a period of 11 min).

$CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) with 10 mM glucose. A single ganglion was placed in a 35 mm petri dish that had been filled with 1.5 mL Sylgard^{TM} and has an RC-37W insert for fluid exchange (Warner Instruments; Holliston, MA, United States). Recordings were performed at room temperature (20–22°C) normal saline while maintaining a constant perfusion rate of approximately 1.5 mL/min using an eight channel valve-controlled gravity perfusion system (VC8xG) and a VWK system (ALA Scientific; Farmingdale, NY, United States). It is not necessary to oxygenate the saline during the recordings. Neurons within the ganglion and the recording electrodes were visualized using a stereomicroscope (Leica SMZ6) with magnification at 70–80× and dark-field illumination (**Figure 1B**).

Within the CNS of *Hirudo* is a ventral nerve cord comprised of 21 linked and almost equivalent segmental ganglia, each of which contain an estimated 400 neurons. The arrangement of these neurons is well-documented, allowing for cells to be selected for analysis based on their size, position in the ganglion, and electrophysiological properties (Muller et al., 1981). This study focused on the nociceptive (N) and pressure-sensitive (P) cells,

each of which as two bilateral pairs in the ganglion. Different P cells innervate different cutaneous receptive fields, while the N cells are characterized as being either mechanical (medial N cells) or polymodal (lateral N cells) nociceptors (Nicholls and Baylor, 1968; Blackshaw et al., 1982; Pastor et al., 1996). Both pairs of N and P cells are accessible through the ventral side of the ganglion. Moreover, the P and N cells also form excitatory, glutamatergic synapses onto a number of shared targets, including the L motor neuron (shown in **Figure 1A**) which contributes to the *Hirudo* whole-body shortening reflex (Nicholls and Purves, 1970; Shaw and Kristan, 1995). Endocannabinoid-mediated potentiation is observed at P cell synapses onto the L motor neuron, as well as P synapses onto other postsynaptic targets (Higgins et al., 2013; Wang and Burrell, 2016).

Drug Application

Drugs were stored in frozen aliquots and were diluted to the desired concentration in *Hirudo* saline shortly before each experiment was conducted. 2-Arachidonoyl glycerol (2-AG), *N*-arachidonylethanolamide (AEA), SB 366791, AM 251, and tetrahydrolipstatin (THL, known also as Orlistat) stocks were dissolved in dimethyl sulfoxide (DMSO). Bicuculline methiodide stocks were dissolved in normal saline. DMSO was obtained from Sigma Aldrich (St. Louis, MO, United States). 2-AG was purchased from Avanti Polar Lipids (Alabaster, AL, United States). AEA, Bicuculline (BIC), THL, and SB 366791 were acquired from Tocris/Bio-Techne (Minneapolis, MN, United States). AM 251 was obtained from Hello Bio Inc (Princeton, NJ, United States).

Electrophysiology

Two-electrode voltage clamp (TEVC) recordings were performed using an Axoclamp 900A amplifier (Molecular Devices, San Jose, CA, United States) as in previous studies (Wang et al., 2015). Neurons were impaled with sharp glass microelectrodes directed by manual micropositioners (Model 1480, Siskiyou, Grants Pass, OR, United States). Microelectrodes were filled with a 2 M potassium acetate (KAc) solution and no KCl was included in the electrode filling solution in order to not disrupt intracellular Cl^- concentrations in *Hirudo* neurons, and therefore, the recordings of tonic inhibition are not from Cl^--loaded cells. Electrodes were fabricated from borosilicate capillary tubing (1.0-mm OD, 0.75-mm ID; FHC, Bowdoinham, ME, United States) with a horizontal puller (Sutter Instruments P-97, Novato, CA, United States). Tip resistances ranged from 35 to 50 MΩ .

P cells were voltage-clamped at −50 mV and holding current monitored using Clampex software (Molecular Devices). Following the protocols used in a variety of published studies, tonic inhibition was measured by recording holding current in normal saline and then in BIC to block tonic GABA receptor activation (Semyanov et al., 2004; Song et al., 2011; Bright and Smart, 2013). The P cell holding current was measured over an 11 min period, the first minute in normal saline followed by a 5 min application of BIC (100 μM) and then 5 min in normal saline to observe the holding current return to baseline (**Figures 1C,D**). For data analysis purposes, a 1 s measurement of holding current was made every 10 s for the duration of the experiment.

To observe changes in tonic GABAergic input to the P cell due to endocannabinoid treatment ganglia were treated with either 2-AG (100 μM), AEA (0.1 μM) or 0.01% DMSO (control) via bath application for 15 min. This was followed by a 30-min washout period with normal saline at which time tonic inhibition was measured to assess whether there had been a persistent effect on GABAergic input. The concentration of 2-AG used in these experiments is comparable to prior experiments using rodent brain slice preparations (Stella et al., 1997; Al-Hayani et al., 2001). In the case of these *Hirudo* studies, it should be noted that an acutely isolated ganglion is essentially an intact segment of the CNS approximately 0.5 mm thick that includes tissues (connective, muscle and glial tissue) that may impede 2-AG diffusion. In addition, this tissue does contain active MAGL that may break down a significant portion of the applied 2-AG (Kabeiseman et al., 2020).

In experiments using high-frequency stimulation (HFS) of the N cell, HFS consisted of 20 trains at a rate of 0.1 Hz, with each train having 10 suprathreshold stimulus pulses at 25 Hz (Wang and Burrell, 2016; Yuan and Burrell, 2019). Following HFS, a 30 min rest period was again used prior to testing the level of tonic inhibition. In experiments that involved application of THL (10 μM), SB 366791 (10 μM), or AM 251 (10 μM), perfusion of the recording chamber with the drug began 5 min prior to N cell HFS and continued throughout HFS period. Once the HFS was ended, the drugs were washed out for 30 min. Co-application of 2-AG and SB 366791 was conducted with the same concentrations used in earlier experiments and incubated for the same 15 min period.

Statistics

The change in P cell holding current (pA) is reported normalized to the average of the first 6 sweeps sampled under perfusion with normal saline. All data is reported as mean ± SEM. The sample size for all experiments refers to the number of animals tested (no replicates of the same treatment in tissue from the same animal). Statistical analyses were performed with Prism GraphPad Prism 9.0 for Mac (GraphPad Software, Inc., San Diego, CA, United States). Comparisons between groups for the maximum negative displacement in holding current were performed with a one-way ANOVA followed by the Tukey's Test for *post hoc* analysis. Results were regarded as significantly different if $p < 0.05$, <0.01, or <0.001.

RESULTS

The first set of experiments assessed whether P cells received tonic GABAergic input. *Hirudo* P cells have been shown to be hyperpolarized by GABA via a negative inward current and this GABA-mediated hyperpolarization/current is blocked by BIC (Sargent et al., 1977; Wang et al., 2015). In mammalian neurons, tonic inhibition has been assessed by measuring the change in holding current before and after BIC application (Semyanov et al., 2004; Song et al., 2011; Bright and Smart, 2013). This approach was used here to assess the capacity of BIC (100 μM) to elicit

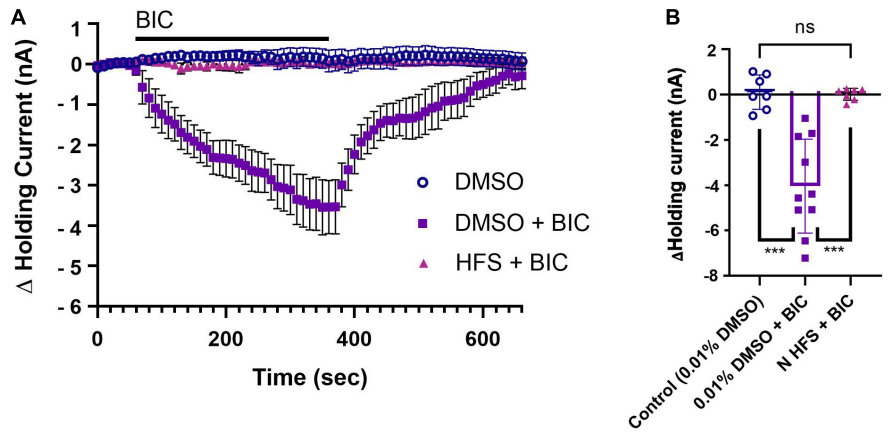

FIGURE 2 | N cell HFS depresses tonic inhibition in the P cell. **(A)** A substantial shift in the P cell holding current is observed during 100 μM BIC application (DMSO + BIC group) compared to the control group that simply had the holding current monitored in vehicle control, saline with 0.01% DMSO (referred to as the DMSO group). Recall that the 0.01% DMSO in saline is applied 30 min prior to BIC application as a vehicle control experiment (see section "Materials and Methods" and **Figure 1C**). N cell HFS applied 30 min before BIC application (HFS + BIC) fully prevented the negative shift in P cell holding current in response to BIC and is shown to be overlain on the control group that did not receive BIC. **(B)** When comparing the peak change in holding current during BIC bath-application, changes in holding current were significantly reduced in the HFS + BIC group compared to the only DMSO + BIC group (*** indicates $p < 0.0001$). No difference in peak current was observed between the N cell HFS + BIC group and the DMSO group. All data is shown as mean ± SE.

a similar shift in the P cell holding current, although we also looked at recovery of the holding current back to pre-treatment levels during the BIC washout phase. The downward shift in holding current during BIC application (note that these are not chloride-loaded cells) following pretreatment with normal saline containing 0.01% DMSO (DMSO + BIC group, $N = 10$) indicates that the P cell does receive tonic inhibitory input that is mediated by a $GABA_A$-like receptor (**Figure 2A**). During washout from BIC, the P cell holding currents return to baseline levels. In control experiments that received the 0.01% DMSO pretreatment, but no BIC application (referred to as the DMSO group, $N = 7$), the holding current was stable throughout the entire duration of the experiment (11 min). The BIC-induced changes in the P cell holding current were significantly different from controls in which BIC was withheld (**Figure 2B**; one-way ANOVA $F_{2,21} = 24.16$, $p < 0.0001$; DMSO + BIC vs. DMSO, *post hoc* $p < 0.0001$).

Previous studies have shown that P cell synapses experience persistent (30–60 min), heterosynaptic potentiation following HFS of the N cells, that requires endocannabinoid signaling and GABAergic signaling (Higgins et al., 2013; Wang and Burrell, 2016). It is our hypothesis that endocannabinoids depress tonic GABAergic input to the P cell, eliciting presynaptic disinhibition that leads to this persistent synaptic potentiation (**Figure 1A**). The subsequent experiments are designed to test whether endocannabinoids elicited a decrease in tonic GABAergic inhibition in the P cell over the same time frame as the previously observed endocannabinoid-mediated synaptic potentiation.

Since N cell HFS elicits endocannabinoid-mediated P synapse potentiation, the first set of experiments examined the effects of such stimuli on tonic inhibition of the P cell. Thirty minutes following HFS of the N cell, the BIC-induced shift in P cell holding current was absent (**Figure 2A**; HFS + BIC, $N = 7$) indicating that tonic inhibition had been depressed by HFS prior to BIC application. The maximum change in holding current was significantly different between BIC application where no HFS was applied (HFS + BIC vs. DMSO + BIC, *post hoc* $p < 0.0001$), but not significantly different from the peak change in holding current in the no-BIC control conditions (**Figure 2B**; HFS + BIC vs. DMSO). To summarize, BIC application produced a negative shift in P cell holding current consistent with the presence of tonic, GABAergic inhibition. HFS of the N cell eliminated the effect of BIC on holding current indicating that this nociceptor activity depressed tonic inhibition onto the P cell.

Next, the potential involvement of endocannabinoids in the depression of tonic GABAergic inhibition onto the P cell was investigated by pretreating the ganglion with either 2-AG or AEA and then monitoring the BIC-induced change in the P cell holding current after a 30 min washout period. Again, the goal here is to observe whether depression of tonic inhibition, and therefore disinhibition of the P cell, coincides with the time frame in which endocannabinoid-mediated P cell synaptic potentiation is observed. Both 2-AG and AEA are present in the *Hirudo* CNS with the concentration of 2-AG being approximately 10 times more than AEA (Matias et al., 2001). Additionally, in the rat brain, the basal levels of 2-AG have been estimated to be up to 1000 times greater than AEA (Buczynski and Parsons, 2010). Therefore, we opted to use a concentration of AEA (0.1 μM) that was 1000 times less than 2-AG (100 μM). 2-AG and AEA were applied for 15 min and then washed out for 30 min so that we could observe potential long-lasting modulatory effects initiated by either endocannabinoid. 2-AG ($N = 8$) or AEA ($N = 7$) treatment reduced BIC-induced shifts in the holding current (**Figure 3A**) indicating that both endocannabinoids persistently

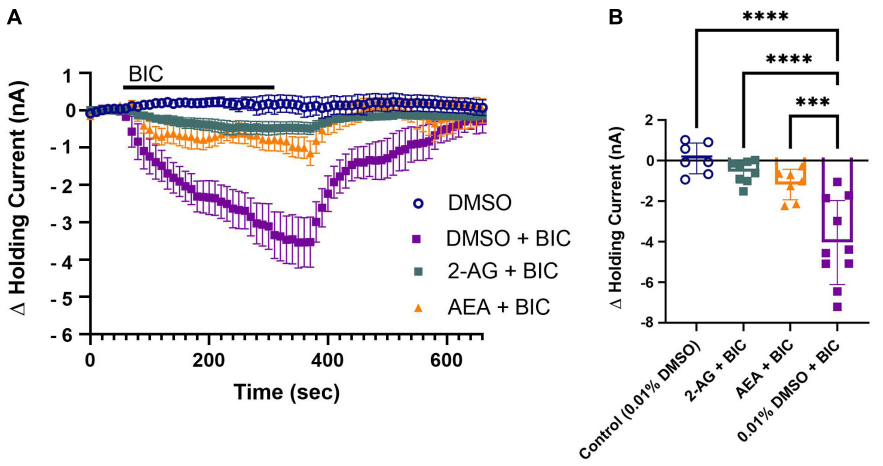

FIGURE 3 | 2-AG and AEA depress tonic inhibition in the P cell. **(A)** 30 min following pretreatment with 2-AG (2-AG+BIC) or AEA (AEA+BIC) produced only a very small change in holding current, indicating depression of tonic GABAergic input. **(B)** In terms of the peak change in holding current, both the 2-AG + BIC and AEA + BIC groups were significantly reduced compared to the DMSO + BIC (*** indicates $p < 0.001$; **** indicates $p < 0.0001$). The DMSO + BIC group was also significantly different from the DMSO group. The 2-AG + BIC and AEA + BIC groups were not significantly different from the DMSO group. All data is shown as mean ± SE.

depressed tonic GABAergic signaling. The peak change in holding currents for the 2-AG- and AEA-treated P cells during BIC application was statistically lower compared to the vehicle control (DMSO group) P cell's response to BIC (**Figure 3B**; $F_{3,28} = 17.58$, $p < 0.0001$; 2-AG + BIC vs. DMSO + BIC, *post hoc* $p < 0.0001$; AEA + BIC vs. DMSO + BIC, $p < 0.0001$; DMSO vs DMSO + BIC, $p < 0.001$). Although there was a small change in holding current during BIC application in the 2-AG and AEA-treated P cells (**Figure 3A**), no significant differences in the peak change in holding current were observed between these groups and the no-BIC control cells (**Figure 3B**; 2-AG + BIC vs. DMSO, AEA + BIC vs. DMSO, 2-AG + BIC vs. AEA + BIC). Thus, both endocannabinoids are able to significantly depress tonic GABA signaling onto the P cell at a time point that coincided with when 2-AG and AEA-elicited P cell synaptic potentiation was observed.

Since N cell HFS substantially depressed tonic inhibition onto the P cell, we next investigated whether this depression was dependent on production of 2-AG and/or TRPV receptors. This was done because both inhibition of 2-AG synthesis and TPRV channels blocked N cell HFS-induced potentiation in P synapses (Wang and Burrell, 2016). We repeated the N cell HFS experiment, but this time pretreating the ganglia with THL/Orlistat, an inhibitor of DAGL. Based on previous experiments (Yuan and Burrell, 2010, 2013b; Wang and Burrell, 2018), we hypothesized that THL/Orlistat would be able to prevent N cell HFS from depressing tonic inhibition. Following THL/Orlistat treatment, the BIC-induced change in holding current was measured 30 min following N cell HFS ($N = 10$). THL/Orlistat did not block the effects of HFS, indicated by the observations that the THL + HFS + BIC group was significantly different from the DMSO + BIC group (**Figure 4**; $F_{4,38} = 9.29$, $p < 0.001$, *post hoc* $p < 0.05$). Since THL/Orlistat treatment did not block the effects of N cell HFS, this suggests that 2-AG synthesis may not play a critical role in this activity-induced depression of tonic inhibition onto the P cell.

In earlier studies with *Hirudo* SB 366791, a TRPV1 receptor antagonist, blocked N cell HFS-induced potentiation of the P cell as well as potentiation produced by exogenous application of 2-AG or AEA (Higgins et al., 2013; Wang and Burrell, 2016). To determine whether a TRPV-like channel mediated N cell HFS-induced depression of tonic GABAergic input, we pretreated ganglia with SB 366791 before N cell HFS and then applied BIC 30 min later to assess tonic inhibition. SB 366791 did prevent depression of P cell tonic inhibition following N cell HFS (**Figures 4A,B**). The SB + HFS + BIC group ($N = 8$) was significantly different from the HFS + BIC group (*post hoc* $p < 0.005$) and not different from DMSO + BIC group. The peak change in holding current in the SB + HFS + BIC group was also significantly greater than the THL/Orlistat group (**Figure 4B**; SB + HFS + BIC vs. THL + HFS + BIC, $p < 0.05$). These findings are consistent with a TRPV-like channel playing a major role in activity-dependent depression of tonic inhibition in *Hirudo*.

The potential involvement of a *Hirudo* CB1-like metabotropic receptor was also examined. We explored the ability of AM251, an inverse agonist of CB1 receptors, to block the N cell HFS-induced depression of tonic inhibition onto the P cell. Ganglia were pre-treated with AM251 followed by N cell HFS and then BIC application 30 min later. While a small change in the in holding current was observed in the AM 251-treated ganglia (**Figure 4A**, $N = 8$), the peak shift in P cell holding current in the AM251 + HFS + BIC was not statistically different from the HFS + BIC group. The AM251 + HFS + BIC group was statistically different from the DMSO + BIC and the SB + HFS + BIC groups administered to control ganglia (**Figure 4B**, $p < 0.05$ for both). These results

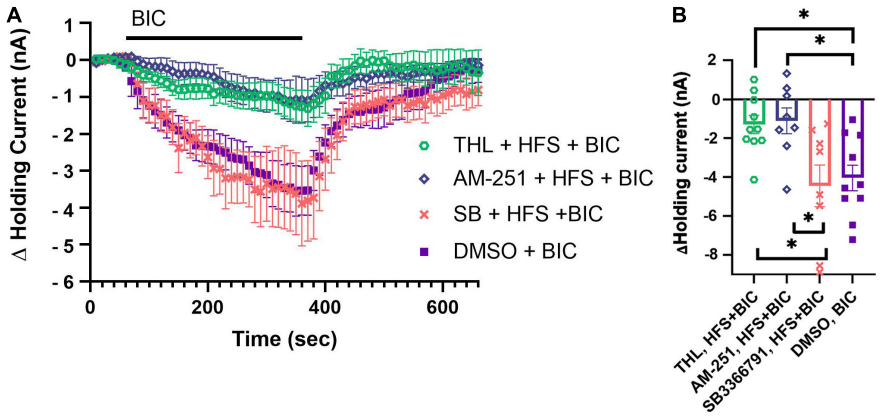

FIGURE 4 | Role of DAGL, TRPV, and CB1 in mediating N cell HFS-induced depression of tonic inhibition. **(A)** Pretreatment with 10 μM SB 366791 (SB) prior to N Cell HFS restore the BIC-induced negative shift in P cell holding current. 10 μM THL/Orlistat (DAGL inhibitor) and 10 μM AM251 (CB1 reverse agonist) did not prevent N cell HFS from depressing tonic inhibition. **(B)** In terms of peak change in holding current significant differences were observed between the THL + HFS + BIC vs. the DMSO + BIC and SB + HFS + BIC groups (* indicates $p < 0.05$). Significant differences were also observed between the AM251 + HFS + BIC vs. the DMSO + BIC and SB + HFS + BIC groups. All data is shown as mean ± SE.

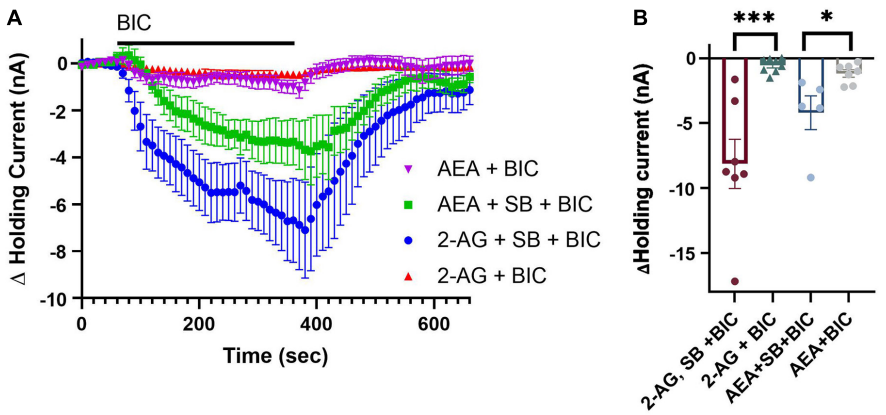

FIGURE 5 | TPRV signaling mediates 2-AG- and AEA-induced depression of tonic inhibition. **(A)** Co-application of 100 μM 2-AG with 10 μM SB is able to prevent 2-AG-induced depression of tonic inhibition onto the P cell. A total of 10 μM SB was also able to block the 0.1 μM AEA-induced depression of tonic inhibition. **(B)** In terms of peak current, the 2-AG + SB + BIC group was significantly different from the 2-AG + BIC group while the AEA + SB + BIC group was significantly different from the AEA + BIC group (* indicates $p < 0.05$; *** indicates $p < 0.001$). All data is shown as mean ± SE.

suggest that a CB1-like receptor does not contribute to HFS-induced depression of tonic inhibition.

Finally, we examined whether SB 366791 would block 2-AG- and AEA-mediated depression of tonic inhibition of the P cell observed in **Figure 3**. Ganglia were pre-treated with SB 366791 followed by SB 366791 plus 2-AG or AEA for 15 min. This was followed 30 min later by BIC application during which the P cell holding current was recorded. In the 2-AG + SB group ($N = 8$), BIC was now able to elicit a change in holding current, indicating that TRPV blocker did prevent depression of tonic inhibition by 2-AG (**Figures 5A,B**; 2-AG + BIC vs. SB + 2-AG + BIC, unpaired t-test $t_{13} = 4.29$, $p < 0.0001$). In the AEA + SB group ($N = 5$), BIC was also able to elicit a change in holding current, indicating that SB 366791 prevented AEA-induced depression of tonic inhibition (**Figures 5A,B**; 2-AG + BIC vs. SB + 2-AG + BIC, unpaired t-test $t_{13} = 2.68$, $p < 0.05$). These results indicate that depression of tonic inhibition by either 2-AG or AEA is mediated by a *Hirudo* TRPV-like channels.

DISCUSSION

In this study we observed endocannabinoid-mediated long-term depression (LTD) of GABAergic input. However, instead of LTD of GABAergic IPSPs as previously reported (Zhu and Lovinger, 2007; Gibson et al., 2008), we observed depression of tonic GABAergic inhibition. The pressure sensitive (P) mechanosensory cells in the *Hirudo* CNS exhibit tonic GABAergic inhibition based on changes in holding current during BIC application similar to how tonic inhibition has been observed in a variety of mammalian neurons (Semyanov et al., 2004; Song et al., 2011; Bright and Smart, 2013). The

source of this putative GABAergic input is not known, but the *Hirudo* CNS does possess GABA-containing neurons that are continuously active (Cline, 1983, 1986). Tonic inhibition has been shown to be an important regulator of excitability and synaptic transmission in both the brain and spinal cord of mice and rats (Semyanov et al., 2004; Pavlov et al., 2014; Gradwell et al., 2017). In *Hirudo*, exogenous application of GABA does hyperpolarize the P cells and disinhibition via BIC application enhances P cell synaptic transmission at the presynaptic level (Wang et al., 2015). This disinhibition plays a critical role in endocannabinoid-mediated potentiation of *Hirudo* P cell synapses. Application of either 2-AG or AEA produces persistent (at least 30–60 min) potentiation in P synapses that is prevented by co-application of BIC or inhibitors of Cl^- transport (Higgins et al., 2013; Wang and Burrell, 2016). HFS of a single *Hirudo* nociceptor (N cell) elicits a heterosynaptic form of synaptic potentiation in P synapses that is 2-AG-mediated (prevented by inhibition of the DAGL inhibitor, THL) and also prevented by blocking disinhibition (Wang and Burrell, 2016).

A lack of change in the holding current following BIC application is indicative that tonic GABAergic input is depressed. In the *Hirudo* P cells, BIC produced little or no change in holding current following 2-AG or AEA treatment, an indication that both endocannabinoids had depressed tonic inhibition to the P cell. This effect was long-lasting and the depression of tonic inhibition coincided with the time when endocannabinoid-mediated disinhibition/potentiation was observed in the previous experiments (Higgins et al., 2013; Wang and Burrell, 2016). The effects of 2-AG and AEA on tonic inhibition were blocked by the TRPV1 inhibitor SB66791. TRPV1 is known to be an important cannabinoid receptor in mammals for both AEA and 2-AG (Zygmunt et al., 1999, 2013; Gibson et al., 2008; De Petrocellis et al., 2011). TRPV-encoding genes are present in *Hirudo* (Heath-Heckman et al., 2021) and pharmacological studies using both activators (capsaicin and resiniferatoxin) and inhibitors (capsazepine and SB366791) support that a TRPV-like channel acts as a cannabinoid receptor in this species (Yuan and Burrell, 2010; Li and Burrell, 2011; Higgins et al., 2013; Wang and Burrell, 2016).

In the present study, it is proposed that depression of tonic inhibition is mediated by endocannabinoids acting on TRPV-like channels located on GABAergic interneurons (**Figure 1A**). In studies of endocannabinoid-mediated LTD of nociceptive (N) synapses in *Hirudo*, which are glutamatergic, intracellular injection of two different TRPV inhibitors (capsazepine and SB366791) demonstrated that a TRPV-like channel was acting presynaptically (Yuan and Burrell, 2010, 2013b). This endocannabinoid-mediated LTD of N synapses is mediated by activation of presynaptic calcineurin and coordinated transcription and translation in the pre- and postsynaptic neurons (Yuan and Burrell, 2012, 2013a). We hypothesize that similar cellular mechanisms are involved in endocannabinoid-mediated depression of tonic inhibition although additional experiments will be needed to confirm this. The identity of the GABAergic neurons undergoing depression is unknown so one cannot definitively prove the involvement of presynaptic TRPV-like channels as was done for the N synapse. However, the P cells, which receive this tonic inhibitory input, lack TRPV-like channels themselves (Summers et al., 2014). Therefore, the GABAergic neurons represent the most likely location for the TRPV-like channels mediating depression of tonic inhibition.

TRPV channels have been found to be required for endocannabinoid-mediated LTD via both presynaptic (Gibson et al., 2008) and postsynaptic mechanisms (Chávez et al., 2010; Grueter et al., 2010) in rodents. It may seem surprising that TRPV channels, which conduct Ca^{2+}, could mediate LTD presynaptically since increases in presynaptic Ca^{2+} would be expected to enhance neurotransmitter release and there are multiple reports of increases in spontaneous EPSP frequency during TRPV1 activation (Baccei et al., 2003; Medvedeva et al., 2008; Peters et al., 2010; Park et al., 2011). How then can presynaptic TRPV channels mediate synaptic depression? One potential explanation is that while TRPV activation can enhance synaptic transmission in the short-term during the period of direct activation, the resulting Ca^{2+} influx can also trigger intracellular signaling cascades that act over longer time frames in which depression is observed (tens of minutes to hours). This is analogous to NMDA-type glutamate receptors which can contribute to an EPSP, but also produce LTP or LTD through Ca^{2+}-dependent activation of intracellular signaling processes. Our studies of endocannabinoid-mediated depression of excitatory synapses and tonic inhibition have all been conducted with a considerable delay/washout period between drug application or HFS/LFS and the actual measurement of synaptic transmission. Furthermore, our past studies have shown that synaptic transmission following endocannabinoid activation of TRPV requires increases in intracellular Ca^{2+}, activation of calcineurin, and new protein synthesis, all at the presynaptic level (Yuan and Burrell, 2012, 2013a). These findings are consistent with a long-lasting modulatory process.

Another factor to consider is that there is a discontinuity in the effects of TRPV activation on spontaneous EPSPs vs. evoked EPSPs. The studies of TRPV1's presynaptic facilitating effects all involved increases in frequency of spontaneous EPSPs. However, one of these studies also reported that capsaicin depressed evoked EPSPs at the same time that spontaneous EPSP frequency was increased (Medvedeva et al., 2008) and there are additional reports of TRPV1 activation resulting in synaptic depression that is mediated presynaptically (Yang et al., 1999; Kusudo et al., 2006). As a possible explanation for how presynaptic TRPV could have opposing effects on spontaneous vs. evoked synaptic transmission, Fawley et al. (2016) have reported that there are distinct Ca^{2+} sources or nano-domains contributing to the spontaneous vs. evoked neurotransmitter release. In their experiments, TRPV1 channels could stimulate spontaneous neurotransmitter release, without contributing to evoked release.

N cell HFS also depressed tonic inhibition to the P cell. We investigated the role of 2-AG mediating this effect by blocking the activity of the DAGL, the primary enzyme involved in 2-AG synthesis (Lee et al., 1995). In previous experiments, THL/Orlistat blocked potentiation of P synapses following N cell HFS (Wang and Burrell, 2016), suggesting

2-AG involvement. Surprisingly, inhibition of 2-AG synthesis did not prevent the effects of N cell HFS on tonic inhibition. While there was a small shift in holding current during BIC application in the THL + HFS + BIC group, this was not significantly different from the HFS + BIC group. This would suggest that even though 2-AG can directly depress tonic inhibition, 2-AG is not required for HFS-induced depression. Perhaps AEA is mediating HFS-induced depression of tonic inhibition, while 2-AG plays some other, unknown role in mediating endocannabinoid-dependent LTP. Future experiments are planned to investigate the role of AEA in more detail.

The TRPV1 channel inhibitor, SB 366791, completely blocked depression of the tonic inhibition following N cell HFS. As noted above, a TRPV-like channel is thought to be a cannabinoid receptor in *Hirudo* and the TRPV inhibitor SB 366791 has also been observed to block HFS-elicited heterosynaptic potentiation of P synapses (Wang and Burrell, 2016). AM 251, a CB1 receptor reverse agonist, did not affect depression of tonic inhibition following N cell HFS. To date, the earliest homolog to the CB1 and CB2 receptors has been observed in *Ciona*, a deuterostome invertebrate (Egertova and Elphick, 2007; Elphick, 2012). Recently however, an endocannabinoid-sensitive metabotropic receptor has been identified in *C. elegans*, a protostome invertebrate (Pastuhov et al., 2016; Oakes et al., 2017). In addition, AM 251 has been found to inhibit putative endocannabinoid modulation in a number of different protostome invertebrates, including *Hirudo* (Lemak et al., 2007; Li and Burrell, 2009; Sunada et al., 2017). It remains to be determined whether these previously observed effects of AM 251 on invertebrates are due to action on a CB1 receptor ortholog or perhaps an alternative cannabinoid-sensitive metabotropic receptor (Paulsen and Burrell, 2019).

To our knowledge, this is the first report of activity-dependent modulation of tonic inhibition and endocannabinoid-mediated depression of tonic GABAergic inhibition. There is considerable evidence of the cannabinoid system being able to modulate activity-dependent synaptic plasticity, in particular LTP (Piette et al., 2020). In many cases, endocannabinoids reduce inhibitory synaptic transmission, thereby lowering the threshold for eliciting LTP (Chevaleyre and Castillo, 2004; Zhu and Lovinger, 2007; Xu et al., 2012), often referred to as metaplasticity (Abraham and Bear, 1996). Tonic inhibition has also been identified as an important modulator of LTP, again by altering the threshold for LTP induction (Martin et al., 2010) or enhancing one form of LTP over another (Dembitskaya et al., 2020). In our previous studies, N cell HFS alone is able to elicit potentiation of P synapses, without the need for the P cell to be directly activated. It is possible that during N cell HFS, endocannabinoid-mediated depression of tonic inhibition in the P cell creates a state where spontaneous/background activity is sufficient to elicit an LTP-like process (see **Figure 1A**). A comparable mechanism has been suggested in which high enough levels of 2-AG promote spike-timing dependent LTP without the need for coordinated pre- and postsynaptic activity in the rat striatum (Cui et al., 2016). Similarly, application of tetrahydrocannabinol alone has been reported to elicit widespread LTP in the mouse hippocampus (Puighermanal et al., 2009). What is the functional relevance of this observed depression of tonic inhibition following HFS of nociceptors and its proposed contribution to potentiation of P synapses? Potentiation of *Hirudo* P synapses is observed in semi-intact preparations following either HFS of a single N cell or delivery of a noxious stimulus to the skin (Wang and Burrell, 2018). This synaptic potentiation is endocannabinoid- and TRPV-dependent and contributes to behavioral sensitization in *Hirudo* in reflexive withdrawal responses to P cell stimulation. These findings in *Hirudo* have relevance to nociception in other species. Both synaptic and tonic inhibition play an important role in regulating activity in nociceptive circuits and disinhibition of these circuits contributes to sensitization to nociceptive and non-nociceptive stimuli following injury in rodents (Torsney and MacDermott, 2006; Kim et al., 2012; Petitjean et al., 2015; Gradwell et al., 2017; Perez-Sanchez et al., 2017). We propose that strong nociceptor activation elicits endocannabinoid synthesis and release (**Figure 1A**). These endocannabinoids, in turn, mediate heterosynaptic depression of inhibitory input with the resulting disinhibition contributing to behavioral sensitization. This hypothesis is supported by both our findings in *Hirudo* (Higgins et al., 2013; Wang and Burrell, 2016, 2018) and those by Pernia-Andrade et al. (2009) in mice. While the latter study involved activation of CB1, another study has shown that TRPV channel activation in the mouse spinal cord can also mediate disinhibition that contributes to allodynia (Kim et al., 2012). This study did not identify the ligand activating TRPV1, but endocannabinoids are a potential candidate.

Activity-dependent depression of tonic inhibition, potentially via endocannabinoids, represents an important, but until now unrecognized, modulatory process that can contribute to plasticity in synaptic circuits. From a functional standpoint, such modulation of tonic inhibition can contribute not only to nociception, but also other neurobehavioral process, e.g., learning memory. These findings are also relevant to understanding endocannabinoids' dual nature in synaptic modulation. That is, their ability to potentiate synapses under some conditions (often, but not exclusively via disinhibition) and depress synapses under other conditions (i.e., depression of neurotransmitter release). Examining how different patterns of activity and different intracellular signaling cascades produce this weakening or strengthening of synapses represents the critical next steps in understanding the endocannabinoid system's role in neurobehavioral plasticity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RP and BB developed the experimental design, carried out the experiments, analyzed the data, and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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