NEW INSIGHTS INTO BEHAVIORAL PHARMACOLOGY

EDITED BY: Nuno Sousa and Hugo Leite-Almeida
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NEW INSIGHTS INTO BEHAVIORAL PHARMACOLOGY

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EDITORIAL
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Editorial: New Insights Into Behavioral Pharmacology

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Keywords: behavior, EBPS young scientist workshop, School of Medicine/University of Minho, phenotypical analysis, neuropharmacology

Editorial on the Research Topic

New Insights Into Behavioral Pharmacology

In August 2019 we hosted at the School of Medicine/Life and Health Sciences research Institute (ICVS), University of Minho, the workshop (WS) "From networks to behavior and back—a EBPS Young Scientist Workshop." It preceded the 2019 European Behavioral Pharmacology Society (EBPS) Biennial Meeting (Braga, Portugal) and the two events were brilliantly bridged by Professor Alcino Silva talk "Molecular Systems Neuroscience of Memory Linking"—see a recently published perspective by the group (De Sousa et al., 2021). The main aim of WS was to provide an encompassing view of the tools used to study behavior including (but not restricted to) the classical paradigms—see for an overview (Cunha et al., 2020)—and a number of techniques to manipulate in real time the experimental setting as a function of animals' performance—(Lopes and Monteiro); see also below—as well as to record/manipulate brain activity. The success of the WS was at all levels evident which prompted us to launch the "New Insights into Behavioral Pharmacology" research topic aiming primarily to resonate the spirit of the workshop.

Articles published under the scope of the research topic indeed tackled an array of behaviors ranging from pain (Jarrin et al.), aversive memory (Guilherme and Gianlorenco), depression (Patricio et al.; Surowka et al.), impulsivity (Esteves et al.), and drug seeking/abuse (Gyawali et al.; Konig et al.) as well as a number of systems and (patho)physiological processes that have profound implications in behavior including in the former monoaminergic (Amalric et al.; Guilherme and Gianlorenco) and endocannabinoid (Lujan and Valverde) systems and in the latter neurogenesis (Lujan and Valverde; Patricio et al.), epilepsy (Dare et al.) and neurodegeneration (Amalric et al.). While most of these studies used rodent models, particularly mice, Dare et al., describe a drosophila high throughput model (*para*^{bss} mutant) to test anti-epileptic drugs (Dare et al.). In addition to the obvious screening value that authors took advantage of, the trans-species validation of physiological and disease mechanisms can offer potentially new insights and avenues of research.

In a different perspective, but of a wide interest for behavioral neuroscience, Lopes and Monteiro, introduce readers to the principles and applications of the visual programming language Bonsai (Lopes et al., 2015), an open access tool that permits the simultaneous control of different data streams. They provide the reader with a number of examples and step-by-step tutorials that can be readily implemented by researchers with elementary programming competences. Specifically, when applied to behavioral settings, Bonsai can be used to extract in real time, relevant information regarding animals' behavior (e.g., position, movement, interaction with elements of the setting). More importantly, it can be used to precisely pair electrophysiological information with behavioral readouts. On the top of that, the system can be programmed to trigger instructions as a function of

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behavior. In this regard possibilities are immense ranging from the presentation of a cue, delivery of a reward or even optogenetic activation just to name a few.

All in all "New Insights into Behavioral Pharmacology" provides an holistisc view of the present Behavioral Neuropharmacology field. It projects to the future new capabilities that will help researchers to navigate through the complexity of many of todays' neuroscience questions that some years ago seemed technically out of reach. Animals' behavior is a powerful tool in Neuroscience but claims of behavior to brain function causality are still often made on the basis of loose associations. In this vein, we hope that this research topic, challenged, even if to a small extent, a reductionist bias as it has been called by other authors (Krakauer et al., 2017)—see also (Yartsev, 2017). Finally, it was gratifying to receive and edit manuscripts from many laboratories across the globe, particularly from several WS attendees and faculty.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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The Pro-neurogenic Effects of Cannabidiol and Its Potential Therapeutic Implications in Psychiatric Disorders

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During the last decades, researchers have investigated the functional relevance of adult hippocampal neurogenesis in normal brain function as well as in the pathogenesis of diverse psychiatric conditions. Although the underlying mechanisms of newborn neuron differentiation and circuit integration have yet to be fully elucidated, considerable evidence suggests that the endocannabinoid system plays a pivotal role throughout the processes of adult neurogenesis. Thus, synthetic, and natural cannabinoid compounds targeting the endocannabinoid system have been utilized to modulate the proliferation and survival of neural progenitor cells and immature neurons. Cannabidiol (CBD), a constituent of the *Cannabis Sativa* plant, interacts with the endocannabinoid system by inhibiting fatty acid amide hydrolase (FAAH) activity (the rate-limiting enzyme for anandamide hydrolysis), allosterically modulating CB1 and CB2 receptors, and activating components of the "extended endocannabinoid system." Congruently, CBD has shown prominent pro-neurogenic effects, and, unlike Δ^9-tetrahydrocannabinol, it has the advantage of being devoid of psychotomimetic effects. Here, we first review pre-clinical studies supporting the facilitating effects of CBD on adult hippocampal neurogenesis and available data disclosing cannabinoid mechanisms by which CBD can induce neural proliferation and differentiation. We then review the respective implications for its neuroprotective, anxiolytic, anti-depressant, and anti-reward actions. In conclusion, accumulating evidence reveals that, in rodents, adult neurogenesis is key to understand the behavioral manifestation of symptomatology related to different mental disorders. Hence, understanding how CBD promotes adult neurogenesis in rodents could shed light upon translational therapeutic strategies aimed to ameliorate psychiatric symptomatology dependent on hippocampal function in humans.

Keywords: cannabidiol, drug addiction, substance use disorder, endocannabinoid system, neurogenesis, hippocampus

INTRODUCTION

Neuropsychiatric disorders such as schizophrenia, mood disorders, or drug addiction, represent a huge burden on society, greatly impairing the health of those affected. During the last half-century, considerable progress has been made to understand, prevent, and treat such conditions. However, treatment options are still far from optimal in terms of efficacy and specificity, and there remain important untreatable maladaptive phenotypes and treatment-resistant patients. To solve this issue, basic and applied research has tried to identify new altered neuropsychological mechanisms suitable to promote new therapeutic strategies (Cuthbert, 2014). In this quest, the discovery of adult hippocampal neurogenesis (Altman and Das, 1965) and its health implications (Kempermann, 2012) has opened new vistas upon innovative pharmacotherapies that could ameliorate impaired hippocampal function. Among the many ways proposed to accomplish such an improvement, cannabidiol (CBD) has recently stood out as a promising compound to be taken into consideration. In light of this, the following mini-review article aims to: (1) summarize the available evidence describing the modulation of adult hippocampal neurogenesis by CBD; to (2) provide a prospective collection of the responsible mechanisms; and (3) to detail the presumed therapeutic potential of this phytocannabinoid *via* the modulation of adult neurogenesis.

Cannabidiol

CBD is one of the most abundant constituents of the *Cannabis sativa* plant. Unlike Δ^9-tetrahydrocannabinol (THC), CBD is devoid of psychotomimetic and rewarding effects (Ligresti et al., 2016), and is well tolerated in humans (Chesney et al., 2020). CBD is thought to interact with several molecular targets (Campos et al., 2017). Its main targets within the central nervous system are comprehended by the activation of 5-hydroxytryptamine 1A (5-HT_{1A}), transient potential vanilloid 1 (TRPV1), G-protein 55 (GPR55) and peroxisome proliferator-activated gamma (PPARγ) receptors, as well as the antagonism of adenosine reuptake (Turner et al., 2017). Despite initial controversy about its endocannabinoid targets (Zlebnik and Cheer, 2016), recent evidence also supports CBD as a negative allosteric modulator of cannabinoid receptors 1 and 2 (CB1, CB2) at physiologically relevant concentrations (Laprairie et al., 2015; McPartland et al., 2015; Martínez-Pinilla et al., 2017; Navarro et al., 2018; Tham et al., 2019). Also, CBD reduces anandamide (AEA) metabolism by inhibiting fatty acid amide hydrolase (FAAH) activity (De Petrocellis et al., 2011). Consequently, CBD is an efficient anxiolytic (Fogaça et al., 2018) and there is evidence suggesting that it possesses anti-inflammatory (Atalay et al., 2019), neuroprotective (Campos et al., 2016), antidepressant (Sales et al., 2019), anti-relapse (Gonzalez-Cuevas et al., 2018), pro-cognitive (Osborne et al., 2017) and antipsychotic (Renard et al., 2017) effects. Accordingly, CBD has been proposed as a novel therapeutic strategy for different mental disorders such as drug addiction (Calpe-López et al., 2019), depression (Silote et al., 2019), or schizophrenia (Elsaid and Le Foll, 2020). Notwithstanding the foregoing, CBD has a formidably complex pharmacology, and therefore, we lack a clear understanding of the molecular and neuroplastic consequences of CBD treatments. With such a pool of targets, numerous hypotheses have tried to explain CBD's therapeutic mechanisms in each of the psychiatric models addressed. The modulation of neuronal network dynamics in the mesolimbic system *via* 5-HT_{1A} activation (Norris et al., 2016) is positioned as the best approximation to CBD's anti-craving actions (Katsidoni et al., 2013; Bi et al., 2019; Galaj et al., 2020). On the other hand, the presumed motivational consequences of *in vivo* CBD's CB1 effects remain unclear. Recent reports show that CBD modulation of cocaine-seeking reinstatement, but not operant intake, depends on CB1 receptor activation (Galaj et al., 2020; Lujan et al., 2020). Therefore, indirect CB1 activation through FAAH blockade, rather than CB1 negative allosteric modulation, is a more plausible mechanism for the anti-craving effects of CBD. In the case of mood and anxiety-related disease models, the activation of ventromedial prefrontal cortex 5-HT_{1A} and CB1 receptors (Linge et al., 2016; Sartim et al., 2016), and the neuroprotection against inflammatory and oxidative brain insults (Campos et al., 2016) are the main mechanism candidates. Lastly, diverse studies have also pointed to the pro-neurogenic effects of CBD, as reviewed below.

Adult Hippocampal Neurogenesis in the Mammalian Brain

Adult hippocampal neurogenesis encompasses a complex, multistep process comprehending the proliferation, survival, differentiation/maturation, and functional integration of newborn neurons residing in the subgranular zone (SGZ) of the dentate gyrus (DG; Kuhn et al., 2018; **Figure 1A**). It is detailed in most mammals (Amrein, 2015), but its existence in humans has been hotly debated due to the critical dependence on ^{14}C labeling (Sorrells et al., 2018). However, the latest evidence suggests that adult hippocampal neurogenesis in humans is abundant even in the senescence (Boldrini et al., 2018; Tobin et al., 2019) and that previous discrepancies were probably due to tissue processing protocols or neurological illness of the tissue donors (Moreno-Jiménez et al., 2019).

During their development, adult-born neurons modulate DG functions that orchestrate diverse behaviors. Newborn neurons act as independent encoding units that can inhibit the activity of mature granule cells (Drew et al., 2016) and dampen overall DG excitability (Ikrar et al., 2013). Given the participation of hippocampal function in mood, cognition, and motivation, adult hippocampal neurogenesis is involved in different neuropsychological processes in physiological and pathological conditions (Mandyam and Koob, 2012). For example, patients with depression exhibit decreased levels of neurogenesis (Lucassen et al., 2010). Neurogenesis ablation increases innate anxiety-like behaviors (Revest et al., 2009) and depressive-like symptoms (Wu et al., 2014) in animal models. And more importantly, anti-depressant drugs increase neurogenesis, an effect that is required to observe some of its behavioral effects in rodents (Santarelli et al., 2003).

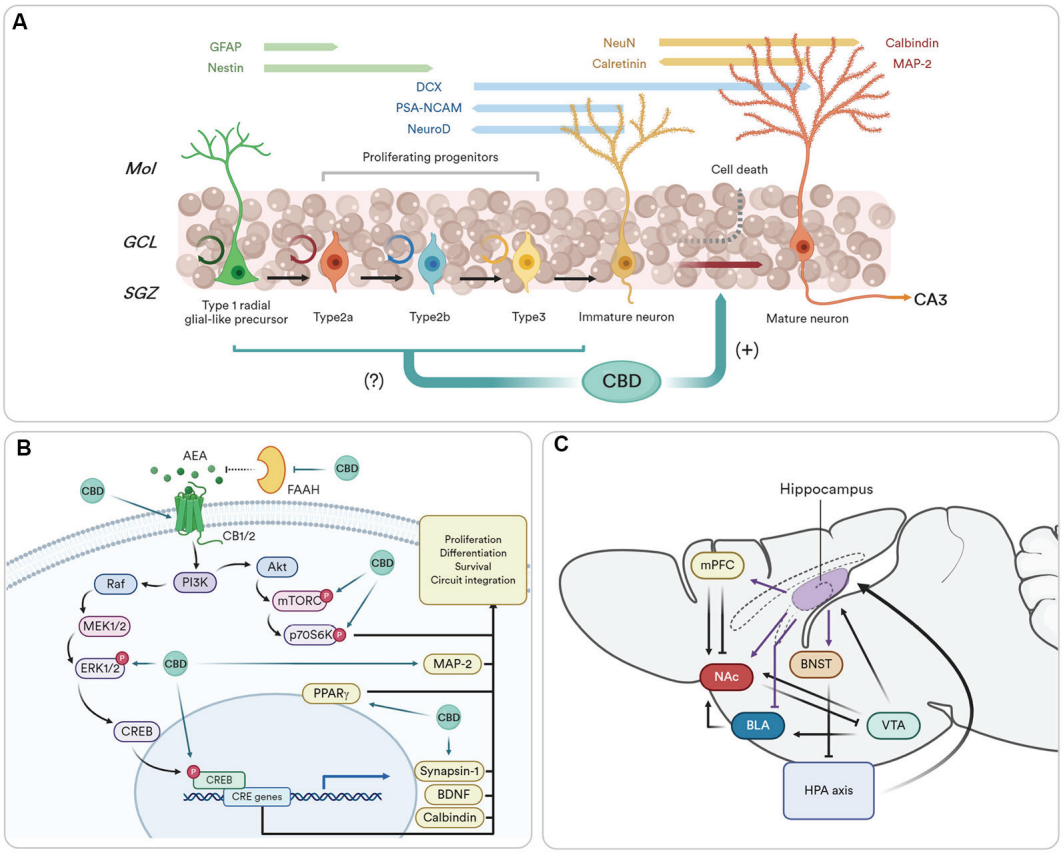

FIGURE 1 | The pro-neurogenic effects of cannabidiol (CBD) and its functional relevance. **(A)** Adult hippocampal neurogenesis originates from type 1 precursor cells that might differentiate into granule neurons (Kriegstein and Alvarez-Buylla, 2009). The newly generated cells can be stimulated *via* GABAergic, endocannabinoid- and serotonin-dependent mechanisms (Encinas et al., 2006; Prenderville et al., 2015). The proliferation phase ends just after precursor cells exit the cell cycle. As early as 1 day after such an event, newborn neurons start expressing the postmitotic marker NeuN, which then declines as most newborn cells are eliminated before they become functional (survival phase; Kempermann et al., 2015). Within days after generation, newborn neurons send their axons to cornu ammonis 3 (CA3), where they form proper synapses (Sun et al., 2013). In the next phase, newborn neurons mature as the excitatory nature of GABA inputs shift into the standard depolarizing profile. Finally, new mature neurons go through a phase of increased synaptic plasticity, which in turn promotes its final integration into the hippocampal circuitry (functional integration phase; Ge et al., 2007). The effects of CBD are preferentially focused on the post-mitotic stages of the neurogenic process, whenever it facilitates neuronal maturation and impedes early neuronal death. **(B)** The pro-neurogenic effects of CBD are orchestrated by the eCB system. Furthermore, CBD upregulates different molecular components of downstream pathways usually associated with the eCB-driven facilitation of adult neurogenesis. Final molecular effectors of the protein synthesis and survival machinery of the hippocampus such as brain-derived neurotrophic factor (BDNF), calbindin, MAP-2, synapsin 1, and the activation of protective peroxisome proliferator-activated gamma (PPARγ) receptors, are also found upregulated after CBD subchronic treatments. **(C)** The figure represents a simplified vision of the hippocampal neurocircuitry functionally coupled to the neurogenic state of the dentate gyrus (DG). The hippocampus (ventral part) sends direct projections to the GABAergic interneurons of the BNST that, in turn, tune-down the hypothalamus–pituitary–adrenal (HPA) axis (Snyder et al., 2011). Direct projections from the hippocampus to the mPFC promote stress sensitivity (Padilla-Coreano et al., 2016), and mediate antidepressant effects (Bagot et al., 2015). Ventral hippocampal outputs to the BLA are involved in the feedforward inhibition of fear and anxiety-related responses (Bazelot et al., 2015). Finally, the hippocampus can indirectly influence VTA DA release in motivated tasks by activating medium spiny neurons of the NAc (Britt et al., 2012). Abbreviations: GFAP, glial fibrillary acidic protein; Mol, molecular layer; GCL, granule cell layer; AEA, anandamide; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area. Created with Biorender.com.

Such relations are thought to be due to the newborn neuron regulation of hippocampal inhibitory control over the hypothalamus–pituitary–adrenal (HPA) axis. The ventral part of the hippocampus has also been related to emotional control. This region shares regulatory projections to canonical emotional-processing structures such as the basolateral amygdala or the medial prefrontal cortex that are key to modulate fear-associated memories and anxiety (Felix-Ortiz et al., 2013; Padilla-Coreano et al., 2016) and are regulated by neurogenesis (Temprana et al., 2015; **Figure 1C**). Interestingly, it is now described that pattern separation, a brain computational mechanism dependent on newborn neurons (Leutgeb et al., 2007; Sahay et al., 2011) also allows for the discrimination of emotional states experienced during memory creation (Redondo et al., 2014), thus granting adult hippocampal neurogenesis a way to modulate emotional memories retrieval and storage (Anacker and Hen, 2017). There is debate as to whether such functional implication could represent a caveat of pro-neurogenic therapeutics, as enhanced

pattern separation processes may promote proactive interference (see Epp et al., 2016; Tello-Ramos et al., 2019).

Additionally, the circuitry involved in the regulation of mood and stress overlaps with the brain circuitry affected by motivation disorders (Koob, 2015; Volkow et al., 2016). Hence, extensive evidence posits adult hippocampal neurogenesis as an additional component of drug addiction etiology (Castilla-Ortega et al., 2016; Barr et al., 2018). In this sense, rats with experimentally-reduced neurogenesis consume more cocaine and work harder to obtain the drug (Noonan et al., 2010; Deroche-Gamonet et al., 2019). Conversely, pharmacological induction of adult neurogenesis facilitates the forgetting of cocaine-contextual memories (Ladrón de Guevara-Miranda et al., 2019). Other drugs, such as alcohol, induce persistent reductions in adult neurogenesis in rodents (Spear, 2018), primates (Taffe et al., 2010), and humans, as shown by *post mortem* samples from alcohol abusers (Dhanabalan et al., 2018). Finally, the neonatal ventral hippocampal lesion rat model, which irreversibly lessens adult neurogenesis, has been used to reveal the participation of adult neurogenesis in the pathogenesis of dual diagnosis schizophrenia (Chambers and Self, 2002). This relation suggests that neurogenic deficits may also underlie positive-like, negative, and cognitive symptoms of schizophrenia in rodent models (Chambers, 2013; Sentir et al., 2020). Overall, a wealth of literature supports the relevance of adult neurogenesis in preclinical models of mood and anxiety disorders, as well as drug addiction or schizophrenia, while opens a new window of therapeutic opportunities aimed to ameliorate impaired hippocampal function.

PROMOTING NEUROGENESIS WITH CANNABIDIOL

Preclinical Evidence

Considering that the endocannabinoid (eCB) system exerts important functions in the regulation of neuronal generation and survival (Aguado et al., 2005), the Kempermann's group firstly explored the possibility that a cannabinoid like CBD could enhance the survival of DG newborn neurons in mice (Wolf et al., 2010). The pioneering study showed that a CBD-enriched diet increased co-localized immunoreactivity of 5-bromo-2′-deoxyuridine (BrdU) and *neuronal nuclei* (NeuN). Moreover, the authors reported an interesting opposition to the effects of THC on this measure. Months later, Demirakca et al. (2010) similarly proposed these pro-neurogenic actions of CBD in humans. Since then, researchers have echoed these investigations, finding a remarkable result consistency in the pro-neurogenesis induced by CBD (**Table 1**).

Much of the preclinical work aimed at delineating the pro-neurogenic profile of CBD has mainly utilized two immunostaining observables: doublecortin (DCX) and BrdU/NeuN. Due to the prolonged presence of both markers in different stages of the neurogenesis process (for a review see Kempermann et al., 2015), it is difficult to elucidate the phase specificity of CBD changes. CBD increased BrdU/NeuN co-localization from 1 month after the injection of the thymine incorporation tracer (Wolf et al., 2010; Fogaça et al., 2018), a measure of late survival, to as early as 7 days (Luján et al., 2018, 2019), a correlate of early differentiation. The same consistency has been found using DCX. Following the same treatment protocol, CBD increased DCX staining from 7 days (Luján et al., 2019) to 1 month (Luján et al., 2018) after the last CBD injection. But in the work of Wolf et al. (2010), CBD did not enhance, and even reduced, neural progenitor cell (BrdU/Nestin-expressing type 1/2 cells) proliferation. More studies analyzing markers of neural progenitor cell proliferation are needed but, these results could imply that CBD pro-neurogenic effects would take place after newborn neurons are generated, and not before (**Figure 1B**). This goes in agreement with molecular findings reflecting the facilitating effects of CBD on postmitotic neuronal survival, differentiation, and maturation. The brain-derived neurotrophic factor (BDNF) positively regulates newborn neuron survival in the DG (Waterhouse et al., 2012), and CBD increases BDNF protein content within the hippocampus (Mori et al., 2017; Luján et al., 2018; Sartim et al., 2018; Sales et al., 2019). Calbindin, a Ca^{2+}-binding protein used as a marker of mature neurons (Brandt et al., 2003), is also increased in the hippocampus of CBD-treated rats (Esposito et al., 2011). This idea has been further corroborated by the discovery that CBD activates different survival and synaptic remodeling cascades such as ERK1/2-CREB (Luján et al., 2018), GSK3β and PSD95 (Campos et al., 2013) or PI3K/mTOR/p70S6K (Renard et al., 2016; Giacoppo et al., 2017; Lanza Cariccio et al., 2018).

CBD pro-neurogenesis also shows great consistency across doses. Literature findings report increases in neuronal proliferation and differentiation after CBD doses ranging from 3 to 30 mg/kg, usually after prolonged treatments (≥10 days; **Table 1**). Despite this, at least two studies point to an inverted U-shaped dose-response curve effect. *in vitro*, Campos et al. (2013) described that CBD enhanced neuronal proliferation at medium concentrations (100, 250 nM), but these effects disappeared at lower (50 nM) or higher doses (500 nM). Similarly, Schiavon et al. (2016) showed that neuronal proliferation enhancement (here assessed by DCX) could only be observed after low (3 mg/kg) but not high (30 mg/kg) doses *in vivo*. Inverted U-shaped dose-response curves usually suggest the participation of multiple pharmacological mechanisms. In this way, it has already been described that CBD also exerts a similar anxiolytic dose-response curve (for a review see Jurkus et al., 2016) and that it is due to the interaction of 5-HT_{1A} and TRPV1 mechanisms (Campos and Guimarães, 2009). Therefore, one of the first neurogenic mechanisms that were evaluated consisted of the activation of 5-HT_{1A} receptors. However, CBD-induced proliferation in HiB5 hippocampal progenitor cells was not blocked by a 5-HT_{1A} antagonist (Campos et al., 2013) and so, an alternative candidate was considered: the eCB system.

Evaluating CBD's Endocannabinoid Mechanisms to Promote Neurogenesis

The eCB system stands out as a key regulator of newborn neuron generation, survival, maturation, and functional

TABLE 1 | Literature assessing the effects of cannabidiol (CBD) in adult hippocampal neurogenesis.

Reference	Animal, cell line	CBD treatment protocol	Markers	Effect	Experimental condition
Wolf et al. (2010)	C57BL/6 female mice	CBD-enriched diet, 6 weeks	BrdU/Nestin/DCX$^-$ (early proliferation)	–	–
			BrdU/Nestin/DCX$^+$ (late proliferation)	↓	–
			BrdU/NeuN (late survival)	↑	–
Esposito et al. (2011)	Sprague–Dawley male rats	10 mg/kg, i.p., 15 days	DCX	↑	Aβ-inoculated rats
Campos et al. (2013)	C57BL/6 male mice	30 mg/kg, i.p., 14 days	DCX	↑	Naive, chronic unpredictable stress
			BrdU/NeuN	↑	Control
	Hippocampal HiB5 progenitors	50, 100, 250, 500 nM, 18 h	BrdU/NeuN (proliferation)	100, 250 mg/kg: ↑ 50, 500 mg/kg: –	–
Shinjyo and Di Marzo (2013)	Mouse neural stem/progenitor cells	1 μM, 2 days	Nestin	↑	–
Schiavon et al. (2016)	Swiss CD-1 male mice	3 and 30 mg/kg, i.p., 15 days	DCX (proliferation)	3 mg/kg: ↑ 30 mg/kg: ↓	–
Mori et al. (2017)	C57BL/6 male mice	10 mg/kg, i.p., 3 days	DCX	↑	Ischemic mice
			MAP-2 (dendritic maturation)	↑	Ischemic mice
Fogaça et al. (2018)	C57BL/6 male mice	30 mg/kg, i.p., 14 days	DCX	–	Control
				↑	Chronic unpredictable stress
			DCX-tagged cell migration	–	Control
				↑	Chronic unpredictable stress
			BrdU/NeuN	–	Control
				↑	Chronic unpredictable stress
Luján et al. (2018)	Swiss CD-1 male mice	20 mg/kg, i.p., 10 days	BrdU/NeuN	↑	Control and cocaine-consuming mice
			DCX	↑	Cocaine-consuming mice
Luján et al. (2019)	Swiss CD-1 male mice	10, 20 mg/kg, i.p., 10 days	BrdU/NeuN	↑	Control and cocaine-consuming mice
			DCX	↑	Control and cocaine-consuming mice
Bis-Humbert et al. (2020)	Sprague-Dawley male rats	3, 10, 30 mg/kg, i.p., 6 days	NeuroD	–	–

In vivo measures of BrdU incorporation or Ki67 not accompanied by a neuronal marker (e.g., NeuN) were not considered, given the difficulty to differentiate from the proliferation of non-neuronal cellular lineages. Abbreviations: BrdU, 5-bromo-2ı-deoxyuridine; NeuN, neuronal nuclei; DCX, doublecortin; MAP-2, microtubule-associated protein 2. –, no change found; ↑, increase; ↓, decrease.

integration in the adult hippocampus. Neural progenitor cells, and their descendants, express a functional eCB system and are subject to the effects of endocannabinoid signaling (Prenderville et al., 2015). CB1 agonists induce neural proliferation and differentiation in the DG (Andres-Mach et al., 2017), which are also attenuated in $CB1^{-/-}$ mice (Aguado et al., 2007; Zimmermann et al., 2016). The same has been detailed for CB2 receptors (Palazuelos et al., 2012; Avraham et al., 2014), although in a more complicated fashion (Rodrigues et al., 2017; Mensching et al., 2019). That is, CB1 receptors participate in the maintenance of adult neurogenesis, whereas CB2 receptors seem to promote the recovery from allostatic neurogenic states (Oddi et al., 2020). Furthermore, the intricate downstream cellular pathways engaged by cannabinoid receptors, mainly converging in Akt/mTORC and MAPK/CREB pathways, are critically involved in cell proliferation, differentiation, and survival and are required for endocannabinoids to exert its pro-neurogenic effects (Prenderville et al., 2015).

Given the mechanistic interactions between CBD and eCB system, a plausible hypothesis originated stating that CBD increases adult neurogenesis by modulating the eCB system. Accordingly, *in vitro* and *in vivo* evidence has suggested such interplay. The first evidence was given by Wolf et al. (2010). In their study, a CBD-enriched diet facilitated newborn neuron survival, an effect prevented in $CB1^{-/-}$ mice. The seminal work of Campos et al. (2013) further explored this idea and tested which molecular mechanisms could be involved *in vitro*. CB1 and CB2 antagonists prevented the pro-neurogenic effect of CBD in hippocampal HiB5 progenitor cells. Furthermore, CB1 and CB2 receptor agonists, as well as eCB degradation inhibitors mimicked the pro-neurogenic effects of CBD. Interestingly, CBD effects were abrogated when the FAAH was inhibited. Combined, these results imply that the pro-neurogenic effects of CBD depending on the increase of AEA concentration. Crucially, CBD is an inhibitor of the FAAH and is well known to increase AEA concentration (Bisogno et al., 2001; De Petrocellis et al., 2011; Leweke et al., 2012; Petrosino et al., 2018). Note that, in this case, the CBD-induced negative allosteric modulation of CB1 receptors should not account for these results, as they rely on the facilitation of CB1 function. Alternatively, CBD can also increase the protein content of CB1 receptors in the hippocampus (Luján et al., 2018). Recently, a similar mechanism was revealed *in vivo*. After a CBD treatment in chronically stressed mice, neuronal differentiation, and late survival were found to be increased in CBD-treated mice (Fogaça et al., 2018). Such pro-neurogenic effects depended on CB1 and CB2 receptor activation, insofar respective antagonists abolished said increase. Intriguingly, CB1 antagonism only prevented the DCX-labeled neuronal differentiation increase whereas the CB2 antagonist precluded the increment of both, neuronal differentiation and late survival (BrdU/NeuN; Fogaça et al., 2018). Regarding this divergence, previous works have indicated that CB1 receptors may be implicated in maintaining basal adult neurogenesis, while CB2 receptors might be more physiologically relevant in coping with neurotoxic brain insults (Oddi et al., 2020). In the study of Fogaça et al. (2018), possibly the CB2 outshined CB1 receptors because its relative contribution was exclusively performed in chronically-stressed mice. So far, the differential role of CB1 and CB2 receptors in the pro-neurogenic effects of CBD in normal and allosteric conditions has not been explored enough, and more studies are needed to address this question. Altogether, studies interrogating the eCB system in conditions in which CBD produced pro-neurogenic effects have all encountered a suggesting implication. Although promising, there remain important gaps to be filled. For instance, no data is available as to the eCB-specific downstream signaling pathways recruited by cannabinoid receptors that would be facilitating neuronal survival and differentiation, despite some approximations in this regard (Luján et al., 2018). Furthermore, there also remain some unexplored CBD mechanisms with potential pro-neurogenic properties, such as GPR55 activation for coping reduction of neurogenesis in response to inflammatory insults (Hill et al., 2019). Noteworthy, a protective interaction involving neuroinflammation processes has been already observed, showing that CBD-mediated activation of PPARγ is associated with increased neurogenic activity, as well as reduced reactive gliosis, in the granule cell layer of the hippocampal DG (Esposito et al., 2011).

THERAPEUTIC INSIGHTS FROM PRECLINICAL PSYCHIATRIC MODELS

A considerable number of studies have reported the pro-neurogenic effects of CBD, and some among them have even related these with an eCB mechanism of action. But, can the pro-neurogenic effects of CBD account for some of its therapeutic applications? Answering this question requires specialized experimental strategies designed to rule out CBD pro-neurogenesis, leaving intact its other pharmacological mechanisms and so, fewer experiments have been conducted. Nonetheless, a handful of studies have addressed this question, presenting evidence for a potential implication in the protection against neurodegenerative diseases (Esposito et al., 2011), anxiety- and mood-related disorders (Campos et al., 2013; Fogaça et al., 2018), as well as drug addiction (Luján et al., 2019).

Neurodegenerative and ischemic conditions are among the circumstances in which hippocampal function can manifest greater impairments (Shah et al., 2019). It was Esposito et al. (2011) who firstly reported the pro-neurogenic effect of CBD in a neuropathological disease model. In their work, they showed how CBD could restore the neuronal differentiation levels after β amyloid peptide inoculation in a rat model of Alzheimer's disease. This effect was shown dependent on the activation of PPARγ receptors. Significantly, when a PPARγ antagonist was co-administered, CBD did not induce neuronal differentiation and, consequently, its neuroprotective effects were prevented (Esposito et al., 2011). Although suggestive, these results will need to be further verified, given the alternative protective

consequences of PPARγ receptor activity by itself (Hughes and Herron, 2019). Anxiety- and mood-related disorders symptomatology is also critically dependent on hippocampal function (Anacker and Hen, 2017). For this reason, Campos et al. (2013) tested if the action of CBD on hippocampal neurogenesis accounted for its anxiolytic and antidepressant effects. Using a genetic-pharmacological approach, they were able to report that the blockade of adult neurogenesis accounted for the anxiolytic and antidepressant effects of CBD on the elevated plus-maze and novel suppressed feeding tests. Using a more indirect approach, Fogaça et al. (2018) have recently supported these implications. The co-administration of CBD and a CB1 or CB2 antagonist prevented both the increase in adult hippocampal neurogenesis and the anxiolytic effects of CBD. Again, this pharmacological strategy does not allow to discard beneficial changes induced by CB1 or CB2 receptor activity by itself, but the replication of the causal discovery of Campos et al. (2013) is certainly meaningful. Finally, our group also tried to unravel the participation of adult neurogenesis in the protective actions of CBD on cocaine self-administration, a rodent model of cocaine abuse. Based on the findings that CBD-induced attenuation of cocaine voluntary intake was accompanied by increased adult neurogenesis, as well as MAPK/CREB pathway activity in the hippocampus (Luján et al., 2018), we developed a pharmacological strategy aimed to prevent the increases in adult neurogenesis induced by CBD with the anti-mitotic agent temozolomide (Niibori et al., 2012). Similar to Campos et al. (2013), we found that such an increase was crucially required by CBD to reduce cocaine voluntary intake in mice (Luján et al., 2019). Overall, available data supports that CBD-induced adult neurogenesis can account for the protective effects of CBD in certain psychiatric conditions. The role of CBD neurogenesis in other mental diseases remains largely unexplored. The case of schizophrenia is especially suggesting. Decreased hippocampal neurogenesis is observed in schizophrenic patients compared with control subjects (Reif et al., 2006), and it is rescued by atypical antipsychotics in rodents (Kusumi et al., 2015). Noteworthy, the pro-neurogenic effects of CBD in mice exposed to chronic unpredictable stress suggestively resembles that of atypical antipsychotics such as clozapine in the same model (Campos et al., 2013; Chikama et al., 2017; Morais et al., 2017; Fogaça et al., 2018). Based on this observation, studies dissecting the importance of CBD pro-neurogenic effects on its antipsychotic properties are promising, as well as highly needed.

CONCLUSION AND FUTURE DIRECTIONS

A significant amount of animal and human data has emerged relating the neuro-modulatory role of adult hippocampal neurogenesis, its interactions with broader hippocampal circuits, and its implications on altered behaviors in different neuropsychiatric disorders (Beckervordersandforth and Rolando, 2020). Meanwhile, some pro-neurogenic compounds have been experimentally employed to counteract maladaptive neuroplasticity and improve hippocampal function. In the last decade, there has been an increased interest in the psychiatric therapeutic potential of CBD. Its protective brain effects, as well as its endocannabinoid mechanisms, have been related to its ability to facilitate the survival and differentiation of newborn neurons of the DG. Crucially, key studies have emerged linking this pro-neurogenic effect with reduced anxiety-like states and improved emotional and motivational processing in animal models of stress-, mood-, and substance use-related disorders. Albeit convincing, investigations of CBD's pro-neurogenic effects are still in an early stage, and further experimental efforts are required to answer several open questions. Only two studies have so far fully addressed the causal implication of such a CBD mechanism (Campos et al., 2013; Luján et al., 2019). This lack of studies also leads to several replication needs. For example, most of the work has been developed in male mice, which hinders possible interpretations regarding sex- or species-specific effects. Also, evidence regarding the effects of CBD in the pre-mitotic stages of neuronal proliferation is scarce. On the other end, we still lack a direct electrophysiological confirmation of the functional integration of maturing neurons in conditions of elevated neurogenic state induced by CBD. Answering such a question is vital to clarify the functional relevance of CBD-induced neurogenesis and rule out an epiphenomenon effect. From a theoretical perspective, we also needed to better conceptualize the therapeutic potential of increased neurogenic states in adults. Newborn neurons necessarily remodel hippocampal circuitries upon functional integration. Thus, increased neurogenesis can destabilize consolidated memories (Chambers et al., 2004; Deisseroth et al., 2004), which may promote forgetting (Akers et al., 2014; but see Epp et al., 2016). Finally, indirect information supportive of the occurrence of hippocampal neurogenesis in humans treated with CBD is not yet available. Measures of the 1.28 ppm neurogenesis-specific peak using magnetic resonance spectroscopy (Manganas et al., 2007) could be incorporated in future clinical trials working with CBD treatments to shed more light on the functional and therapeutic relevance of these CBD's neurogenic changes.

AUTHOR CONTRIBUTIONS

ML and OV were responsible for the study concept and design. Both authors drafted the manuscript and approved the final version for publication.

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frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
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miR-409 and miR-411 Modulation in the Adult Brain of a Rat Model of Depression and After Fluoxetine Treatment

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Depression is a chronic debilitating disorder predicted to affect around 20% of the world population. Both brain and peripheral changes, including neuroplastic changes have been shown to occur in the brains of depressed individuals and animal models of depression. Over the past few decades, growing evidence has supported the role of miRNAs as regulators of critical aspects of brain plasticity and function, namely in the context of depression. These molecules are not only highly expressed in the brain, but are also relatively stable in bodily fluids, including blood. Previous microarray analysis from our group has disclosed molecular players in the hippocampal dentate gyrus (DG), in the context of depression and antidepressant treatment. Two miRNAs in particular—miR-409-5p and miR-411-5p—were significantly up-regulated in the DG of an unpredictable chronic mild stress (CMS) rat model of depression and reversed by antidepressant treatment. Here, we further analyzed the levels of these miRNAs along the DG longitudinal axis and in other brain regions involved in the pathophysiology of depression, as well as in peripheral blood of CMS-exposed rats and after fluoxetine treatment. The effects of CMS and fluoxetine treatment on miR-409-5p and miR-411-5p levels varied across brain regions, and miR-411-5p was significantly decreased in the blood of fluoxetine-treated rats. Additional bioinformatic analyses revealed target genes and pathways of these miRNAs related to neurotransmitter signaling and neuroplasticity functions; an implication of the two miRNAs in the regulation of the cellular and molecular changes observed in these brain regions in depression is worth further examination.

Keywords: chronic stress, depression, fluoxetine, miR-409, miR-411, neural plasticity

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INTRODUCTION

Major Depression (MD) is a chronic debilitating disorder predicted to affect more than 300 million people worldwide. Moreover, the World Health Organization has rated it as the leading cause of disease burden in developed countries since 2017 (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Despite the available pharmacological treatments, less than 70% of all patients that are currently treated with available antidepressants present full remission (Insel and Wang, 2009).

Depression most likely results from a complex interaction between genetic predisposition and environmental factors, such as early life experiences, life events, and chronic stress (Mandelli and Serretti, 2013). Though imbalances in the levels of monoaminergic neurotransmitters have long been assumed as central in the pathophysiology of depression, current knowledge puts forward many other systems as relevant for its pathophysiology and treatment. Both central and peripheral substrates, such as neuroimmune and neuroendocrine systems, neuroplasticity, and the gut microbiome are acknowledged substrates of the disease; however, it is still challenging to integrate the observed imbalances to interpret the full spectrum of behavioral outcomes observed in depressed individuals (Krishnan and Nestler, 2010; Schmidt et al., 2011; Galea et al., 2013).

Neuroplasticity changes, in particular, have been consistently described in the brain of depressed individuals and animal models of depression (Stockmeier et al., 2004; Sahay et al., 2011). These changes occur in several adult brain regions that play an important role in behavioral processes somehow related to the pathophysiology of the disease, from learning and memory to anxiety and mood. The hippocampus, one of the most widely studied brain regions in this context, is a glucocorticoid receptors-rich region extending along a Spatio-temporal axis, and particularly sensitive to the effects of chronic stress, a major precipitating factor for depression (Anacker et al., 2011; Egeland et al., 2015). Within this brain region lies the hippocampal dentate gyrus (DG), a functionally heterogeneous structure and one of the adult brain regions where new cells can be generated from resident progenitors (Kheirbek et al., 2013; Tanti and Belzung, 2013).

Previous microarray analysis from our group has disclosed a set of molecular players differently expressed in the hippocampal DG in the context of depression and antidepressant treatment (Patrício et al., 2015). Two miRNAs in particular—miR409 and miR411—were shown to be significantly up-regulated in the DG of an unpredictable chronic mild stress (CMS) rat model of depression, and reversed by different antidepressants treatment, namely fluoxetine, imipramine, tianeptine and agomelatine (Patrício et al., 2015). These two miRNAs, in particular, have not yet been reported in human studies of depressed patients.

miRNAs are a family of small (19–25 nucleotides) highly conserved non-coding RNAs, that regulate gene expression at the post-transcriptional level. miRNAs bind primarily to the 3' UTR of mRNAs leading to mRNA destabilization or repressing translation. Over the past few years, growing evidence has supported the role of miRNAs as regulators of critical aspects of neuroplasticity and brain function (Dreyer, 2010; Im and Kenny, 2012; O'Carroll and Schaefer, 2013; Dubes et al., 2019). Additionally, alterations in miRNAs levels have been reported in several neuropsychiatric disorders, including depression and as targets for antidepressant treatment (Hansen and Obrietan, 2013; Issler et al., 2014; Gururajan et al., 2016; O'Connor et al., 2016). These molecules are not only highly expressed in the brain, but are also relatively stable in bodily fluids, including blood (Gheysarzadeh et al., 2018). The association between changes in miRNAs in bodily fluids, such as blood and cerebrospinal fluid (CSF), and brain tissue has generated great interest in the field, as these may represent potential biomarkers for disease (Li et al., 2013; Camkurt et al., 2015; Gururajan et al., 2016; Lopez et al., 2018).

Hence, the present work aimed to further analyze the levels of these two miRNAs along the septotemporal axis of the DG as well as in other brain regions involved in the pathophysiology of depression, including the *cornu ammonis* (CA) regions of the hippocampus (HPC), the prefrontal cortex (PFC) and the nucleus accumbens (NAc). Moreover, we assessed the expression of these miRNAs in peripheral blood samples of the same animals. Bioinformatics analyses were also performed to get insights into possible gene targets of these miRNAs as well as the pathways and functions in which they are involved.

MATERIALS AND METHODS

The array data in which the two herein presented miRNAs were identified are publicly accessible from NCBI/GEO (GSE56028).

Animals

Male Wistar rats (2-month old; Charles River Laboratories) were maintained under standard laboratory conditions (lights on 08:00–20:00 h; 22°C, relative humidity of 55%, *ad libitum* access to food and water).

Unpredictable Chronic Mild Stress (CMS)

Rats (n = 10–12/group) were randomly assigned to one of the following groups: non-stressed control (CT) + vehicle (NaCl 0.9%); stress-exposed (CMS) + vehicle; and CMS+fluoxetine (CMS+FLX). A validated CMS protocol was applied for 6 weeks, as previously described (Bessa et al., 2009). During the last 2 weeks of the CMS protocol, animals were injected daily with fluoxetine (intraperitoneal injection; 10 mg/kg in ultra-pure water; Kemprotec, Middlesborough, UK) or vehicle. Fluoxetine dose was chosen based on previous studies (Bessa et al., 2009; Patrício et al., 2015). All procedures were carried out following the EU Directive 2010/63/EU and the Portuguese guidelines on animal care and experimentation.

Behavioral Assessments

The development of behavioral signs akin to human depression was assessed using different behavioral tests. Specifically, anxiety-like behavior and anhedonia were evaluated at the end of the CMS protocol, as previously described (Mateus-Pinheiro et al., 2014; Patrício et al., 2015). Anxiety-like behavior was assessed in a novelty suppressed feeding (NSF) paradigm, 72 h before sacrifice and, brain and blood samples collection. Briefly, food-deprived (18 h) animals were placed in an open-field arena with a single food pellet positioned in the center, for a maximum of 10 min. After reaching the pellet, each rat was individually placed in a cage to feed for 10 min. The latency to feed in the open-field arena was used as an anxiety-like behavior index, and the food consumption in the cage provided a measure of appetite drive. Anhedonic behavior was assessed using a modified version of the Sweet Drive test (SDT; Mateus-Pinheiro et al., 2014), 36 h before sacrifice and sample collection. In this test, animals were food-deprived for

10 h following which they were placed in a 3-chamber box and could choose between sugared pellets (Cheerios®, Nestlé) or isocaloric regular chow during a 10 min trial. Decreased preference for sugared pellets was taken as a measure of anhedonic behavior.

Blood Collection and Corticosterone Levels Measurement

Blood sampling (tail venipuncture) was performed during the diurnal nadir (N, 08:00–09:00) and the diurnal zenith (Z, 20:00 −21:00), at the end of the CMS protocol, on the day before sacrifice. Corticosterone levels were measured in the collected blood serum using a Corticosterone ELISA Kit (ab108821, Abcam), according to the manufacturer's instructions.

Brain Regions Macrodissection

Dorsal (dDG; $n = 6$–9) and ventral DG (vDG; $n = 6$–9), remaining hippocampus (HPC; $n = 3$), prefrontal cortex (PFC; $n = 3$) and nucleus accumbens (NAc; $n = 3$) were collected 24 h after the last stressor/fluoxetine injection. Animals were first anesthetized with pentobarbital and transcardially perfused with 0.9% saline. Immediately after dissection, tissues were frozen and stored at −80°C until further analysis. To avoid experimenter-dependent bias, brains were macrodissected by a single investigator.

RNA Purification and Real-Time PCR

Total RNA, including miRNAs, was isolated from the macrodissected brain regions and blood samples (collected during diurnal nadir, on the day of sacrifice) using the Direct-zol™ RNA MiniPrep (ZymoResearch), according to the manufacturer's instructions. RNA samples were treated with qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences) to generate cDNA. QRT-PCR was performed using PerfeCTa microRNA assay for miR-411-5p and miR-409-5p (Quanta Biosciences) and PerfeCTa Universal PCR primer (Quanta Biosciences). Samples were analyzed using 5xHOT FIREPol® EvaGreen® qPCR Mix Plus (ROX, Solis BioDyne), according to the manufacturer's instructions, in an AB7500 Fast Real-Time PCR system (Applied Biosystems). U6 small nuclear RNA (RNU6) was used as an internal reference (Control Assay, Quanta Biosciences). The results are presented as fold change ($2\Delta\Delta CT$) of control samples.

miRNA Target Prediction and Pathway Analysis

For the computational prediction of miRNAs target genes, the mirWalk web platform database was used[1] (Sticht et al., 2018). This tool incorporates databases from other established programs for miRNA target prediction. In the present study, the following databases were included for target prediction: mirWalk, miRanda, miRDB, and TargetScan. For functional analysis, we considered the target genes commonly predicted by at least three out of the four databases for each mature miRNA (Accession numbers: miRNA 409-MIMAT0003204 and miRNA 411-MIMAT0005312), using the following parameters for target prediction: Gene region—3′UTR binding site; 2,000 bp upstream flanking region (assumed promoter); minimum seed length: 7 nucleotides; $p < 0.05$). For the functional annotation of miRNA predicted targets, we used the PANTHER database and for interaction analysis, we used the STRING database.

[1]http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The underlying assumptions of all statistical procedures were assessed. The normal distribution was tested using the Kolmogorov–Smirnov test. One-Way ANOVA with Sidak *post hoc* multi comparisons test was used to assess differences between experimental groups in NSF, SDT, and miRNA levels. Two-way ANOVA repeated measures with Sidak *post hoc* multi comparisons test was used to assess differences between groups in corticosterone levels. Test statistics are presented in the text and *post hoc p*-values are shown in the Figures. Significance was set at $p < 0.05$.

RESULTS

To assess the levels of the previously identified miRNAs in different brain regions and blood of depressive-like and antidepressant treated animals, we used a validated rat model of depression, the Chronic Mild Stress (CMS; **Figure 1A**). The behavioral analysis confirmed the development of anxiety-like and anhedonic behavior after CMS exposure, two important components of depressive-like behavior phenotype in this animal model, as shown by the increased latency time to reach the pellet in the NSF test (**Figure 1B**; $F_{(2,32)} = 51.29$, $p < 0.001$), and by a decreased preference for the sugared pellet in the SDT test (**Figure 1C**; $F_{(2,32)} = 6.293$, $p = 0.0052$), respectively. On the other hand, chronic treatment, for 2 weeks, with fluoxetine reversed both anxiety-like and anhedonic behavior (**Figures 1B,C**). In line with these behavioral deficits, and also a relevant hallmark of this animal model, CMS-exposed rats presented significantly higher corticosterone levels in the blood serum during the diurnal Nadir (N; **Figure 1D**), as compared to control (CT) rats. Fluoxetine treatment was not able to completely reverse the effects of CMS on the diurnal Nadir corticosterone levels back to those of CT animals (N; **Figure 1D**; Interaction: $F_{(2,33)} = 3.583$, $p = 0.0391$; Timepoint of blood collection: $F_{(1,33)} = 10.29$, $p = 0.0030$).

miR-409-5p and miR-411-5p Levels Change in the Brain and Blood After Chronic Mild Stress and Fluoxetine Treatment

The levels of miR-409-5p and miR-411-5p were analyzed in brain regions traditionally associated with depression pathophysiology, namely the hippocampus (DG and CA regions), the PFC and the nucleus accumbens (NAc), upon exposure to CMS and after treatment with fluoxetine. Given the functional heterogeneity of the hippocampal formation, we sought to investigate the levels of these miRNA along the DG Spatio-temporal axis. Analysis of miR-409-5p levels in the dorsal (dDG) and ventral DG (vDG) revealed no statistically significant differences between

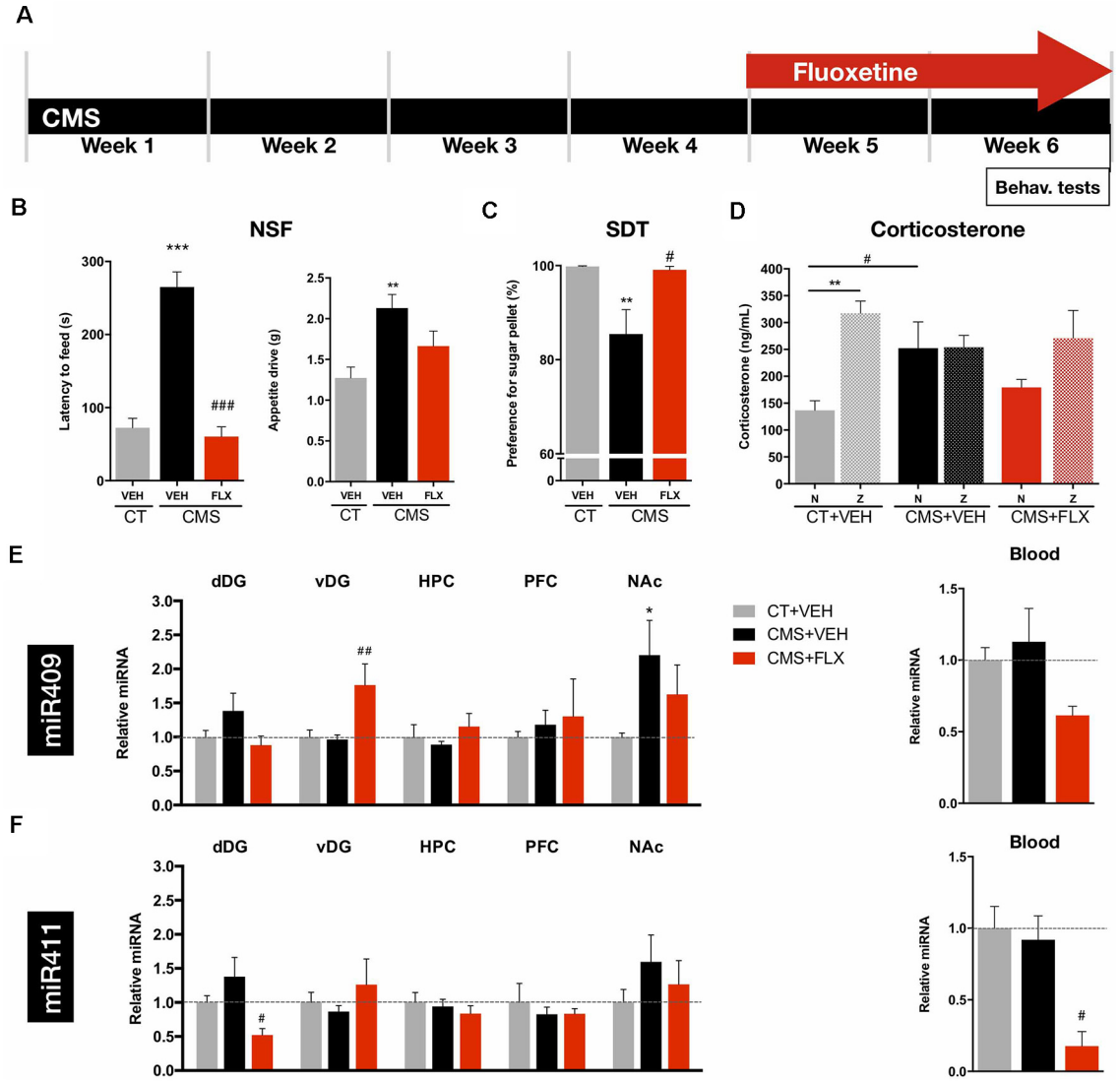

FIGURE 1 | Behavioral analyses and miRNA levels after CMS exposure and fluoxetine treatment. **(A)** CMS protocol was applied for 6 weeks and fluoxetine treatment was performed during the last 2 weeks. Behavioral analyses and blood collection were performed at the end of the CMS protocol. **(B)** Novelty suppressed feeding (NSF) test was applied to evaluate anxiety-like behavior; left panel: latency to feed and right panel: appetite drive. **(C)** Sweet Drive Test (SDT) was used to assess anhedonia. **(D)** Corticosterone levels measured in the blood serum of rats between 8:00 and 9:00 (basal levels, Nadir, N) and between 20:00 and 21:00 (peak levels, Zenith, Z). **(E)** Relative levels of miRNA-409-5p in the dorsal (dDG) and ventral dentate gyrus (vDG), CA regions of the hippocampus (HPC), prefrontal cortex (PFC), nucleus accumbens (NAc), and in the blood. **(F)** Relative levels of miR-411-5p in the dDG, vDG, HPC, PFC, NAc, and in the blood. *Denotes differences between CT and CMS; #Denotes differences between CMS+VEH and CMS+FLX. Abbreviations: CT, Control; CMS, unpredictable chronic mild stress; NSF, Novelty Suppressed Feeding test; SDT, Sweet Drive test. Data presented as mean ± SEM. FLX, fluoxetine; N, Nadir; Z, Zenith. $^{*/\#}p < 0.05$; $^{**/\#\#}p < 0.01$; $^{***/\#\#\#}p < 0.001$.

CT and CMS-exposed animals, whereas fluoxetine treatment significantly increased the levels of this miRNA in the vDG, specifically (**Figure 1E**; dDG: $F_{(2,20)} = 1.843$, $p = 0.1843$; vDG: $F_{(2,20)} = 7.176$, $p = 0.0045$). The levels of miRNA-409-5p were also significantly increased in the NAc of CMS-exposed animals, but not reversed by fluoxetine treatment (**Figure 1E**; $F_{(2,6)} = 7.246$, $p = 0.0251$). The levels of this miRNA in the CA regions of the HPC, in the PFC (**Figure 1E**; HPC: $F_{(2,5)} = 0.5157$, $p = 0.6257$; PFC: $F_{(2,6)} = 0.2143$, $p = 0.8130$), or in the blood (**Figure 1E**, right panel; $F_{(2,6)} = 3.256$, $p = 0.1103$) were not significantly impacted by CMS exposure or fluoxetine treatment.

Regarding miR-411-5p levels, we could not disclose statistically significant differences between CMS-exposed and CT animals in the dDG, though a statistically significant decrease in the levels of this miRNA was observed upon chronic treatment with fluoxetine (**Figure 1F**; $F_{(2,20)} = 4.107$, $p = 0.0320$). The levels of this miRNA were not impacted by CMS or fluoxetine treatment in any of the remaining brain regions—vDG, CA regions of the HPC, PFC and NAc (**Figure 1F**;

vDG: $F_{(2,20)} = 0.9404$, $p = 0.4071$; HPC: $F_{(2,5)} = 0.4372$, $p = 0.6684$; PFC: $F_{(2,6)} = 0.3124$, $p = 0.7429$; NAc: $F_{(2,6)} = 2.566$, $p = 0.1566$). Strikingly, and in line with the results for the dDG, the levels of this miRNA were significantly decreased in the blood of fluoxetine-treated CMS animals (**Figure 1F**, right panel; $F_{(2,6)} = 10.06$, $p = 0.0121$).

Bioinformatics Analysis Reveals Specific Target Genes and Pathways for miR-409-5p and miR-411-5p

To identify possible target genes for each of these miRNAs, we used 4 different online target prediction tools: miRanda, miRDB, miRWalk, and TargetScan (**Figure 2A**). Venn diagrams depict the number of predicted genes by each tool (**Figures 2B,C**). For further bioinformatics analyses, we considered the experimental target genes predicted by at least three out of the four databases. This strategy unveiled 62 and 145 target genes for miR-409-5p and miR-411-5p, respectively (**Supplementary Tables S1, S2**). Both gene lists were further computed into the PANTHER classification system for functional annotation analysis of the miRNAs target genes. This clustering of the predicted target genes was performed through pathway enrichment and protein class analyses.

Regarding mir-409-5p, we found very few genes assigned to each pathway, with the integrin signaling pathway and nicotine pharmacodynamics pathway being the most represented (two genes in each pathway; **Figure 2B**). The majority of the predicted target genes for mir-409-5p fell into the enzyme modulator and transferase protein class categories (six genes per category), followed by the transporter, hydrolase, oxidoreductase, and transcription factor protein classes (five genes per category).

This target gene prediction analysis for mir-409-5p also identified several genes of the solute carrier family, namely *Slc26a1*, *Slc36a1*, *Slc4a4*, and *Slc6a7* and the Zinc finger protein (ZFP) family, namely *Zfp384*, *Zfp403* and *Zfp672* (**Supplementary Table S1**). Moreover, some of these genes were represented in the interaction analysis, including *Slc4a4* interacting with *Slc26a1*, and Zfp384, interacting with *Lzts1* and *Fez1* (**Figure 3A**).

Concerning mir-411-5p target genes, the pathway enrichment analysis identified an over-representation of the integrin signaling, angiogenesis, inflammation mediated by chemokine and cytokine, Huntington disease, and gonadotropin-releasing hormone receptor, related genes (**Figure 2C**). The most represented protein classes in the protein enrichment analysis for the mir-411-5p target genes were transferase (13 genes), hydrolase (nine genes), and receptor (eight genes; **Figure 2C**). In the list of 145 mir-411-5p target genes identified in three out of four target prediction databases, several solute carrier family genes, including *Slc12a1*, S*cl12a2*, *Slc18a1*, and *Slc5a7* was also detected, similar to what was found for mir-409-5p. Also, three genes of the transmembrane protein family were represented, *Tmem106c*, *Tmem30a*, and *Tmem97* (**Supplementary Table S2**). The STRING analysis revealed a few clusters of interacting genes, including: (i) *Rab9a*, *Rab21, Rab4a, Stx12* and *Tmem30a*; (ii) *Dusp1, Ptprr, Map2k1* and *Gadd45b*, (iii) *Acsl4, Acat1, Cpt1a, Acox2, Hsd17b*4 and Pex11a (**Figure 3B**).

DISCUSSION

In a previous genome-wide analysis, we identified two pre-miRNAs—miR-409 and miR-411—to be significantly upregulated in the whole hippocampal DG of CMS-exposed rats when compared to the Control rats. Moreover, their levels were restored by chronic treatment with antidepressants from four different classes (fluoxetine, imipramine, tianeptine, and agomelatine; Patrício et al., 2015). Here, we investigated the levels of miR-409-5p and miR-411-5p along the septotemporal axis of the DG and in other brain regions associated with depression pathophysiology, including the CA regions of the hippocampus, the PFC and the NAc, and in peripheral blood samples. To further explore the relevance of these miRNAs for molecular regulation and brain function, we performed bioinformatics analysis to identify possible gene targets and functional analysis of pathways and protein functions.

First, we observed that the impact of CMS exposure on the levels of both miRNAs in each of the brain regions analyzed was very modest, with a similar pattern observed for both miRNAs and with only statistically significant increases in mir-409-5p levels in the NAc. Whereas our previous microarray data had shown increased levels of both miRNAs in the DG upon CMS exposure, here we could only detect a moderate trend for this increase in the dDG for both miRNAs, suggesting this subregion to be the major contributor for the effect previously observed in the whole DG samples (Patrício et al., 2015). Chronic fluoxetine treatment effects were more pronounced than those of the CMS alone, and its impact on the levels of the two miRNAs among brain regions was very heterogeneous. We show that fluoxetine treatment significantly increased the levels of mir-409-5p in the vDG while inducing major decreases in the levels of mir-411-5p in both the dDG and in the blood. This contrasting impact of fluoxetine on the two DG poles was not unexpected, in light of the functional and molecular dichotomy along the hippocampal longitudinal axis (Strange et al., 2014) and may reflect also functional differences between both miRNAs. Moreover, and though the main goal of this work was to understand the changes induced by antidepressants in a pathological context, by modeling the human depressive condition, these fluoxetine effects encourage the analysis of mir-411-5p levels in the brain of naïve animals (not exposed to stress) treated with fluoxetine.

The potential use of miRNAs as biomarkers of disease and treatment response (Dalton et al., 2014), in part due to their stability in bodily fluids that can be collected by minimally invasive procedures, is encouraged by gene expression studies showing similarities between blood and brain samples (Liew et al., 2006; Chen et al., 2008; Jasinska et al., 2009). Here, we show similarities between fluoxetine impact on the dDG and peripheral blood, though the origin and functional role of these miRNAs in the blood are yet to be fully determined (Scott et al., 2015).

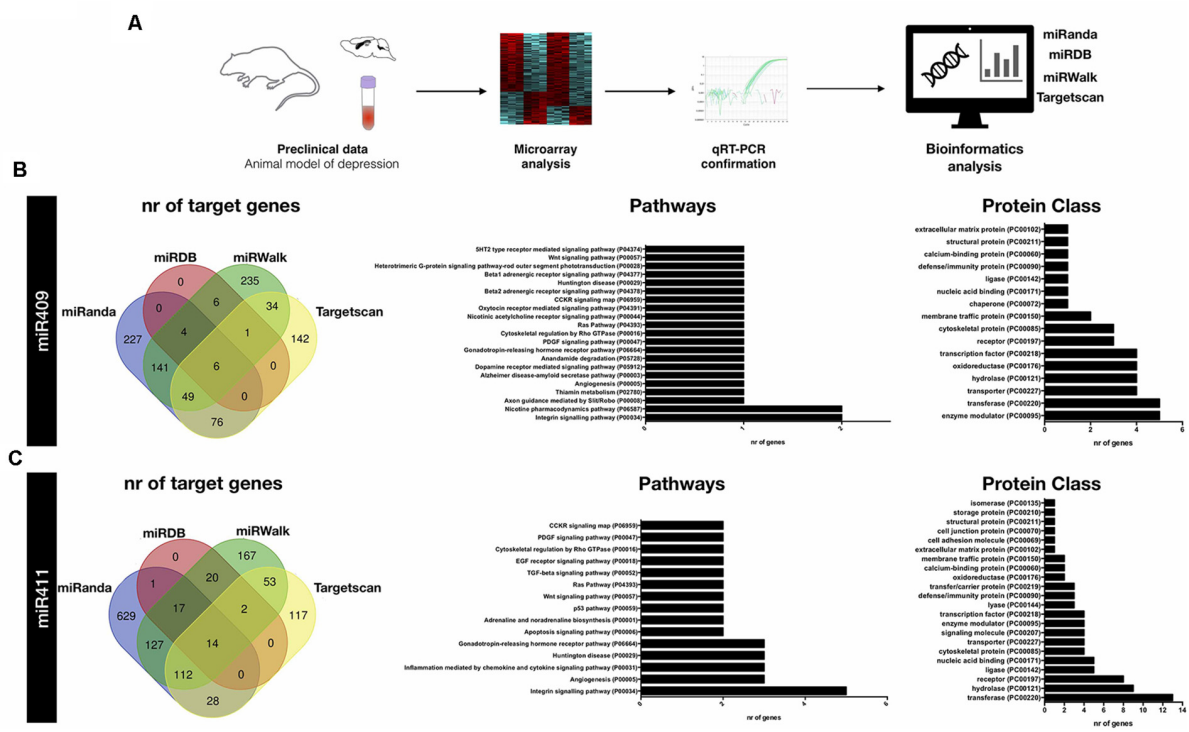

FIGURE 2 | Bioinformatics analyses of miR-409-5p and miR-411-5p target genes. **(A)** Schematic representation of the experimental approach used to identify and analyze miR-409-5p and miR-411-5p target genes. **(B,C)** Venn diagrams depicting the number of target genes identified by each of the miRNA target prediction tools: miRanda, miRDB, miRWalk and TargetScan (left panel), and list of the top significantly enriched pathways and protein classes identified by PANTHER database (right panel) for **(B)** miR-409 and **(C)** miR-411 target genes, respectively. The target genes predicted by three out of the four miRNA target genes predicting tools were used as input in the PANTHER database.

Previous studies have shown how miRNAs can regulate antidepressant treatment, namely selective serotonin reuptake inhibitors (SSRIs), as is the case of fluoxetine, the antidepressant used in this study (Baudry et al., 2010; Issler et al., 2014). At least two other miRNAs, mir-16 and mir-135, were shown to be involved in the action of SSRIs in depressed individuals, by targeting genes related to brain serotoninergic system, including *Slc6a4* [serotonin (5-HT) transporter, responsible for 5-HT reuptake (SERT) and *Htr1a* (5-HT inhibitory receptor 1a; Issler et al., 2014)]. Interestingly enough, *Slc18a1*, or vesicular monoamine transporter 1 (VMAT1), which is involved in the packaging and storage of serotonin in presynaptic terminals, is among the mir-411-5p predicted target genes, identified in our *in silico* analysis. Moreover, *Slc18a1* has been implicated in the development and treatment of psychiatric disorders (Lohoff et al., 2006; Lin et al., 2011), further suggesting a functional role of mir-411-5p in this context.

Functional annotation analysis of miR-409-5p target genes also revealed, a group of Zinc-finger protein (ZFP) genes. The ZFP family is a large group of proteins, capable of binding nucleic acids, proteins, or small molecules, involved in the regulation of many cellular processes (Cassandri et al., 2017). Many of these genes have been associated with neuropsychiatric disorders including schizophrenia, bipolar disease, and intellectual disability (Sun et al., 2015). In particular, the expression of one of these target genes—*Zfp672*- has been previously reported to be increased in the brains of Low Anxiety Behavior (LAB) mice, an animal model of anxiety-trait behavior, compared to High Anxiety Behavior mice (Czibere et al., 2011). In the CMS model of depression, we also observe the concomitant emergence of anxiety-like behavior, which is reversed by fluoxetine treatment. This behavioral outcome and the increased levels of miR-409-5p in the vDG, a brain region that has been implicated in anxiety behavior, upon fluoxetine treatment, reinforce an involvement of *Zpf672* in the development or treatment of this trait. Another ZFP identified here was *Zfp384*, which has been shown to contribute to the regulation of the dendritic growth of newborn hippocampal neurons (Kang et al., 2011). Our previous studies with this animal model have consistently shown the impact of both chronic stress and fluoxetine treatment in dendritic morphology (Bessa et al., 2009, 2013; Mateus-Pinheiro et al., 2013b; Patrício et al., 2015). This is true, namely in the brain regions where changes in the levels of this miRNA were observed, the vDG and the NAc. In line with the possible role of these miRNAs in regulating neural plasticity, one of the identified target genes for mir-411-5p was Dual specificity protein phosphatase 1 (*Dusp1*), a negative regulator of the MAP kinase pathway (Huang and Tan, 2012). This gene was found to be upregulated upon

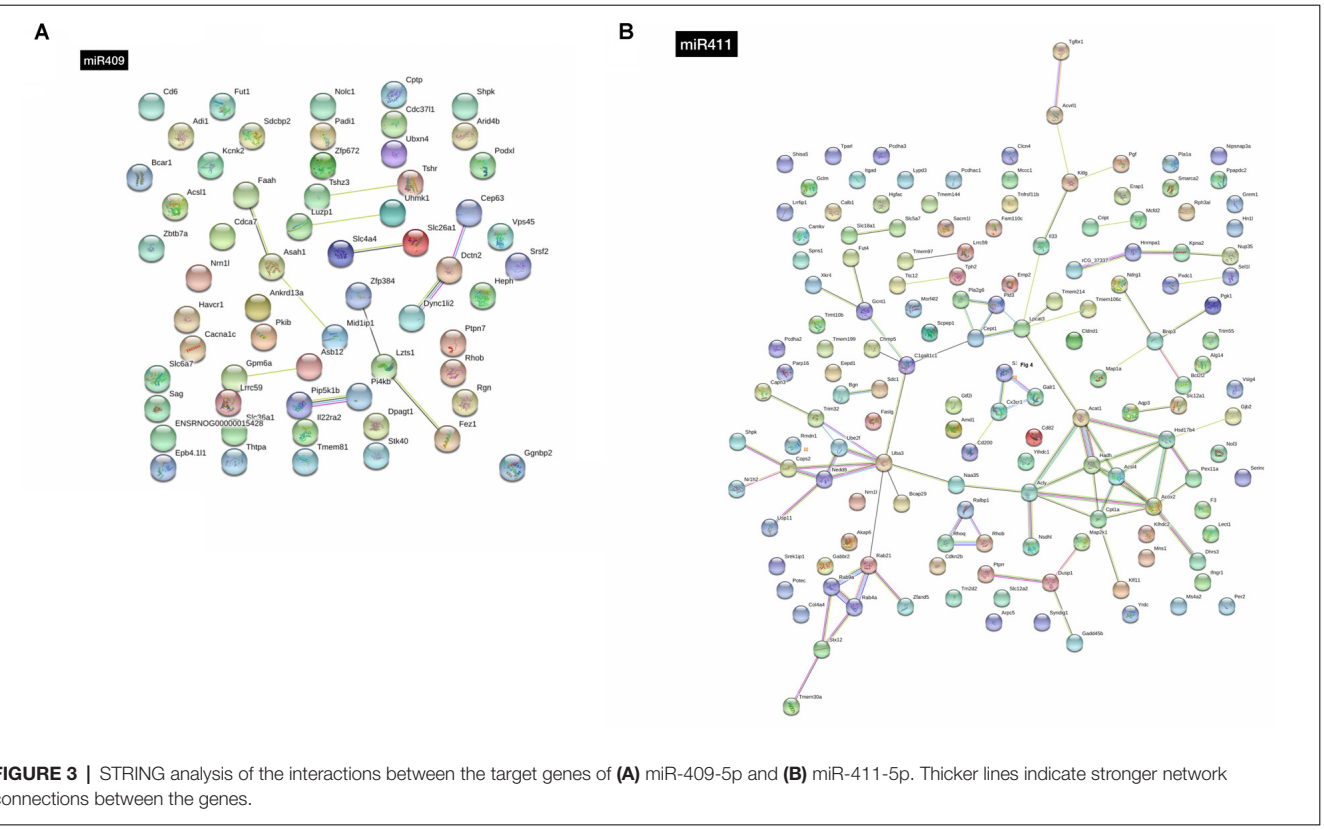

FIGURE 3 | STRING analysis of the interactions between the target genes of **(A)** miR-409-5p and **(B)** miR-411-5p. Thicker lines indicate stronger network connections between the genes.

CMS exposure and reversed by antidepressant treatment, in our previous microarray analysis (Patrício et al., 2015), and has been associated with depression pathophysiology in human patients and in an animal model of depression (Duric et al., 2010). String analysis showed that *Dusp1, Gadd45b*, and *Map2k1*, three predicted target genes of miR-411-5p, clustered together in terms of their possible interactions. Gadd45b is a DNA demethylating agent that regulates expression of *Bdnf* and *Fgf1* and has been shown to influence synaptic plasticity and memory processes, while also mediating the effects of social stress in the mesolimbic dopamine circuit (Ma et al., 2009; Mateus-Pinheiro et al., 2011; Labonté et al., 2019). *Map2k1 (Mek1)* encodes a kinase of the MAPK/ERK pathway, that has been implicated in synaptic plasticity processes and memory, through activation of MAPK signaling (Kelleher et al., 2004). A recent study also reported MAP2K1 to regulate Neuronal Per Arnt Sim domain protein 4 (NPAS4), an immediate-early gene that controls a transcriptional program involving neural activity-regulated genes (Lin et al., 2008; Yun et al., 2013), including *Bdnf*, thus promoting neural circuitry plasticity, learning, and memory (Funahashi et al., 2019). Interestingly, our previous work revealed that *Npas4* is epigenetically regulated in a conditional *Tet3* KO mouse model that presents anxiety-like behavior and cognitive deficits (Antunes et al., 2020). Changes in epigenetic regulators have long been described in the context of stress, depression and antidepressant treatment (Nestler, 2014), with chronic stress being suggested to interact with susceptibility genes *via* epigenetic mechanisms to produce long-lasting changes in the brain that may partly explain the heterogeneity of depression etiology (Tsankova et al., 2007; Mateus-Pinheiro et al., 2011; Menke and Binder, 2014). miRNAs, in particular, have emerged as an important form of epigenetic regulation of gene expression in the context of depression, with growing evidence suggesting their role in both the pathophysiology and treatment of this disorder (Hansen and Obrietan, 2013; Issler et al., 2014; Maffioletti et al., 2014).

Here, we do not provide enough experimental evidence to support the causal relationship between these changes in the levels of miRNAs and the neural plasticity changes that have been previously shown to underlie some of the behavioral deficits observed in this animal model, including neuronal morphology changes and decreased hippocampal cytogenesis (Bessa et al., 2009, 2013; Mateus-Pinheiro et al., 2013a). In this work, we have not analyzed the expression of putative target genes identified in the *in silico* analysis. Though this is a valid and common approach to address the functional role of miRNAs, it might also raise some questions and limit the interpretation of the data, because there may not be a direct association between the levels of miRNAs and of their target genes. First, miRNAs may act either as enhancers or repressors of gene expression by binding to distinct regulatory regions of the genome (O'Brien et al., 2018). In fact, under certain circumstances and if interacting at the promoter level, miRNAs can also activate transcription (Dharap et al., 2013). Second, because it is not straightforward to negatively or positively associate the levels of a regulatory molecule *in vivo* with the levels of its targets or effectors

as one might not exclude the possibility of compensatory mechanisms. High target gene levels may either trigger an increase in the cognate miRNA levels or be the reflection of that miRNA low levels. Nevertheless, by combining miRNAs levels assessment with bioinformatics analysis of the predicted targets, we hypothesize that these miRNAs, may be important mediators of the effects of chronic stress and fluoxetine, in neural plasticity. Functional assays, using pre-miRs and anti-miRs, would help disclose any causal effect. We additionally highlight the need to further explore the levels of the mature miR-3p form of both miRNAs in this context.

Despite the animal model used herein is based in a very broad stimulus known to induce depressive-like signs in rodents—chronic stress—and the fact that the levels of these two miRNAs had been previously shown to be impacted by different classes of antidepressants, may reflect that these effects may be generalized to other stress-based animal models and antidepressants. Thus, these findings need further validation in other animal models, including in female subjects, and also in clinical samples. Still, they encourage further investigation of miRNAs as targets for disruption and treatment prediction in the context of depression, towards a better understanding of the neurobiology of the disease and more precise diagnosis and directed treatment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56028.

ETHICS STATEMENT

The animal study was reviewed and approved by ORBEA EM/ICVS-I3Bs School of Medicine, University of Minho, Braga, Portugal.

AUTHOR CONTRIBUTIONS

PP and LP designed the study. PP and AM-P performed all the experiments. NA and MM assisted in the CMS protocol, sacrifice, brain, and blood samples collection and processing. PP wrote the manuscript. JB, AR, NS, and LP revised the manuscript.

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Part of the dataset included in this article, namely part of the samples included in the analysis of miR-411-5p levels in the dDG and vDG, and in the peripheral blood, are included in the first author's doctoral thesis identified in the references list as Patrício (2016).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00136/full#supplementary-material.

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Differential Role of Anterior Cingulate Cortical Glutamatergic Neurons in Pain-Related Aversion Learning and Nociceptive Behaviors in Male and Female Rats

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Pain is comprised of both sensory and affective components. The anterior cingulate cortex (ACC) is a key brain region involved in the emotional processing of pain. Specifically, glutamatergic transmission within the ACC has been shown to modulate pain-related aversion. In the present study, we use *in vivo* optogenetics to activate or silence, using channelrhodopsin (ChR2) and archaerhodopsin (ArchT) respectively, calmodulin-kinase IIα (CaMKIIα)-expressing excitatory glutamatergic neurons of the ACC during a formalin-induced conditioned place aversion (F-CPA) behavioral paradigm in both female and male adult Sprague-Dawley rats. Expression of c-Fos, a marker of neuronal activity, was assessed within the ACC using immunohistochemistry. Optogenetic inhibition of glutamatergic neurons of the ACC abolished F-CPA without affecting formalin-induced nociceptive behavior during conditioning. In male rats, optogenetic activation of ACC glutamatergic neurons decreased formalin-induced nociceptive behavior during conditioning without affecting F-CPA. Interestingly, the opposite effect was seen in females, where optogenetic activation of glutamatergic neurons of the ACC increased formalin-induced nociceptive behavior during conditioning. The abolition of F-CPA following optogenetic inhibition of glutamatergic neurons of the ACC was associated with a reduction in c-Fos immunoreactivity in the ACC in male rats, but not female rats. These results suggest that excitatory glutamatergic neurons of the ACC play differential and sex-dependent roles in the aversion learning and acute sensory components of pain.

Keywords: anterior cingulate cortex, glutamate neurons, optogenetics, inflammatory pain, formalin, rat, c-Fos, conditioned place aversion

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INTRODUCTION

Pain is comprised of both sensory-discriminative and affective-motivational components, which have distinct roles in the pain experience and can often modulate one another. Thus, it is not surprising that chronic pain and anxiety disorders are frequently co-morbid, with approximately 45% of chronic pain patients exhibiting a comorbid anxiety disorder (Lenze et al., 2001;

McWilliams et al., 2003; Kessler et al., 2005; Korff et al., 2005; Roy-Byrne et al., 2008; Asmundson and Katz, 2009). Being able to target specifically the affective component of pain would be therapeutically beneficial for patients with chronic pain. In order to develop these improved treatments for chronic pain, there is a need for a better understanding of the neural circuitry involved in pain-related negative affect or aversion.

The anterior cingulate cortex (ACC) is a key brain region in the affective-motivational component of pain (Price, 2000). It has been found that lesion of the ACC reduces both formalin-induced conditioned place aversion (F-CPA) and visceral pain-induced CPA, pre-clinical behavioral paradigms used to investigate the affective component of pain, without affecting nociceptive responding (Johansen et al., 2001; Gao et al., 2004; Yan et al., 2012). Glutamatergic transmission and the expression of glutamatergic receptors in the ACC are increased in animal models of pain (Xu et al., 2008; Chen et al., 2014; Li W. et al., 2014; Yi et al., 2014; Hubbard et al., 2015; Liu et al., 2015), as well as clinically in patients with chronic pain conditions (Kameda et al., 2017; Lv et al., 2018). Studies have found that optogenetic activation of glutamatergic neurons in the ACC elicits mechanical allodynia in male mice while having no effect on nociceptive responding following an injection of complete Freund's adjuvant (CFA). Conversely, optogenetic inhibition of glutamatergic neurons in the ACC has an antinociceptive effect of increasing paw withdrawal threshold in the mouse CFA-induced inflammatory pain model (Kang et al., 2015). It has been found that microinjections of the ionotropic glutamate receptor antagonist, kynurenic acid, into the ACC (Johansen and Fields, 2004) reduce aversion behavior in an F-CPA paradigm, while microinjection of the excitatory amino acid, homocysteic acid, into the ACC produces avoidance learning in the absence of a noxious stimulus in a CPA paradigm (Johansen and Fields, 2004). Thus, glutamatergic transmission within the ACC plays an important role in CPA.

Although chronic pain has a greater prevalence in women than in men (Fayaz et al., 2016), the vast majority of pre-clinical pain studies have only been conducted in males, with 79% of behavioral non-human animal pain experiments in papers published between 1996 and 2005 using male rodents only (Mogil and Chanda, 2005; Mogil, 2012). The inclusion of both sexes in pain studies is important because sex differences in pain have been observed, both in animal models and clinically (Berkley, 1997; Mogil and Bailey, 2010; Rhudy et al., 2010; Mogil, 2012; Sorge and Totsch, 2017). Due to the scarcity of pre-clinical pain studies performed in both males and females, little is known about sex differences in the role of glutamatergic neurons in the ACC in regulation of the sensory and affective components of pain.

In this study, we investigated the hypothesis that the glutamatergic neurons of the ACC have a facilitatory effect on pain-induced aversive behavior, possibly in a sex-dependent manner. The specific aims of the study were (1) to determine the role of glutamatergic neurons of the ACC in both formalin-induced nocifensive and aversive behaviors in female and male rats using optogenetic methodology and (2) to examine if behavioral changes are associated with alterations in expression of the marker of neuronal activity, c-Fos, in the ACC using fluorescent immunohistochemistry.

MATERIALS AND METHODS

Animals

Experiments were carried out on adult male and female Sprague-Dawley rats (**Table 1**; Charles River, United Kingdom) maintained at a constant temperature (21 ± 2°C and relative humidity ranged from 36 to 49%) under standard lighting conditions (12:12 h light: dark, lights on from 08.00 to 20.00 h). All surgeries and behavioral trials and testing were carried out during the light phase between 08.00 and 19.00 h. Animals were group housed with three rats per cage until surgery after which they were singly housed. Cages were 42 cm × 26 cm × 13 cm and filled with 3Rs paper bedding (3Rs Lab, United Kingdom). A rectangle plastic insert was placed into the cage under the food hopper allowing animals to access food but preventing animals getting too close to the cage top which may result in damage to the optical fiber implants. Food (14% protein rodent diet, Harlan, United Kingdom) and water were available *ad libitum*. The experimental protocol was carried out following approval (Filing ID: 15/FEB/01) from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license (project authorization number AE19125/PO28) from the Health Products Regulatory Authority in the Republic of Ireland and in accordance with EU Directives 86/609 and 2010/63 and were in accordance with ARRIVE guidelines from the National Centre for the Replacement Refinement and Reduction of Animals in Research (Kilkenny et al., 2010).

Virus Construction and Packaging

Recombinant adeno-associated viral (AAV) vectors were serotyped with AAV5 coat proteins and packaged by the viral vector core at the University of Pennsylvania, Philadelphia, PA, United States. Viral titer were 5×10^{12} particles/mL for AAV5.CAMKII.ChR2-mCherry.WPRE.hGH, AAV5.CAMKII.ArchT.eYFP.WPRE.hGH, and AAV5.CAMKII.mCherry.WPRE.hGH. Plasmids were provided by the Deisseroth lab, Stanford, United States.

Stereotaxic Intracranial Viral Injections and Optical Fiber Implantation

Following delivery, rats were left to acclimatize to the animal unit for at least 4 days prior to surgery. They were then placed under isoflurane (2–3% in O_2, 0.5 L/min) anesthesia and 0.5 μL of virus as specified above was bilaterally injected into the ACC (AP: + 1.5 mm; ML: ± 1.3 mm; DV: -1.3 mm at an angle of 12° toward the midline) at a rate of 0.5 μL/min. The microinjection needle was left in place for an additional 3 min prior to its removal. Rats were then bilaterally implanted with optical fibers (0.39 NA, 200 μm core multimode, Thorlabs, Germany) into the ACC (AP: + 1.5 mm; ML: ± 1.3 mm; DV: -1.0 mm at an angle of 12°toward the midline). Optical fiber implants were permanently fixed to the skull using stainless steel screws and

glass ionomer dental cement (GC Europe, Kortrijk, Belgium). The non-steroidal anti-inflammatory drug, carprofen (2.5 mg/kg, s.c., Rimadyl, Pfizer, Kent, United Kingdom), was administered before the surgery to manage postoperative analgesia. To prevent postoperative infection, rats received a single daily dose of the antimicrobial agent enrofloxacin (5 mg/kg, s.c., Baytril, Bayer plc, Berkshire, United Kingdom) on the day of surgery and a subsequent 4 days.

Formalin-Induced Conditioned Place Aversion

Animals were randomized to treatment groups and an experimenter blind to treatment carried out behavioral scoring. Behavioral testing was carried out 4 weeks after stereotaxic intracranial opsin encoding AAV injections. A two-chamber apparatus (each chamber 30 cm × 30 cm × 40 cm, l × w × h) with distinct odor (peppermint or strawberry) and visual (black and white balanced stripes or black dots on a white background) contexts was used for the F-CPA behavioral testing (**Figure 1**). The apparatus was tested and optimized in pilot experiments prior to the presented study so that there was no statistically significant difference in the time spent in the two chambers. The behavioral paradigm F-CPA combines the formalin test of tonic, persistent pain with the place-conditioning paradigm to measure pain-related aversion learning in rodents (Johansen et al., 2001). The test was run over 4 days: Day 1: pre-conditioning, Days 2 and 3: conditioning, and Day 4: post-conditioning. The pre-conditioning day consisted of a 20-min trial in which the rat was allowed free access between both chambers and the time spent in each chamber was recorded. The conditioning days consisted of a 60-min trial in which the rat was restricted to one of the chambers on each of the 2 days. On the second conditioning day (formalin conditioning), the preferred chamber from the pre-conditioning trial was paired with an intra-plantar injection of 50 µl formalin (2.5% in 0.89% saline, Sigma, Ireland) into the right hind-paw under brief isoflurane anesthesia (2% in O_2; 0.5 L/min) as well as bilateral optogenetic stimulation at 10 Hz (15 ms pulse) with 2 s inter-pulse intervals for ChR2 groups and continuous stimulation for ArchT groups with a 465 nm LED light source (Plexon, United States) for the full 60-min conditioning trial. The behavior during the formalin-conditioning trial was recorded. The post-conditioning day consisted of a 20-min trial in which the rat was again allowed free access between the two chambers and the time in each chamber was recorded. The chambers were cleaned with warm soapy water and dried between each animal to remove odor cues. Male and female animals were tested on separate days, in the same apparatus, by the same experimenter, and under identical conditions.

Behavioral Analysis

Behavioral trials were recorded and analyzed off-line using the EthoVisionXT11.5 software (Noldus, Netherlands) by a trained observer blind to the experimental conditions. Formalin-evoked nociceptive behavior was scored for the 60-min post formalin administration (day 3) according to the weighted composite pain scoring (CPS) technique (Watson et al., 1997). According to this method, pain behaviors are categorized as time spent elevating the formalin-injected paw above the floor without contact with any other surface (Pain 1), and holding, licking, biting, shaking or flinching the injected paw (Pain 2) to obtain a CPS [CPS = (Pain 1 + 2(Pain 2))/(duration of trial)]. For F-CPA, time spent in each chamber during the pre- and post-conditioning trials, as well distance moved and durations of rearing and grooming during the 60-min formalin conditioning trial were also assessed

TABLE 1 | Summary of experimental groups.

Sex	Supplier	Weights	Age at surgery	Control (*n*)	ChR2 (*n*)	ArchT (*n*)
Female	Charles River, United Kingdom	200–300 *g*	9–10 weeks	7	11	10
Male	Charles River, United Kingdom	400–500 *g*	9–10 weeks	9	8	10

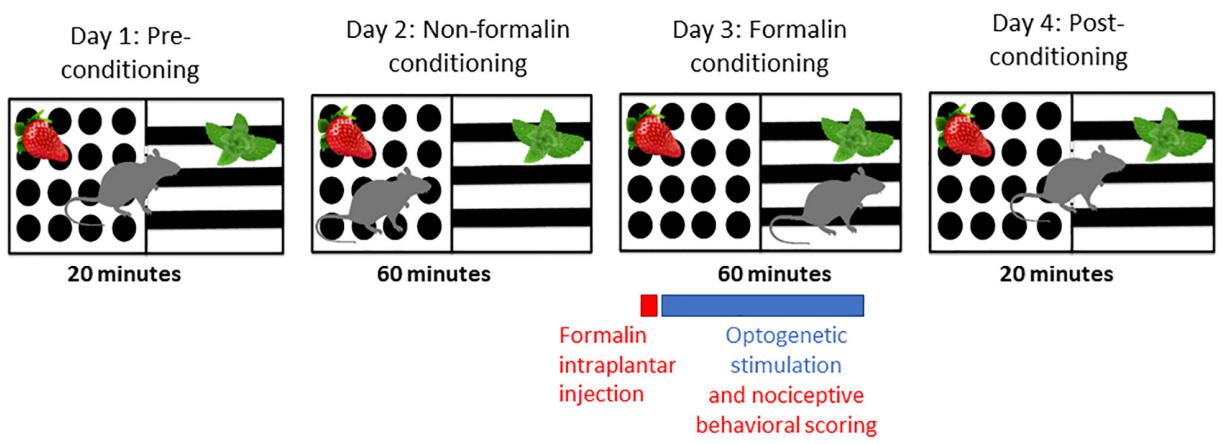

FIGURE 1 | Schematic of F-CPA apparatus and behavioral testing timeline.

using EthoVisionXT11.5. The number of defecation pellets were counted during the 60-min formalin trial. F-CPA was calculated as duration spent in the formalin-paired chamber during the post-conditioning trial (day 4) minus the duration spent in that same chamber during the pre-conditioning trial (day 1). Therefore, a negative F-CPA score indicates an aversion to the formalin-paired chamber.

Histology

Animals were euthanized and perfused immediately after completion of the post-conditioning trial on day 4 of testing. Brains were removed and post-fixed in 4% PFA in 0.1 M PBS for 24 h at 4°C before being transferred into 25% sucrose and 1% sodium azide in 0.1 M PBS. The ACC was later coronally sectioned (30 μm) using a freezing microtome and collected in 0.1 M PBS with 1% sodium azide (Sigma-Aldrich, Ireland). The positions of optical fiber tracts were noted during sectioning to locate and confirm placement in the ACC. Fluorophore-tagged opsin expression was confirmed for each brain by mounting sections onto gelatine-coated slides, cover slipping with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Labs, United Kingdom), and imaging them using an Olympus wide field inverted fluorescence microscope (Olympus, Tokyo).

Fluorescent Immunohistochemistry

For confirmation of opsin expression and analysis of c-Fos immunoreactivity in the ACC, immunohistochemical staining was performed on free-floating sections. Sections were given 3 × 10 min washes in PBS, followed by an incubation for 1 h in 20% normal goat serum (Sigma Aldrich, Ireland) in PBS to block non-specific binding of the secondary antibody. Sections were then incubated in polyclonal rabbit anti-c-Fos antibody (Abcam, United Kingdom) at a concentration of 1:2,000 and rat anti-red fluorescent protein (RFP) antibody (Chromotek, Germany) at a concentration of 1:1,000 made up in PBS, 0.2% (v/v) Triton X, and 1% (w/v) normal goat serum for 24 h at room temperature under constant agitation. The sections were then given 3 × 10-min washes in PBS to remove the primary antibody and were then incubated for 3 h in 1:200 goat anti-rabbit secondary antibody in 10 μl/ml NGS in PB (Abcam, United Kingdom), tagged with either Alexa Fluor 488 for mCherry control and mCherry-tagged ChR2 sections or Alexa Fluor 594 for eYFP-tagged ArchT sections in order to distinguish the c-Fos labeling from the fluorophore-tagged opsin. Sections were kept in the dark and washed 3 × 10 min in PB and stored at 4°C until mounted onto gelatin-coated slides cover slipped with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Labs, United Kingdom).

Sections were imaged using an Olympus widefield inverted fluorescence microscope (Olympus, Tokyo). The number of c-Fos immunoreactive neurons within a 1 mm^2 area in the ACC were counted for at least 5 sections per rat. The mean number of c-Fos expressing cells was then calculated for each rat that had at least 5 non-damaged sections for analysis followed by the overall group means. Counting was performed with the aid of NIMH Image J software (Bethesda, MD, United States).

Statistical Analysis

IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, United States) was used to perform two-way repeated measures analysis of variance (ANOVA) and GraphPad Prism statistical package (Graphpad Prism version 8.02 for Windows, GraphPad Software, La Jolla, CA, United States) was used to perform all other analyses, including two-way ANOVAs and *post hoc* pairwise comparisons. Normality and homogeneity of variance were assessed using Shapiro–Wilk and Brown–Forshythe test, respectively. Two-way repeated measures ANOVA was used to analyze CPS in the formalin test and two-way ANOVA was used to analyze F-CPA with sex and optogenetic modulation as factors. Immunohistochemistry results were analyzed with two-way ANOVA. Kruskal–Wallis test was used to analyze non-parametric data. *Post hoc* pairwise comparisons were made using Fisher's LSD and corrected Dunn's tests where appropriate. Data were considered significant when $p < 0.05$. Correlation analysis on c-Fos and F-CPA data was performed using Pearson's correlation. Results are expressed as group means ± standard error of the mean (±SEM).

RESULTS

Histological Verification of Implant Locations and Opsin Expression

After histological verification, 90% of males and 88% of females had implant tracts that were found to be within the borders of both the left and the right ACC. The remaining implants were placed in the corpus callosum, or outside the borders of the ACC. Only data from rats where optical fibers were accurately placed in both the left and the right ACC and that had opsin expression within the ACC have been included in the analysis (**Figure 2**).

Formalin-Evoked Nociceptive Behavior

Intra-plantar administration of formalin into the right hind paw produced robust nociceptive behavior in both male and female SD rats as evidenced by the composite pain score. A two-way repeated measures ANOVA revealed significant effects of time [$F_{(1,49)} = 13.83$; $p < 0.0001$], sex × optogenetic modulation interaction [$F_{(2,49)} = 5.56$; $p < 0.01$], and time × sex × optogenetic modulation interaction [$F_{(22,49)} = 1.83$; $p < 0.05$] on formalin-evoked nociceptive behavior across the 60-min formalin trial but no effects of optogenetic modulation, sex, time × sex, or time × optogenetic modulation interactions (**Figure 3**).

Post hoc analysis revealed that optogenetic activation (ChR2) of glutamatergic neurons in the ACC significantly reduced ($p < 0.05$ or 0.01) formalin-evoked nociceptive behavior in male rats compared to controls at time bins 9 and 11 of the formalin trial (**Figure 3A**). By contrast, in female rats, optogenetic activation (ChR2) of glutamatergic neurons significantly increased ($p < 0.05$ or 0.01) formalin-evoked nociceptive behavior compared to controls at time bins 7–9 (**Figure 3B**). Moreover, formalin-evoked nociceptive behavior

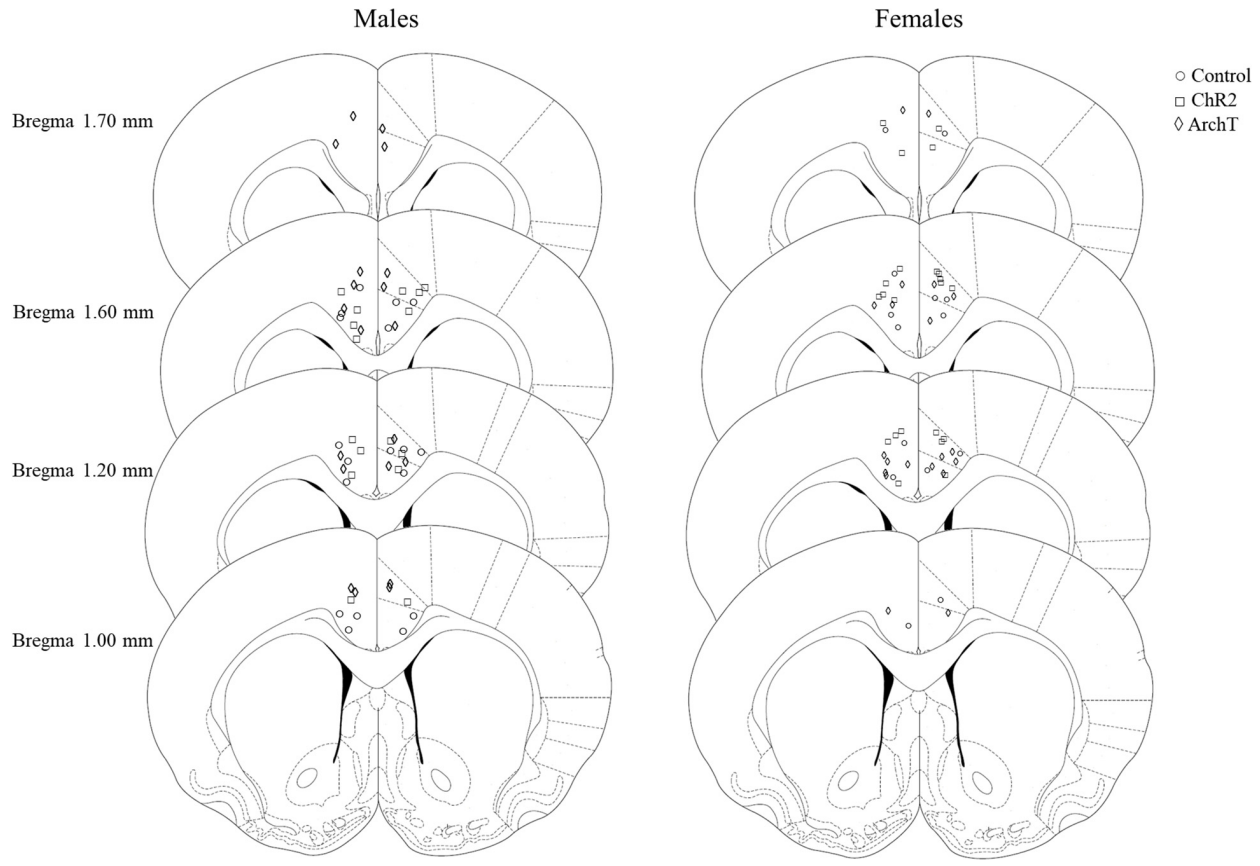

FIGURE 2 | Schematic depicting sites of optical fiber implantation in the **left** and **right** ACC of male and female rats, adapted from Paxinos and Watson (2007).

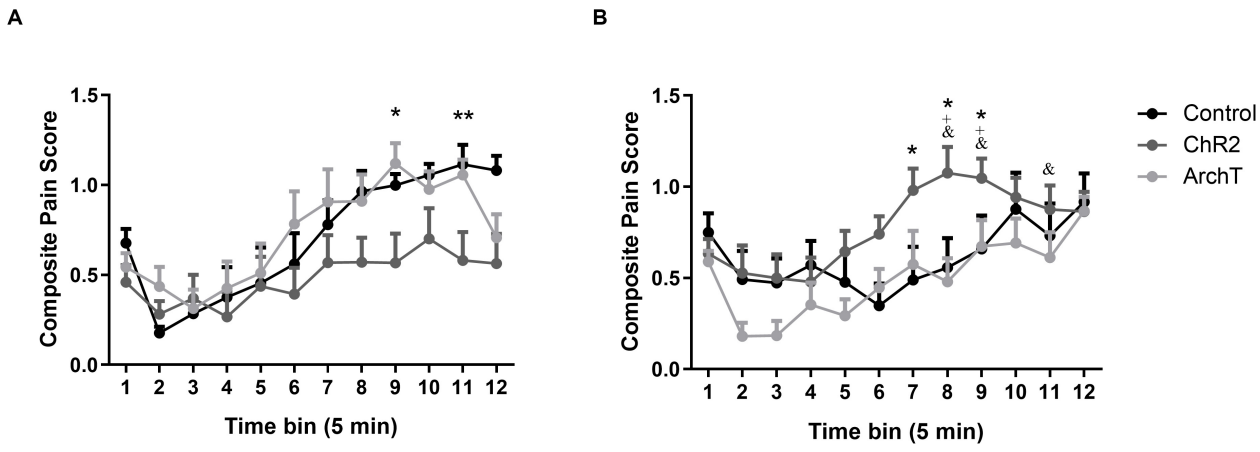

FIGURE 3 | (A) Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on formalin-evoked nociceptive behavior of male Sprague-Dawley rats. Data are mean ± SEM (n = 8–10 per group). $^{**}p < 0.01$, $^{*}p < 0.05$ ChR2 vs control. **(B)** Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on formalin-evoked nociceptive behavior of female Sprague-Dawley rats. Data are mean ± SEM (n = 7–11 per group). $^{*}p < 0.05$ ChR2 vs control. $^{+}p < 0.05$ ChR2 female vs ChR2 male, and $^{\&}p < 0.05$ ArchT female vs ArchT male.

in the Female-ChR2 group was significantly higher than in Male-ChR2 counterparts at time bins 8 and 9 ($p < 0.05$). Optogenetic inhibition (ArchT) of glutamatergic neurons in the ACC had no significant effect on formalin-evoked nociceptive behavior in male or female rats, however, the Female-ArchT group exhibited significantly less formalin-evoked nociceptive behavior compared to Male-ArchT counterparts at time bins 8, 9, and 11 ($p < 0.05$; **Figure 3**).

Effects of Optogenetic Modulation on General Locomotor Activity and Defecation in Formalin–Treated Rats

Two-way ANOVAs revealed that there were significant effects of sex on distance moved [$F_{(1,49)} = 7.858$; $p < 0.01$] and grooming [$F_{(1,49)} = 15.82$; $p < 0.01$] during the formalin trial, but no effects of optogenetic modulation of glutamatergic neurons in the ACC or sex × optogenetic modulation interaction (**Table 2**). Distance moved ($p < 0.01$) and duration of grooming ($p < 0.001$) were significantly greater in males than females regardless of optogenetic manipulation. Two-way ANOVAs revealed that there were no effects of optogenetic modulation or sex or their interaction on rearing or defecation (**Table 2**).

Formalin-Induced Conditioned Place Aversion

Two-way ANOVA revealed an effect of optogenetic modulation of glutamatergic neurons in the ACC [$F_{(2,49)} = 3.910$; $p = 0.03$] on F-CPA behavior, but not of sex or sex × optogenetic modulation interaction (**Figure 4**). Optogenetic inhibition (ArchT), but not stimulation (ChR2), of ACC significantly reduced ($p < 0.01$) F-CPA behavior compared to control fluorophore-expressing rats, regardless of sex (**Figure 4**).

c-Fos Immunoreactive Cells in the ACC

Two-way ANOVA revealed a significant effect of optogenetic modulation of glutamatergic neurons in the ACC during the day 3 formalin conditioning trial [$F_{(2,24)} = 6.289$; $p < 0.01$] and a significant effect of sex [$F_{(1,24)} = 14.47$; $p < 0.01$] on c-Fos-positive immunoreactive cells in the ACC after the day 4 post-conditioning trial (**Figure 5**). *Post hoc* analysis showed that optogenetic inhibition (ArchT) of glutamatergic neurons in the ACC during formalin conditioning significantly reduced the number of c-Fos-positive immunoreactive cells in the ACC of male rats compared to controls (**Figure 5B**; $p < 0.05$) and that by contrast, optogenetic activation (ChR2) significantly increased the number of c-Fos-positive immunoreactive cells in the ACC of females rats compared to controls (**Figure 5C**; $p < 0.01$). A correlation analysis was also performed between F-CPA score and the number of c-Fos-positive cells, for groups that exhibited significant differences in c-Fos expression (i.e., the control and ArchT groups for the male rats and the control and ChR2 groups for females). We found that there was a negative correlation between F-CPA score and c-Fos immunoreactivity in the ACC [$r(9) = -0.59$; $p = 0.05$] among the control and ArchT male rats, suggesting that greater formalin-induced aversive behavior was coupled with increased neuronal activity in the ACC in males. However, no correlation between F-CPA score and c-Fos was found in the control and ChR2 female groups [$r(8) = 0.29$; $p = 0.41$].

DISCUSSION

The results of the present study reveal differential effects of optogenetic modulation on formalin-evoked nociceptive behavior and F-CPA in male and female rats. We found that while optogenetic inhibition did not affect formalin-evoked nociceptive behavior in either sex, optogenetic activation of glutamatergic neurons of the ACC had a differential effect in males and females, reducing formalin-evoked nociceptive behavior in males during the second phase of the formalin trial and increasing formalin-evoked nociceptive behavior in females during this same stage of the trial. One mechanism suggested for the differences in pain observed between males and females is sex-dependent pathways for analgesia and hyperalgesia (Nemmani et al., 2004; Bryant et al., 2006; Bliss et al., 2016). Non-competitive antagonism of NMDA receptors potentiates morphine analgesia in male but not female mice (Nemmani et al., 2004), and attenuates morphine tolerance in male but not female mice (Bryant et al., 2006). Human studies have found differences in the functional connectivity between the subgenual ACC (sgACC) and various brain regions of the descending pain pathway in men and women (Wang et al., 2014; Monroe et al., 2017). For instance, women have greater functional connectivity between the sgACC and the periaqueductal gray (PAG) than men. It has been suggested that these projections from the ACC to the PAG may be involved in facilitating

TABLE 2 | Effects of optogenetic modulation of glutamatergic neurons in the ACC on general locomotor behavior and defecation during 60-min formalin conditioning trial in male and female rats.

	Grooming (s)	Rearing (s)	Distance moved (cm)	Defecation (pellet number)
Male-Control (*n* = 9)	207.3 ± 40.2	25.4 ± 10.1	3915 ± 719	1.6 ± 0.5
Male-ChR2 (*n* = 8)	165.0 ± 54.4	20.9 ± 9.7	5037 ± 1410	2.5 ± 0.7
Male-ArchT (*n* = 10)	105.4 ± 24.6	33.0 ± 9.9	4884 ± 1255	1.8 ± 0.4
Male-Total (*n* = 27)	157 ± 23.5	26.9 ± 5.6	4606 ± 650	1.9 ± 0.3
Female-Control (*n* = 7)	57.2 ± 15.7	19.7 ± 13.4	2633 ± 289	2.7 ± 0.8
Female-ChR2 (*n* = 11)	63.3 ± 18.6	12.7 ± 2.9	3003 ± 265	1.9 ± 0.4
Female-ArchT (*n* = 10)	62.3 ± 13.7	14.9 ± 3.9	2475 ± 200	1.3 ± 0.3
Female-Total (*n* = 28)	61.5 ± 9.3***	15.1 ± 3.3	2722 ± 147**	1.9 ± 0.3

*Data are mean ± SEM (n = 7–11 per group). ***p < 0.001, **p < 0.01 vs corresponding Male- Total, where Male-Total and Female-Total are the means ± SEM for all rats within each sex.*

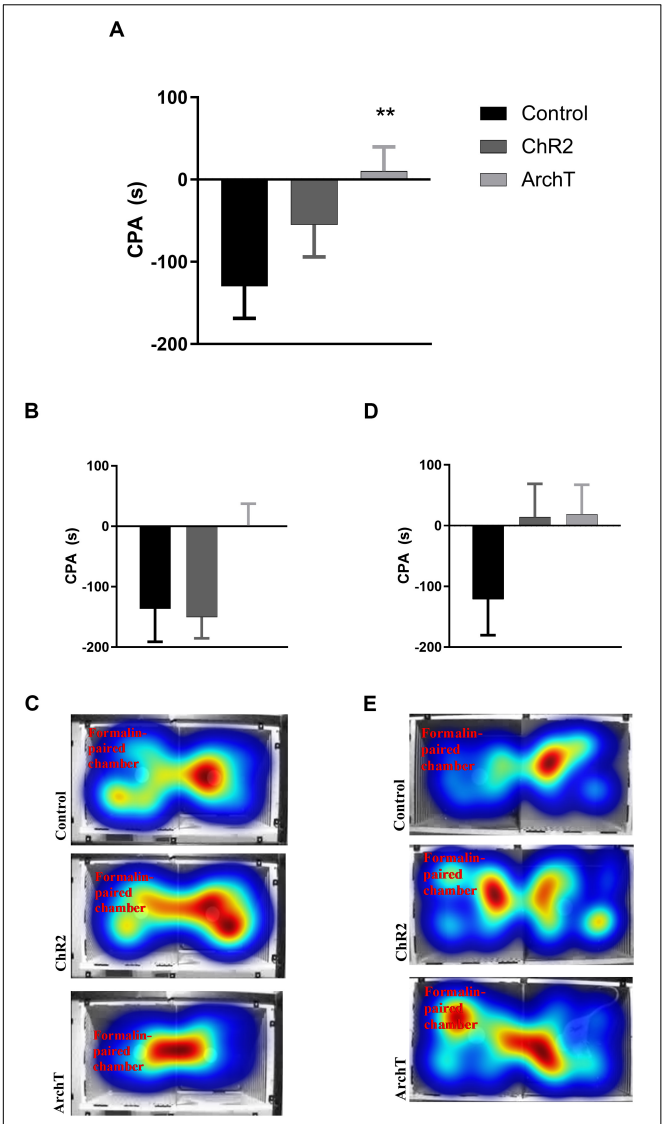

FIGURE 4 | (A) Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on formalin-induced conditioned place aversion (F-CPA) in combined male and female Sprague-Dawley rats. Data are mean ± SEM (n = 7–11 per group). $^{**}p < 0.01$ vs control. **(B)** Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on formalin-induced conditioned place aversion (F-CPA) in male Sprague-Dawley rats. Data are mean ± SEM (n = 8–10 per group). **(C)** Representative heat map images of post-conditioning trials for each treatment group in male rats. **(D)** Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on formalin-induced conditioned place aversion (F-CPA) score of female Sprague-Dawley rats. Data are mean ± SEM (n = 7–11 per group). **(E)** Representative heat map images of post-conditioning trials for each treatment group in female rats.

nociception through the RVM (Calejesan et al., 2000). If the functional connectivity of the ACC to the PAG is stronger in females compared to males, this difference could explain how optogenetic activation of glutamatergic neurons of the ACC increased formalin-evoked nociceptive behaviors in females and not in males.

Another possible mechanism underlying sex differences in pain is the influence of sex hormones (Martin, 2009). In particular, the affective component of pain has been found to be modulated by sex hormones. In female rats, pain-related aversive behavior is attenuated by ovariectomy (Chen et al., 2014; Li L.-H. et al., 2014; Hubbard et al., 2015). Inhibition of estrogen receptors or aromatase androstatrienedione in the ACC blocks F-CPA in both male and female rats, and exogenous estrogen elicits aversive behavior in the absence of a noxious stimulus in both sexes (Xiao et al., 2013). A limitation of the present study is the lack of estrous cycle stage assessment, however, we did not observe any effect of sex on F-CPA. It has been reported that estrogen enhances glutamatergic excitatory postsynaptic currents (EPSCs) in the ACC, suggesting that the mechanism by which estrogen modulates affective pain may be through modulation of glutamatergic transmission in the ACC (Xiao et al., 2013). Similarly, female rats in a high estrogen state exhibit increased pain behaviors and reduced excitatory amino acid transporter (EAAT) function in the ACC compared to rats in a low estrogen (Moloney et al., 2016). These findings may explain why we saw an increase in nociceptive behaviors with optogenetic activation (ChR2) of glutamatergic neurons in the ACC of female, but not male, rats.

Another key finding of the present study is that the inhibition of glutamatergic neurons of the ACC is sufficient to abolish formalin-induced aversion learning without reducing nociceptive behavior. Our results support and extend previous work which showed that administration of an NMDA receptor antagonist into the ACC abolished F-CPA in SD rats, while not affecting formalin-induced nociceptive behavior (Johansen and Fields, 2004; Lei et al., 2004a; Ren et al., 2006), suggesting that glutamatergic neurons in the ACC are active during pain states and are responsible for the aversive component of pain and that it is possible to dissociate this aversive component of pain from the sensory component. Previous work has also demonstrated that the NMDAR subunits NR2A and NR2B, as well as the NMDAR downstream ERK pathway, within the ACC, are necessary for pain-related aversion learning in rats (Cao et al., 2009; Li et al., 2009). Our immunohistochemical analysis revealed a reduction in c-Fos immunoreactive neurons in the ACC during the post-conditioning trial in male rats that received optogenetic inhibition of glutamatergic neurons in the ACC during the formalin conditioning trial, which was not seen in female rats that also received optogenetic inhibition. These results correspond to, and correlate with, the behavioral results observed, in that male rats that received optogenetic inhibition exhibited a reduction in formalin-evoked aversion behavior, which is in agreement with Lei et al. (2004b) who found that Fos immunoreactivity in the ACC is significantly increased after retrieval of formalin-induced conditioned place avoidance. By contrast, in female rats, optogenetic activation of glutamatergic neurons was associated with an increase in Fos immunoreactivity in the ACC. Interestingly, however, this increase was not coupled with the F-CPA behavior in females, as it was in males. There was no effect of optogenetic modulation on general locomotor activity, suggesting that the effect of optogenetic modulation

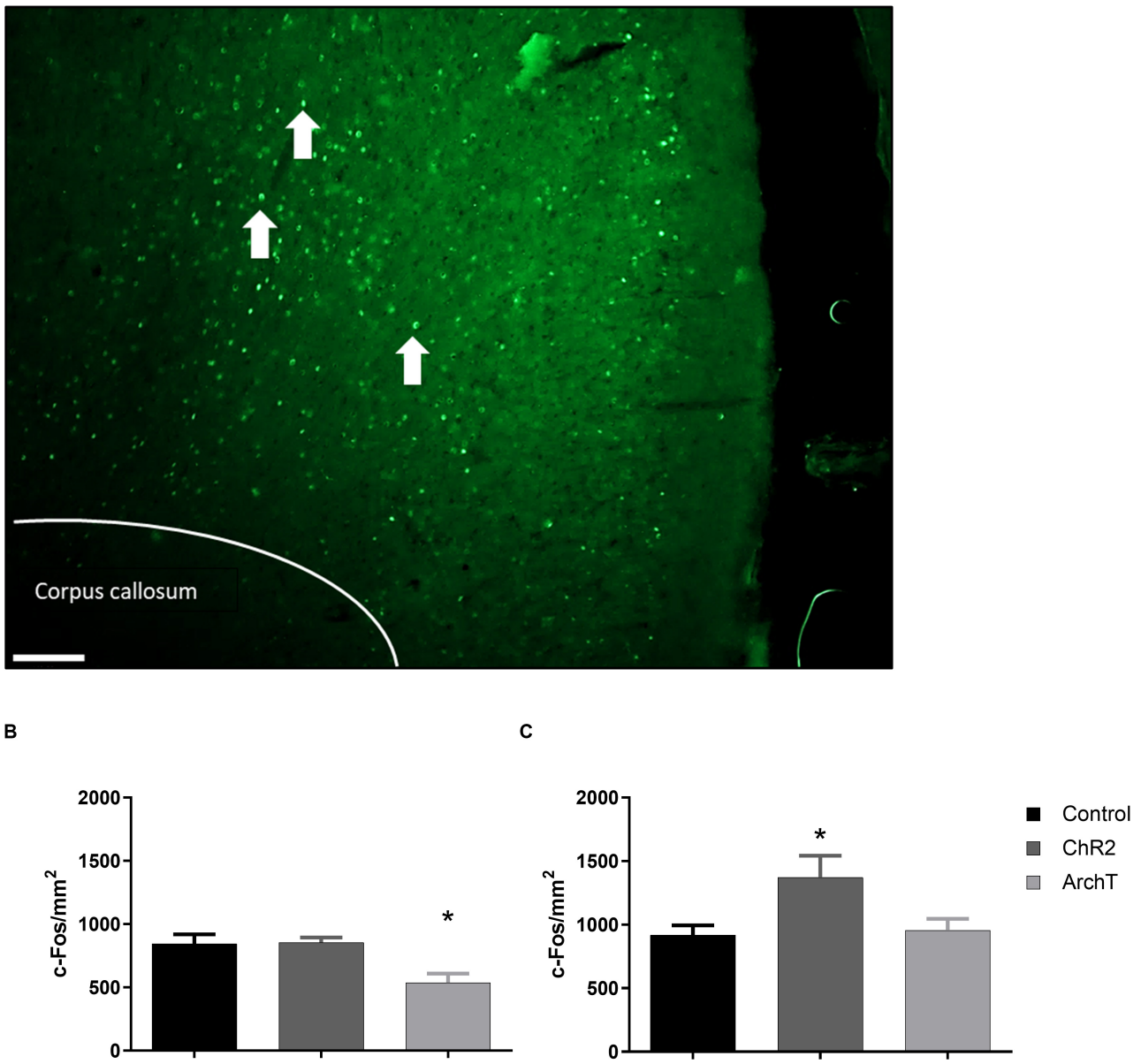

FIGURE 5 | Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on c-Fos positive cells in the ACC of SD rats. **(A)** Representative images of c-Fos (green) expression within the ACC (scale bar = 200 μm). White arrows denote representative c-Fos-positive staining. **(B)** Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on c-Fos positive cells in the ACC of male SD rats. Data are mean ± SEM (n = 5–6 per group). *$p < 0.05$ vs. control. **(C)** Effects of optogenetic stimulation (ChR2) or inhibition (ArchT) of glutamatergic neurons in the ACC on percent of c-Fos positive cells in the ACC of female SD rats. Data are mean ± SEM (n = 4–6 per group). *$p < 0.05$ vs. control.

on formalin-evoked nociceptive behavior was not a result of alterations in motor behavior. Female rats had lower levels of grooming and distance moved compared to males, but as there were no differences between control males and females for formalin-evoked nociceptive behavior, F-CPA or CPS, despite the difference in grooming, and since there were no effects of optogenetic manipulation on grooming, despite its effects on formalin-evoked nociceptive behavior, F-CPA or CPS, it is unlikely that sex differences in grooming have a bearing on the key findings and conclusions of this study.

A feature of the CPA behavioral paradigm is the necessary learning component, and thus it is possible that the effects of optogenetic modulation could result either from an effect on aversion behavior *per se* and/or an effect on general memory and

learning. However, a previous study has found that optogenetic inhibition of glutamatergic neurons in the ACC of mice following induction of complete Freund's adjuvant-induced arthritic pain induces place preference, suggesting that inhibition of these neurons does not inhibit memory acquisition or learning (Kang et al., 2017). It has also been found that optogenetic activation of glutamatergic neurons of the ACC in the absence of pain induces CPA to the stimulation-paired chamber (Tan et al., 2017).

The present study reveals, for the first time, sex differences in the role of glutamatergic neurons of the ACC in sensory and aversive components of pain. A particularly interesting result was the differential effect of activation of glutamatergic neurons of the ACC on formalin-evoked nociceptive behavior in males and females. The results of the study also support and extend the current understanding that inhibition of glutamatergic neurons of the ACC prevents aversion learning without affecting nociceptive behavior. Our findings identify glutamatergic neurons within the ACC as a potentially important substrate influencing sex differences in pain responding.

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 Animal Care and Research Ethics Committee, National University of Ireland Galway.

AUTHOR CONTRIBUTIONS

SJ and DF designed the experiments. SJ conducted the behavioral experiments and analysis of the c-Fos expression by immunohistochemistry, analyzed the data, and wrote the manuscript. DF, MR, and AP reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Trait Sensitivity to Negative and Positive Feedback Does Not Interact With the Effects of Acute Antidepressant Treatment on Hedonic Status in Rats

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Aberrant cognition plays a pivotal role in the development and maintenance of depression. One of the most important cognitive distortions associated with depression is aberrant sensitivity to performance feedback. Under clinical conditions, this sensitivity can be measured using the probabilistic reversal learning (PRL) test, which has also been recently implemented in animal studies. Although the evidence for the coexistence of depression and altered feedback sensitivity is relatively coherent, it is unclear whether this sensitivity can influence the effectiveness of antidepressant treatment. In the present research, we investigated how trait sensitivity to negative and positive feedback interacts with the effects of acute antidepressant treatment on hedonic status in rats. We tested a cohort of rats with a series of 10 PRL tests, and based on this screening, we classified each animal as sensitive or insensitive to negative and positive feedback. Subsequently, in the Latin square design, we evaluated the effects of a single administration of two antidepressant drugs (each at three different doses: agomelatine: 5, 10, and 40 mg/kg; mirtazapine 0.5, 1, and 3 mg/kg) on the hedonic status of rats in the sucrose preference tests. There was no statistically significant interaction between trait sensitivity to feedback and the effects of acute antidepressant treatment on hedonic status in rats.

Keywords: feedback sensitivity, cognitive bias, anhedonia, animal model, antidepressant

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INTRODUCTION

A depressive disorder is a serious mental illness characterized by lowered mood and anhedonia (i.e., the loss of pleasure; Belzung et al., 2015). It has also been associated with sustained widespread cognitive impairments, including abnormal responses to negative (NF) and positive (PF) feedback (Clark et al., 2009). Indeed, several studies have demonstrated that depressed individuals are hypersensitive to NF (punishments) and hyposensitive to PF (rewards), which leads to altered processing of negatively and positively valenced information (Beats et al., 1996; Elliott et al., 1998). Such negatively distorted thinking perpetuates a maladaptive belief system and low mood, leading to a specific state in which criticism or minor errors are overemphasized and major achievements are ignored (Clark et al., 2009). Although clinical evidence for the coexistence of depression

and altered sensitivity to performance feedback is relatively coherent, we still do not know whether increased or decreased sensitivity to NF/PF are associated with better/worse antidepressant treatment outcomes.

One of the most influential, recent theories of the antidepressant drug action, implies that antidepressants may produce their ultimate, clinical effects by early actions on information processing biases, including distorted sensitivity to feedback (Harmer et al., 2003b, 2009). It has been proposed that, at a neuropsychological level, antidepressant drugs remediate the negative affective biases and that, contrary to common opinion, these actions occur relatively quickly, even following a single drug administration (Harmer et al., 2009). This induction of a more positive way of processing environmental stimuli (positive bias) leads to cognitive and psychological reconsolidation and wider antidepressant effect. There is now a growing body of experimental evidence that antidepressants can affect emotional processing very early in treatment. Several studies revealed that in healthy subjects, even single doses of antidepressants increase the recognition of happy facial expressions (Harmer et al., 2003a,b), and increase attention to positive, socially relevant stimuli in a visual probe task (Browning et al., 2007). Studies in animals yielded similar results. It has been demonstrated that acute administration of several widely prescribed antidepressants changes the affective bias of naïve rats in the affective bias test [citalopram, desipramine, fluoxetine, mirtazapine, venlafaxine, reboxetine, clomipramine and ketamine (Stuart et al., 2013, 2015)], modulate cognitive judgment bias in the ambiguous-cue interpretation (ACI) test [citalopram, desipramine (Rygula et al., 2014; Golebiowska and Rygula, 2017) and reboxetine (Enkel et al., 2010; Anderson et al., 2013)], and alter the sensitivity of rats to performance feedback in the preclinical version of the PRL task [agomelatine, mirtazapine (Drozd et al., 2019), citalopram (Bari et al., 2010) and ketamine (Rychlik et al., 2017)].

The recent implementation of the preclinical version of the probabilistic reversal learning (PRL) paradigm (Bari et al., 2010) allowed for the investigation of this question in animal models. The results of our previous studies demonstrated that in rodents, sensitivity to NF and PF are stable and enduring behavioral traits (Noworyta-Sokolowska et al., 2019) and that even single doses of agomelatine or mirtazapine could change this sensitivity in the PRL test (Drozd et al., 2019). In that latter study, acute agomelatine treatment reduced the sensitivity of rats to NF, as indexed by the decreased proportion of lose-shift behaviors, while mirtazapine increased the sensitivity of rats to PF, as indexed by the increased proportion of win-stay behaviors. This decrease in NF sensitivity and the increased sensitivity to PF were hypothesized to manifest antidepressant-induced, positive, information-processing biases, similar to those reported previously in humans following acute antidepressant treatment (Arnone et al., 2009; Rawlings et al., 2010; Komulainen et al., 2016).

In the current study, we build off of these prospective findings by testing a hypothesis that the effects of antidepressant drugs on reward-related processes may be influenced by trait sensitivity to NF or PF. In other words, we designed this study to investigate whether trait sensitivity to NF/PF could boost/diminish the effects of acute antidepressant treatment on the hedonic capacity of rats.

The animals were screened in a series of PRL tests and classified as sensitive/insensitive to NF/PF. Subsequently, the influence of this trait on the effects of acute administration of two antidepressant drugs, namely, mirtazapine and agomelatine (each in three different doses), on the hedonic processing of rats was investigated using sucrose preference (SP) tests.

MATERIALS AND METHODS

Subjects and Housing

In the present study, we used 80 male Sprague–Dawley rats (Charles River, Germany) weighing 175–200 g (about 10 weeks old) upon arrival. Rats were kept in groups (four animals/cage) under controlled temperature (21 ± 1°C) and humidity (40–50%) under a 12/12 h light/dark cycle (lights on at 7:00 h). The cage size was 56 (L) × 35 (W) × 21 (H) cm.

During the entire experiment, rats were mildly food restricted to 85% of their free-feeding weight (according to normal growth curve recommended by the laboratory rodent supplier—Charles River Research Models and Services Catalogue) by providing 15–20 g of food pellets per rat per day (standard laboratory chow). Food restriction began 1 week before behavioral training. Water was available *ad libitum*.

The experiments were performed during the light phase of the light/dark cycle.

Apparatus

The PRL training and testing was performed in 16 computer-controlled operant conditioning boxes (Med Associates, St. Albans, Vermont, VT, USA). Boxes were equipped with a fan, light, speaker, a food dispenser set to deliver a sucrose pellet (Dustless Precision Pellets, 45 mg; Bio-Serv, Flemington, NJ, USA), and two retractable levers which were located on opposite sides of the feeder. We have programmed the experimental protocols using Med State notation code (Med Associates). The data were analyzed using a custom-written R programme. The experimental procedure for the PRL task used in this study was a modified version of the procedures used and described previously by Bari et al. (2010) and has been described in detail elsewhere (Noworyta-Sokolowska et al., 2019).

Measuring Feedback Sensitivity Using the PRL Test

PRL Training and Testing

After the initial instrumental training described elsewhere (Noworyta-Sokolowska et al., 2019), the rats were trained in the PRL paradigm. Each PRL training session consisted of 200 trials, and each trial lasted for a maximum of 22 s. The start of a trial was signalled by the house light, which remained on until the end of the trial. Two seconds after the trial had started, both levers were presented, and one of

them was randomly assigned as the "correct" lever, which delivered a reward 80% of the times it was pressed. A press on the other lever—the "incorrect" lever—would result in a rewarding outcome only 20% of the times it was pressed. No response in 10 s triggered the ITI and was counted as an omission. The same ITI directly followed a punishing outcome, i.e., no reward on 20% of the "correct" and 80% of the "incorrect" lever presses. After every eight consecutive "correct" lever presses (regardless of the outcome), the criterion for the reversal of the outcome probabilities was reached. The previously "correct" lever now became "incorrect" and vice versa. This pattern was followed until the end of the session.

This training phase was repeated daily until the individual animals achieved sufficient performance levels. The criteria to be met were a minimum of three reversals completed during three consecutive training sessions, with less than 15% omissions per session.

Parameters Measured in the PRL Test

To monitor the sensitivity of rats to PF and NF, the animals' decisions were tracked on a trial-by-trial basis. To evaluate the sensitivity to NF we assessed the ability of animals to ignore infrequent and misleading, punished (non-rewarded) outcomes on the "correct" lever. For this, the animal's decisions to switch levers following such a misleading punishment (probabilistic lose-shifts), were scored and expressed as a ratio of all punished (unrewarded) outcomes on that lever. To evaluate sensitivity to PF, all rewarded outcomes (true and misleading) followed by a decision to stay with the lever that delivered them (win-stays) were counted jointly for the "correct" and "incorrect" levers and expressed as a ratio of all rewarded outcomes.

Measuring Hedonic Capacity Using the SP Test

The preference for palatable sweet solutions is the most frequently used test to measure sensitivity to rewards/hedonic capacity in rodents (Papp et al., 1991; Willner et al., 1992). In this test, animals can choose between a palatable sweet solution and plain water, and the decreased or increased preference for the palatable solution is considered to reflect the decrease or increase in hedonic capacity respectively. The advantages of this test, which explain its popularity in laboratories throughout the world, are its simplicity and reliability. The method itself has been used in our laboratory for several years and has been thoroughly validated using various behavioral and pharmacological manipulations (Rygula et al., 2005, 2006, 2008, 2013; Noworyta-Sokolowska et al., 2019). During the SP test, the rats were separated into single cages and were offered a voluntary choice between two bottles for 1 h, where one bottle contained a 2% (w/v) sucrose solution and the other bottle contained tap water. To prevent potential effects of side preference in drinking, the position of the bottles was switched after 30 min. The consumption of water and sucrose solution was measured by weighing the bottles. The preference for sucrose was calculated from the amount of sucrose solution consumed and is expressed as a percentage of the total amount of liquid that was consumed.

Experimental Design and Drugs

The experimental schedule is presented in **Figure 1**. Initially, the rats were trained for the PRL test as described above. After achieving a stable performance, animals that reached the criterion were subsequently tested in 10 consecutive PRL tests over 10 days. Based on this "sensitivity screening," the rats were divided using a median split into sensitive and insensitive to NF and PF. The division according to sensitivity to NF was made based on the average ratio of lever changes following misleading punishment (probabilistic lose-shifts) made by the animals across all 10 screening tests. The division according to the sensitivity to PF was made based on the average ratio of pressing the same lever (win-stays) following both true and misleading rewards across all 10 screening tests. To confirm the stability of the feedback sensitivity traits, we additionally analyzed the "frequency of sensitivity," expressed as the number of the PRL tests (out of the 10 comprising screening) in which an animal displayed sensitivity to feedback. After the feedback sensitivity screening, the effects of acute administration of agomelatine and mirtazapine on the hedonic capacity of rats were evaluated using SP tests in the fully randomized Latin square design, which means that on any given day all treatments were represented and were balanced across the tests. This within-subject study design, contrary to the between-subject designs, allowed us to reduce the variance of the data and the number of animals used. The use of a Latin square design is a common and valid method in pharmacological research (Howell, 1997). It has been also successfully applied in a number of our previous studies (Drozd et al., 2017, 2019; Golebiowska and Rygula, 2017; Rychlik et al., 2017).

The drugs and their doses were chosen based upon our previous study, which demonstrated that even single doses of agomelatine or mirtazapine could change the sensitivity to the feedback of experimental animals in the PRL test (Drozd et al., 2019). Agomelatine (TCI Europe, Zwijndrecht, Belgium, HPLC −98%), a relatively novel antidepressant drug that acts as a potent agonist of melatonin MT1/MT2 receptors (Yous et al., 1992; Ying et al., 1996) and an antagonist of the 5-HT2C receptor subtype (Millan et al., 2003) was dissolved in 1% hydroxyethyl cellulose and applied in doses 5, 10, and 40 mg/kg). Mirtazapine (TCI Europe, Zwijndrecht, Belgium, HPLC −98%), a tetracyclic antidepressant modulating noradrenergic and serotonergic neurotransmission *via* blockade of central α2-adrenergic auto- and heteroreceptors, stimulation of 5-HT1A receptors (Berendsen and Broekkamp, 1997), and blockade of 5-HT2A, 5-HT2C and 5-HT3 receptors (de Boer, 1995), was dissolved in an equimolar solution of citric acid and injected in doses 0.5, 1, and 3 mg/kg. The drugs were administered intraperitoneally in a dose volume of 1 ml/kg 30 min. before the SP test. Control animals received corresponding injections of vehicle solutions. The wash-out period between administrations of different drug doses in the Latin square design was 1 week, what considering the pharmacokinetics of tested drugs (the

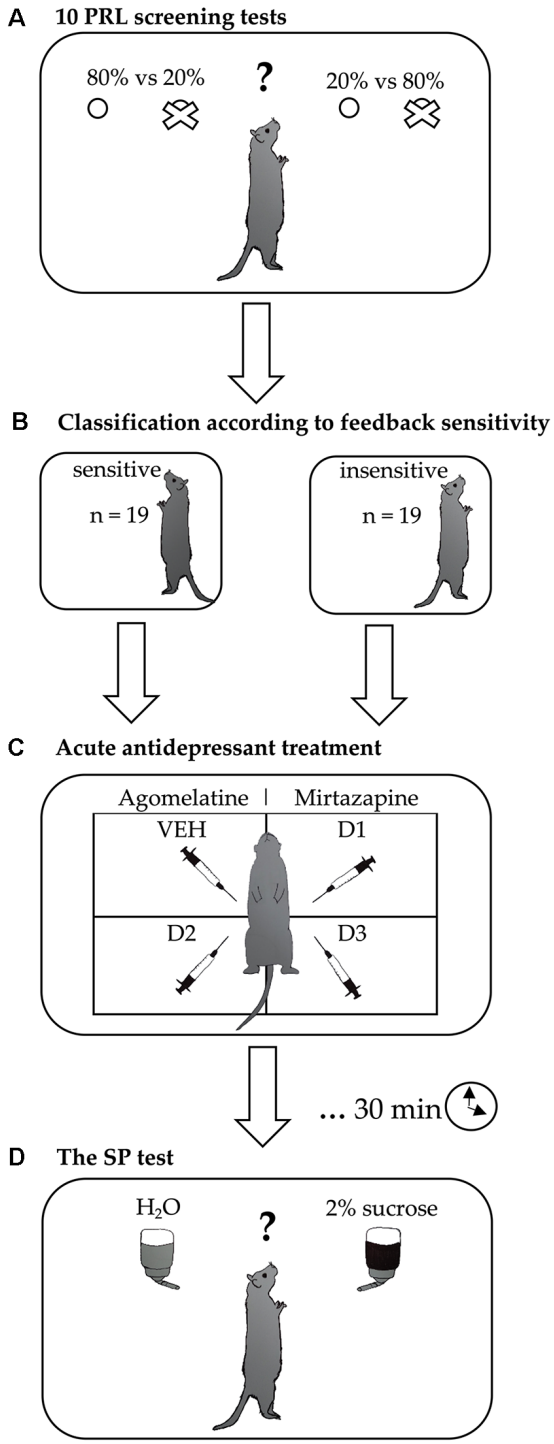

FIGURE 1 | Experimental design. After achieving a stable performance in the probabilistic reversal learning (PRL) training sessions, the rats were subjected to **(A)** feedback-sensitivity screening, consisting of 10 PRL tests carried out over 10 consecutive days. Based on the results of this screening, each rat was **(B)** classified as insensitive or sensitive to negative and positive feedback. Subsequently, differences in the effects of **(C)** acute treatment with two different antidepressant drugs (agomelatine and mirtazapine, each in three doses) on hedonic processing were evaluated using **(D)** sucrose preference (SP) tests conducted in a fully randomized Latin square design.

elimination half-lives of a few hours), was more than enough to avoid the accumulation of drug effects (Zupancic and Guilleminault, 2006; Rouini et al., 2014; He et al., 2018).

Statistics

The data were analyzed using SPSS (version 25.0, SPSS Inc., Chicago, IL, USA). The distribution of the experimental data was tested using the Kolmogorov–Smirnov test. The effects of trait sensitivity to NF/PF and the effects of antidepressant treatment on parameters measured in the SP test were investigated using two-way repeated-measures ANOVAs with the between-subject factor of sensitivity (two levels: "sensitive," "insensitive") and the within-subject factor of dose (four levels: vehicle, D1, D2, and D3). Homogeneity of variance and sphericity of ANOVA were verified using Levene's and Mauchly's tests, respectively. For pairwise comparisons, the values were adjusted using the Sidak correction (Howell, 1997). All of the tests of significance were performed at $\alpha = 0.05$.

RESULTS

Effects of Acute Agomelatine Administration on Sucrose Preference in Rats Classified as Sensitive/Insensitive to Negative and Positive Feedback

All animals fulfilled the training criteria and qualified for PRL screening. Two rats were removed from the analysis due to fluid leakage during the SP test.

Negative Feedback Sensitivity Screening

For the animals classified as NF-insensitive, the average proportion of lose-shift behaviors following misleading NF ranged from 0.341 to 0.501, with an average of 0.461 ± 0.009. For those classified as NF-sensitive, the average proportion of probabilistic lose-shift behaviors ranged from 0.503 to 0.734 with an average of 0.561 ± 0.013. The sensitivity to NF in both subgroups was stable across the screening period (nonsignificant Screening day × NF sensitivity interaction ($F_{(9,324)} = 0.913$, $p = 0.514$, **Figure 2A**).

The average number of reversals made by the animals classified as insensitive to NF during the screening period ranged from 4.6 to 9.5, with an average of 6.12 ± 0.28; for animals classified as sensitive to NF, the average number of reversals ranged from 3.6 to 7.7, with an average of 5.60 ± 0.24. Reversal performance in both groups was stable [there was a nonsignificant interaction between screening day and NF sensitivity ($F_{(9,324)} = 0.662$, $p = 0.743$), **Figure 2B**].

The average frequency of NF sensitivity in animals classified as NF-insensitive ranged from 0 to 5, with an average of 3.4 ± 0.3; in those classified as NF sensitive, the average frequency of NF sensitivity ranged from 5 to 10, with an average of 6.6 ± 0.4. The animals classified as NF-insensitive were significantly less sensitive to NF than the rats classified as NF-sensitive ($t = 6.853$, $df = 36$, $p < 0.001$, **Figure 2C**).

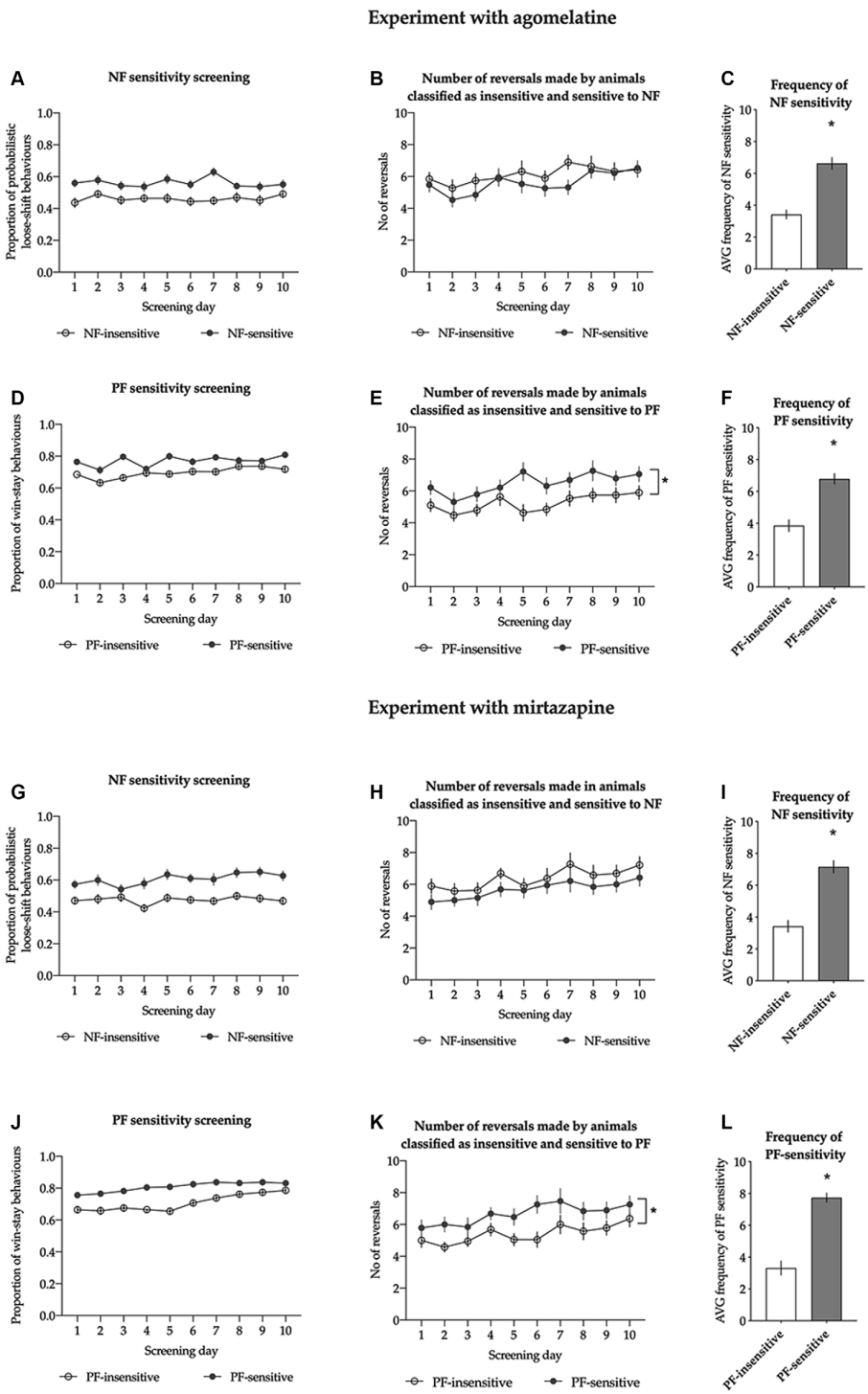

FIGURE 2 | Results of the sensitivity to feedback screening in animals treated with agomelatine **(A–F)** and mirtazapine **(G–L)**. Panels **(A,G)** show the average proportion of lose-shift behaviors following misleading punishment in rats classified as insensitive (open circles, *N* = 19) and sensitive (filled circles, *N* = 19) to negative feedback (NF) across all 10 screening probabilistic reversal learning (PRL) tests in cohorts of rats treated with agomelatine and mirtazapine, respectively. Panels **(B,H)** show the average number of reversals in rats classified as insensitive (open circles, *N* = 19) and sensitive (filled circles, *N* = 19) to NF across all 10 screening PRL tests in cohorts of rats treated with agomelatine and mirtazapine, respectively. Panels **(C,I)** show the average frequency of sensitivity to NF in a cohort of rats treated with agomelatine and mirtazapine respectively. Panels **(D,J)** show the average proportion of win-stay behaviors following a reward in rats

(*Continued*)

FIGURE 2 | Continued
classified as insensitive (open circles, *N* = 19) and sensitive (filled circles, *N* = 19) to positive feedback (PF) across all 10 screening PRL tests. Panels **(E,K)** show the average number of reversals in rats classified as insensitive (open circles, *N* = 19) and sensitive (filled circles, *N* = 19) to PF across all 10 screening PRL tests in cohorts of rats treated with agomelatine and mirtazapine, respectively. Panels **(F,L)** show the average frequency of sensitivity to PF in a cohort of rats treated with agomelatine and mirtazapine respectively. The frequency is expressed as the number of PRL tests (out of the 10 comprising screening) in which an animal displayed the value of given feedback sensitivity located above the median of the values from the entire cohort. Data are presented as the mean ± SEM. *Indicates $p < 0.05$ compared to the insensitive group.

Positive Feedback Sensitivity Screening

The average proportion of win-stay behaviors in the animals classified as PF-insensitive ranged from 0.635 to 0.725, with an average of 0.696 ± 0.006. The average proportion of win-stay behaviors in the animals classified as PF-sensitive ranged from 0.733 to 0.865, with an average of 0.770 ± 0.008. The sensitivity to PF in both subgroups was stable across the screening period [there was a nonsignificant interaction between screening day and PF sensitivity ($F_{(9,324)} = 1.740$, $p = 0.079$, **Figure 2D**)].

The average number of reversals made by the animals classified as insensitive to PF during the screening period ranged from 3.6 to 6.7, with an average of 5.24 ± 0.19. This average was significantly ($p < 0.05$) lower than that for animals classified as sensitive to PF, where it ranged from 4.6 to 9.5 with an average of 6.48 ± 0.26. Reversal performance in both groups was stable [there was a nonsignificant interaction between screening day and PF sensitivity ($F_{(9,324)} = 0.740$, $p = 0.672$, **Figure 2E**)].

The average frequency of PF sensitivity in animals classified as PF-insensitive ranged from 1 to 7, with an average of 3.8 ± 0.4; in those classified as PF-sensitive, the average frequency of PF sensitivity ranged from 5 to 10, with an average of 6.8 ± 0.3. The animals classified as PF-insensitive were statistically significantly less sensitive to PF than those classified as PF-sensitive ($t = 5.958$, $df = 36$, $p < 0.001$, **Figure 2F**).

Sucrose Preference Test

The animals classified as NF-insensitive and NF-sensitive did not differ in sucrose preference either basally or after acute treatment with agomelatine [nonsignificant effect of NF sensitivity ($F_{(1,36)} = 0.014$, $p = 0.907$) and not significant Treatment × NF sensitivity interaction ($F_{(3,108)} = 1.733$, $p = 0.164$)]. Acute agomelatine treatment itself also had no statistically significant effects on sucrose preference [there was a nonsignificant effect of treatment ($F_{(3,108)} = 0.504$, $p = 0.680$), **Figure 3A**].

Similarly, PF sensitivity had no statistically significant effects on sucrose preference either basally or after acute treatment with agomelatine [the effect of PF sensitivity was nonsignificant ($F_{(1,36)} = 3.181$, $p = 0.083$), and there was a nonsignificant interaction between treatment and PF sensitivity ($F_{(3,108)} = 1.500$, $p = 0.219$, **Figure 3B**)].

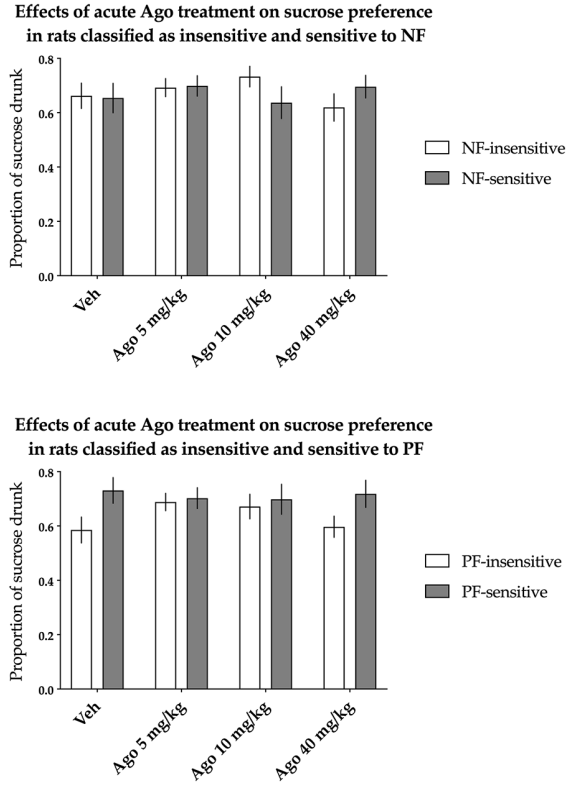

FIGURE 3 | Effects of acute administration of agomelatine (Ago) on the hedonic status of rats measured in the sucrose preference test. The data represent the average sucrose preference of rats classified as insensitive (open bars) and sensitive (filled bars) to **(A)** negative feedback (NF) and **(B)** positive feedback (PF) following acute administration of three different doses (5, 10 and 40 mg/kg) of agomelatine and vehicle solution. Data are presented as the mean ± SEM. *N* = 19 rats per group.

Effects of Acute Mirtazapine Administration on Sucrose Preference in Rats Classified as Sensitive/Insensitive to Negative and Positive Feedback

All animals fulfilled the training criteria and qualified for PRL screening. Two rats were removed from the analysis due to fluid leakage during the SP test.

Negative Feedback Sensitivity Screening

For the animals classified as NF-insensitive, the proportion of lose-shift behaviors following misleading NF ranged from 0.390 to 0.518, with an average of 0.475 ± 0.008. For those classified as NF-sensitive, the proportion of probabilistic lose-shift behaviors ranged from 0.521 to 0.722, with an average of 0.607 ± 0.015. The sensitivity to NF in both subgroups was stable across the screening period [there was a nonsignificant screening day × NF sensitivity interaction ($F_{(9,324)} = 0.833$, $p = 0.586$, **Figure 2G**)].

The number of reversals made by the animals classified as NF-insensitive during the screening period ranged from 3.5 to

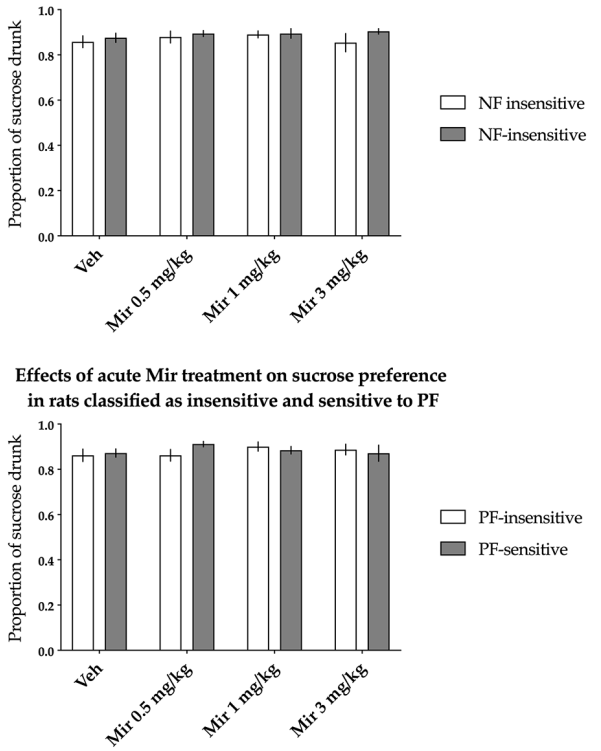

FIGURE 4 | Effects of acute administration of mirtazapine (Mir) on the hedonic status of rats, measured in the sucrose preference test. The data represent the average sucrose preference of rats classified as insensitive (open bars) and sensitive (filled bars) to **(A)** negative feedback (NF) and **(B)** positive feedback (PF) following acute administration of three different doses of mirtazapine (0.5, 1 and 3 mg/kg) and vehicle solution. Data are presented as the mean ± SEM. *N* = 19 rats per group.

9.9, with an average of 6.38 ± 0.33; for animals classified as NF-sensitive, the number of reversals ranged from 3.8 to 8.1, with an average of 5.68 ± 0.24. Reversal performance in both groups was stable [there was a nonsignificant interaction between screening day and NF sensitivity ($F_{(9,324)}$ = 0.174, p = 0.997, **Figure 2H**)].

The frequency of NF sensitivity in animals classified as NF-insensitive ranged from 0 to 7, with an average of 3.4 ± 0.4; in those classified as NF-sensitive, the frequency of NF sensitivity ranged from 5 to 10, with an average of 7.16 ± 0.38. The animals classified as NF-insensitive were statistically significantly less sensitive to NF than the rats classified as NF-sensitive (t = 7.011, df = 36, $p < 0.001$, **Figure 2I**).

Positive Feedback Sensitivity Screening

The proportion of win-stay behaviors in the animals classified as PF-insensitive ranged from 0.649 to 0.755, with an average of 0.708 ± 0.007. The proportion of win-stay behaviors in the animals classified as PF-sensitive ranged from 0.759 to 0.875, with an average of 0.808 ± 0.007. The sensitivity to PF in both subgroups was stable across the screening period [there was a nonsignificant screening day × PF sensitivity interaction ($F_{(9,324)}$ = 1.726, p = 0.082, **Figure 2J**)].

The number of reversals made by the animals classified as PF-insensitive during the screening period ranged from 3.5 to 7.1, with an average of 5.41 ± 0.21. This average was significantly ($p < 0.05$) lower than that for animals classified as PF-sensitive, where it ranged from 4.3 to 9.9 with an average of 6.65 ± 0.30. Reversal performance in both groups was stable [there was a nonsignificant interaction between screening day and PF sensitivity ($F_{(9,324)}$ = 0.427, p = 0.920, **Figure 2K**)].

The frequency of sensitivity to PF in animals classified as PF-insensitive ranged from 0 to 7, with an average of 3.3 ± 0.4; in those classified as PF-sensitive, the frequency of sensitivity ranged from 6 to 10, with an average of 7.7 ± 0.3. The animals classified as PF-insensitive were statistically significantly less sensitive to PF than those classified as PF-sensitive (t = 8.268, df = 36, $p < 0.001$, **Figure 2L**).

Sucrose Preference Test

The animals classified as NF-insensitive and NF-sensitive did not differ in sucrose preference either basally or after acute treatment with mirtazapine [nonsignificant effect of NF sensitivity ($F_{(1,36)}$ = 0.883, p = 0.354) and nonsignificant treatment × NF sensitivity interaction ($F_{(3,108)}$ = 0.514, p = 0.674)]. Acute mirtazapine treatment itself also had no statistically significant effects on sucrose preference [nonsignificant effect of treatment ($F_{(3,108)}$ = 0.645, p = 0.588, **Figure 4A**)].

Similarly, PF sensitivity had no statistically significant effects on sucrose preference either basally or after acute treatment with mirtazapine [nonsignificant effect of PF sensitivity ($F_{(1,36)}$ = 0.088, p = 0.769) and nonsignificant treatment × PF sensitivity interaction ($F_{(3,108)}$ = 1.302, p = 0.278, **Figure 4B**)].

DISCUSSION

The results presented here confirmed that in rats, sensitivity to NF and PF can be considered stable and enduring behavioral traits. They also confirmed that these traits do not interact with basal hedonic capacity. Most importantly, they demonstrated that trait sensitivity to feedback does not determine the effects of acute administration of two antidepressant drugs, agomelatine and mirtazapine, on hedonic processing in rats.

Over the past years, behavioral research has revealed that a concept of cognitive/behavioral traits exists and can be measured in animals (Gosling, 2001). It also revealed that there exists considerable cross-species overlap for some of these traits and that the assessment of these traits in animals has numerous practical applications that can contribute to a better understanding of psychiatric disorders (Rygula et al., 2018). For instance, recent studies using animal models have demonstrated that trait "pessimism," which has been previously linked with increased sensitivity to NF (Rygula and Popik, 2016), is associated with a "pro-depressive profile" that predicts increased vulnerability to stress-induced anhedonia (Rygula et al., 2013) and motivational deficits (Drozd et al., 2017). They also showed that biased judgement, as a trait, is

associated with alterations in the effectivity of antidepressant drug treatment (Drozd et al., 2019). Other studies suggested that trait sensitivity to NF and/or PF could be candidates for a cognitive biomarker of depression (Noworyta-Sokolowska et al., 2019). Although we did not observe statistically significant interactions between trait sensitivity to feedback and the hedonic capacity of tested animals in the present study, this result was not surprising and has already been explained elsewhere (Noworyta-Sokolowska et al., 2019) using Beck's cognitive model of depression (Beck, 1987, 2008). According to this theory, although biased acquisition and processing of information has a primary role in the maintenance and recurrence of depression, the development of depressive symptoms usually requires environmental triggers, e.g., stress. Thus, studies using animal models of depression based on chronic stress will be required to ultimately confirm whether the sensitivity to feedback (especially to PF) is a latent trait that could determine the hedonic capacity of rats.

It has also been recently proposed that antidepressant drugs may produce their ultimate clinical effects by early actions on information processing biases (Harmer et al., 2003b, 2009). Indeed, in our previous study, we demonstrated that both agomelatine and mirtazapine produce rapid effects on feedback sensitivity in the PRL paradigm (Drozd et al., 2019). In that study, acute agomelatine treatment reduced the sensitivity of rats to NF, as indexed by the decreased proportion of lose-shift behaviors, while mirtazapine increased the sensitivity of rats to PF, as indexed by the increased proportion of win-stay behaviors. This decrease in NF sensitivity and the increased sensitivity to PF were hypothesized to manifest antidepressant-induced, positive, information-processing biases, similar to those reported previously in humans following acute antidepressant treatment (Arnone et al., 2009; Rawlings et al., 2010; Komulainen et al., 2016). Building off these prospective findings, in the current study, we tested a hypothesis that the basal valence of individuals' sensitivity to feedback, measured as a stable and enduring behavioral trait, could moderate the effects of these two antidepressant drugs on hedonic processing in rats.

The fact that the results of the conducted experiments did not confirm this hypothesis, at least concerning the acute effects of antidepressants on sucrose preference, might suggest various effects of these drugs on the "wanting" and "liking" of rewards. Indeed, according to incentive sensitivity theory, brain mechanisms that determine how much a reward is "wanted" are separate from those that determine how much the reward is "liked" (Berridge and Robinson, 1998). "Wanting," which was expressed herein by the ratio of win-stay behaviors, is generated by the mesolimbic dopamine system, while "liking," or the actual pleasurable impact of reward consumption, which is indexed herein by the sucrose preference, is mediated by other, dopamine-independent and mainly opioidergic mechanisms (Berridge and Robinson, 1998). Although they were not investigated in our study, these various mechanisms could contribute to the observed differences in the effects of mirtazapine on win-stay behaviors in the PRL test, as previously observed by Drozd et al. (2019), and the effects on sucrose preference, as reported in the present study.

There are several limitations to this study that need to be mentioned. When considered in the context of depression, the first limitation would be the use of naïve rats. According to Beck (2008) and Harmer et al. (2003b), altered processing of information may play an important role in the effectiveness of antidepressant treatment; however, as mentioned above, a truly naturalistic animal model would require the use of environmental triggers, e.g., stress. Based on the present results, we cannot exclude that the expected interaction between trait sensitivity to feedback and the effects of antidepressants on hedonic processing in the SP test would become more salient if investigated in a model of depressive-like symptoms based on chronic psychosocial stress (Rygula et al., 2005). This limitation, however, in our opinion, does not undermine the validity of the present data, since the antidepressant efficacy of drugs is being widely and commonly studied in naïve animals [e.g., in the forced swim test (Porsolt et al., 2001)] and because both antidepressant drugs tested in the present study have been demonstrated previously to produce "antidepressive-like" effects in naïve animals (Stuart et al., 2013; Drozd et al., 2019).

The second limitation would be the use of only male subjects. Indeed, since the prevalence of the depressive disorder is significantly higher in women than in men, it seems more accurate to investigate the associated processes in females. However, the decision to use only male subjects was based on practical reasons: males do not have an oestrous cycle that could quite likely, by itself, affect the sensitivity to feedback. Thus, to avoid this additional confounding factor, we decided to test only male rats.

The third limitation would be the use of only one method for the measurement of the hedonic capacity of rats. Although indeed testing the hedonic capacity in a variety of other tests e.g., the cookie test (Surget et al., 2011) or the sweet drive test (Mateus-Pinheiro et al., 2014) could make the results more robust, we are convinced that the results obtained in the SP test are valid and reliable. The advantages of this test, which explain its popularity in laboratories throughout the world, are its simplicity and reliability. The method itself has been used in our laboratory for several years and has been thoroughly validated using various behavioral and pharmacological manipulations (Rygula et al., 2005, 2006, 2008, 2013).

Finally, it might be interesting to test the impact of trait sensitivity to feedback on hedonic processing in animals subjected to chronic antidepressant treatment. Although the tested compounds were previously reported to be effective in changing sensitivity to feedback following a single administration (Stuart et al., 2013; Drozd et al., 2019), the full antidepressant effects of these compounds (including their effects on hedonic processing) could perhaps be achieved following prolonged treatment.

The results of our study add to the growing body of experimental data regarding the role of cognitive traits in the development, maintenance, and treatment of affective disorders. These results also show that the immediate effects

of some antidepressant drugs on cognitive processing are not immediately conveyed by changes in the hedonic processing of rewards.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experiments were conducted following the European Union guidelines for the care and use of laboratory animals (2010/63/EU). Experimental protocols were reviewed and approved by the 2nd Local Institutional Animal Care and Use Committee at the Maj Institute of Pharmacology Polish Academy of Sciences in Krakow (Permission no. 242/2017).

AUTHOR CONTRIBUTIONS

RR designed the research. PS and KN performed the research. PS, KN, and RR analyzed the data. PS, KN, and RR wrote the article.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Role of BNST CRFR1 Receptors in Incubation of Fentanyl Seeking

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The time-dependent increase in cue-triggered opioid seeking, termed "incubation of opioid craving," is modeled in rodents by examining responding for opioid-associated cues after a period of forced abstinence. With opioid drugs, withdrawal symptoms may heighten cue reactivity by recruiting brain systems involved in both reward seeking and stress responses. Corticotropin releasing factor (CRF) in the bed nucleus of the stria terminalis (BNST) is a critical driver of stress-induced relapse to drug seeking. Here, we sought to determine whether BNST CRF receptor 1 (CRFR1) signaling drives incubation of opioid craving in opioid dependent and non-dependent rats. First, we tested whether BNST CRFR1 signaling drives incubation of opioid craving in rats with short-access fentanyl self-administration experience (2.5 μg/kg/infusion, 3 h/day for 10 days). On Day 1 of forced abstinence, we gave bilateral intra-BNST vehicle injections to all rats and measured lever responding for opioid cues in the absence of fentanyl infusions. On Day 30 of forced abstinence, we gave an identical test after bilateral intra-BNST injections of vehicle or CRFR1 receptor antagonist, R121919 (1 μg/0.3 μL/hemisphere). Vehicle treated rats showed greater responding for opioid associated cues on Day 30 relative to Day 1, and this incubation effect was prevented by intra-BNST R121919 on Day 30. Next, we incorporated an opioid-dependence procedure to investigate whether BNST CRFR1 signaling drives opioid cue-reactivity to a greater extent in opioid-dependent relative to non-dependent rats. We trained rats to self-administer fentanyl for 5 days before initiating the dependence phase and resuming daily fentanyl self-administration sessions for 10 days. We gave intra-BNST R121919 or vehicle injections before testing during acute (Day 5) or protracted (Day 30) withdrawal. During acute withdrawal, antagonizing BNST CRFR1 decreased the number of press bouts without affecting bout size or duration. These patterns of responding with R121919 treatment resulted in less fentanyl-associated conditioned reinforcement during test. Together, these findings suggest a role for BNST CRFR1 signaling in driving cue-reinforced opioid seeking after periods of forced abstinence.

Keywords: incubation, opioid dependence, withdrawal, CRF, BNST

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INTRODUCTION

Substance use disorder (SUD) consists of cycles of compulsive drug seeking and consumption, followed by periods of abstinence, withdrawal, and relapse (Koob and Volkow, 2016). Drug-associated cues trigger intense craving for drugs of abuse even after periods of abstinence (Sinha, 2011; Li et al., 2015). With opioid drugs of abuse, abstinent opioid-dependent individuals also experience aversive withdrawal symptoms that promote relapse (DSM-V). Preclinically, the incubation of drug craving model examines cue-triggered drug seeking in rodents after a period of forced abstinence (Grimm et al., 2001; Pickens et al., 2011; Reiner et al., 2019). During abstinence from opioid drugs of abuse, withdrawal may invigorate cue-driven opioid seeking by recruiting brain systems involved in both stress responses and reward seeking. The bed nucleus of the stria terminalis (BNST) is a critical regulator of both stress states and reward related behavior (Ch'ng et al., 2018). Corticotropin releasing factor (CRF) in the BNST is elevated during acute ethanol withdrawal (Olive et al., 2002) and CRF mRNA is increased in the BNST by stress-induced reinstatement of heroin seeking after periods of forced abstinence (Shalev et al., 2001). We postulate that CRF system in the BNST regulates incubation of opioid craving after forced abstinence in opioid dependent and non-dependent rats.

The CRF system is heavily implicated in opioid dependence and withdrawal (Sarnyai et al., 2001; Logrip et al., 2011). CRF receptor 1 (CRFR1) is necessary for the expression of conditioned behaviors associated with opioid withdrawal states (Contarino and Papaleo, 2005). Systemic CRFR1 antagonism reduces behavioral signs of precipitated opioid withdrawal (Iredale et al., 2000). In addition, CRFR1 antagonist dose dependently reduces heroin intake only in long access rats that are postulated to be heroin dependent (Greenwell et al., 2009). Together, these studies suggest that CRFR1 antagonists could be reducing heroin seeking by alleviating a negative emotional state in heroin dependent rats. When interpreting these CRF system findings with regard to relapse studies, it is critically important to consider whether withdrawal was precipitated or spontaneous. While precipitated opioid withdrawal is insufficient to reinstate heroin seeking, both stress and heroin priming reinstate opioid seeking after spontaneous withdrawal (Shaham and Stewart, 1995).

Several studies point to BNST CRF in stress-induced reinstatement of drug seeking, and specifically, a role of BNST CRFR1 signaling (Erb et al., 2001; Mantsch et al., 2016). Early studies showed intracerebroventricular pretreatment of a CRFR1 antagonist prevents stress-induced reinstatement of morphine conditioned place preference (Lu et al., 2000) and this effect is mediated by BNST (Wang et al., 2006). Central amygdala (CeA) CRF projections to the BNST mediate stress-induced reinstatement of cocaine seeking (Sakanaka et al., 1986; Erb et al., 2001). Stress-induced reinstatement only occurs in the presence of cues associated with drug self-administration suggesting an interplay between stress and drug-associated cues (Shelton and Beardsley, 2005). Incubation of opioid craving studies have yet to address whether spontaneous opioid withdrawal potentiates the motivational properties of drug-associated cues. In the present study, we begin to address this by examining cue-reinforced opioid seeking after forced abstinence in opioid-dependent and non-dependent rats. Further, we seek to identify whether BNST CRFR1 receptors are involved in incubation of opioid craving.

Here, we sought to determine whether BNST CRFR1 signaling drives incubation of opioid craving in opioid-dependent and non-dependent rats. First, using CRFR1 antagonist, R121919 [(2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7 dipropylamino pyrazolo[1,5-a]pyrimidine] (Heinrichs et al., 2002; Jagoda et al., 2003), we tested whether BNST CRFR1 signaling drives incubation of opioid craving in rats with short-access fentanyl self-administration experience. Second, we incorporated an opioid-dependence procedure to investigate if BNST CRFR1 mediates opioid cue-reactivity to a greater extent in opioid-dependent rats compared to non-dependent rats. Finally, to determine whether intra-BNST R121919 affected opioid consumption or the ability to lever press we utilized within-session behavioral economic opioid demand procedures. These experiments allow us to test the role of BNST CRFR1 in mediating incubation of fentanyl seeking after acute and protracted withdrawal in opioid-dependent and non-dependent rats.

MATERIALS AND METHODS

Subjects: We used 8 weeks old male Sprague Dawley rats (Charles River, $n = 124$) weighing 250–350 g before surgery and maintained the rats under a reverse 12:12 h light-dark cycle (lights on and off at 9 AM and 9 PM, respectively) with food and water available *ad libitum*. We performed the experiments in accordance to the "Guide for the care and use of laboratory animals" (8th edition, 2011, US National Research Council) and experimental protocols were approved by the University of Maryland Institutional Animal Care and Use Committee. We excluded rats because of failure of catheter patency ($n = 48$) or incorrect cannula placements ($n = 4$).

Surgery

We performed a single surgery to implant both intravenous catheters and intracranial cannulae.

Catheterization surgery: We anesthetized 9 week old rats with isoflurane (4.5% induction, 2-3% maintenance) and implanted catheters into the right jugular vein as described in Martin et al. (2020). Following surgery, we injected rats daily with 0.05 mL of anti-microbial, anti-bacterial, and anti-coagulant Taurolidine-Citrate (TCS) catheter lock solution i.v. (Cat# TCS-04, Access Technologies, IL, United States) to reduce biofilm and clot formation, to promote catheter patency, and to reduce the risk of microbial infection. We checked catheter patency occasionally by giving intravenous injections of 0.1 mL of methohexital sodium. Rats without a sudden loss of muscle tone were removed from the study.

Intracranial surgery: We implanted bilateral guide cannulae (23 gage; Plastics One) 1.0 mm above the BNST. We implanted cannulae at AP: −0.3 mm, ML: ±3.6 mm, DV: −6.2 mm (18°

angle) from Bregma and anchored the cannula to the skull using dental cement and jeweler's screws. We used the above coordinates based on a previous study (Pomrenze et al., 2019) and pilot experiments. After 1 week of recovery from surgery we started the self-administration training phase.

Intracranial and subcutaneous injections: We intracranially injected 0.3 μL/side of the selective CRF1 receptor antagonist R121919 [3-(6-(dimethylamino)-4-methyl-pyrid-3-yl)-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo(2,3-a)pyrimidin-7-amine], NBI 30775, a gift from Dr. Kenner Rice (Chemical Biology Research Branch, Drug Design and Synthesis Section, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Rockville, MD, United States) into the BNST 15 min before the beginning of the test session. We dissolved R121919 in 4% Kolliphor RH40 in aCSF, pH 5 (Pomrenze et al., 2019). We injected 4% Kolliphor RH40 in aCSF as vehicle (Day 1, Day 5, or Day 30 tests) or R121919 (Day 5 or Day 30 tests). We performed all intracranial injections over 1 min with injectors that extended 1 mm below the tips of guide cannulae. We left the intracranial injectors in place for an additional minute to allow for diffusion.

Apparatus: We trained rats in self-administration chambers housed in sound attenuating cabinets (Med Associates) containing two retractable levers (Active and Inactive) on the same wall located 10 cm above the chamber floor. We counterbalanced the position of the Active and Inactive Levers across rats.

Drugs: We purchased fentanyl citrate from Sigma Aldrich or Cayman Chemical and diluted it in 0.9% sterile saline at 12.5 or 1 mg/mL. We purchased remifentanil from Toronto Research Chemicals, morphine sulfate from Spectrum Chemical, and methohexital sodium from Parchem. We diluted remifentanil and morphine in 0.9% sterile saline at 12.5 and 50 mg/mL, respectively.

Self-administration training: We trained rats in daily sessions to self-administer fentanyl (for dose see Drugs section for detail) for 3 h per day under an FR1-20s timeout reinforcement schedule. Presses on the Active Lever resulted in activation of a syringe pump which delivered a 28 μL infusion of fentanyl over one second paired with a compound 5-s light (7.5 W white light located above the Active Lever) and a tone (2,900 Hz speaker located above the light) cue. A red houselight remained on during the entire session. Presses on the Inactive Lever were recorded but not reinforced.

Day 1, 5 or 30 extinction testing: We started the 90-min extinction tests 15 min after intracranial injections. For experiment 1, rats (n = 28) completed the extinction test on Day 1 and Day 30 while for experiment 2, rats completed the extinction test either on Day 5 only (n = 28) or Day 5 and Day 30 (n = 16). All extinction sessions began with extension of Active and Inactive Levers and illumination of red house light which remained on for the duration of the session. Presses on the Active Lever no longer resulted in drug infusions, but still resulted in contingent tone-light cue on the same FR1 20 s time out schedule of reinforcement used during training. Maintaining the same reinforcement schedule during testing enables us to examine patterns of drug seeking based on previously learned cue reinforcement contingencies in the absence of the drug, instead of newly acquired cue reinforcement contingencies in the absence of the drug. We recorded number of active presses for all experiments as well as the timestamp of each active press during day 5 test. Presses on the Inactive Lever were recorded but had no consequences.

Histology: After testing, we deeply anesthetized rats with isoflurane and transcardially perfused them with 100 mL of 0.1 M PBS followed by 400 mL of 4% paraformaldehyde (PFA) in dH_2O. We extracted the brains and post fixed them in 4% PFA for 2 h before we transferred them to 30% sucrose in PBS for at least 48 h at 4°C. We subsequently froze the brains and stored them in −20°C until sectioning. We sectioned the brains at 40 μm containing BNST on a cryostat (Leica Microsystems) and collected every third section through the cannula placement in a cryopreservant. Finally, we stained the sections with cresyl violet and coverslipped with Permount. We verified cannulae placements under a light microscope.

Statistical analysis: We organized the data in Excel and analyzed it using SPSS, Prism, and Matlab. We used mixed design repeated measures ANOVAs to analyze the self-administration training data, separately examining number of cues + infusions earned and lever press (Active and Inactive) data, using within- and/or between- subject factors of Session and Dependence Group as appropriate. For Experiment 1 incubation data, we separately examined cues earned and lever press data in mixed design repeated measures ANOVA using Withdrawal Day (Day 1, Day 30), Lever (Active, Inactive) and Day 30 Treatment (vehicle, R121919) as within- or between-subject factors as appropriate. For Experiment 2, Day 5 vs Day 30 protracted withdrawal test data we performed similar analyses on cues earned and lever data adding Dependence (dependent, non-dependent) as a factor. For Experiment 2, Day 5 acute withdrawal test data, we examined cues earned and lever data using Day 5 Treatment and Dependence with Lever added as appropriate. Further, for the day 5 test we collected active press timestamp data to conduct a press bout analysis. We defined a press bout as two or more presses for which the interval between successive presses in the bout did not exceed the time out interval of 20 s. We calculated number of bouts, presses per bout, bout duration (time from first to last press in a bout) and inter bout interval (time between last press in bout and first press in next bout).

Demand data analysis: We analyzed demand data as previously described (Martin et al., 2020). In brief, we used drug consumption for each bin as the primary dependent measure. We defined the price of the drug as the number of responses required to reach 20 μg/kg such that price units = number of responses/20 μg/kg. Unlike our previous study, we also included data from the first bin as we did not observe a "loading effect" with remifentanil. We averaged the consumption and price across adjacent 15-min bins, 1–2, 3–4, 5–6, 7–8, 9–10 resulting in five prices and five consumption values for each rat. We fitted the data in Matlab using the "fitnlm" function modeled with an exponential demand equation $Q = Q_0 \times 10^{k\left(e^{-\alpha Q_0 C_{-1}}\right)}$. Q_0

represents the theoretical consumption of drug at low prices when no effort is required, α is the measure of demand elasticity and is inversely related to motivation, Q is the consumption at a given C (price) during a particular bin, and k is the logarithmic range of consumption data. We constrained Q_0 values to three times the maximum consumption to reduce overestimation of Q_0 in sessions where responding falls quickly with increasing price. Constraining Q_0 resulted in insignificant decrease in R^2 values (average R^2 for constrained: 0.948 and unconstrained: 0.950). We used a k value derived from each session's consumption data as described in Martin et al. (2020) to maximize the quality of fit and to avoid systematic errors in α. We excluded three sessions due to low R^2 values ($R^2 < 0.25$) while calculating Q_0 and α from demand graphs. We compared Q_0, α, total Active Lever presses, and total consumption between days rats received intra-BNST vehicle or intra-BNST R121919. We performed paired t test to determine if above parameters were significantly different in drug injected day compared to vehicle injected day.

Experiment 1

After a week of recovery following surgery, we trained rats to self-administer fentanyl for 10 days as described above. For the first cohort of animals (n = 12) we trained rats to self-administer fentanyl (Sigma Aldrich) at 2.5 μg/kg/infusion. For the second cohort (n = 16), fentanyl was not available from our original source. Fentanyl sourced from Cayman Chemical visibly occluded rats from responding at 2.5 μg/kg/infusion. Thus, for cohort 2 we reduced the concentration of fentanyl (Cayman Chemical) to 2.0 μg/kg/infusion for days 3 and 4 of self-administration and to 1.5 μg/kg/infusion for the rest of the self-administration phase. Since the terminal levels of responding for both cohorts were not significantly different (see section "**Supplementary Results**"), we pooled the data together. During training, Active Lever presses resulted in fentanyl infusion paired with a compound 5s tone-light cue located above the Active Lever.

Incubation extinction tests: After 10 days of training, we injected vehicle intracranially into the BNST of all rats 15 minutes prior to Day 1 of forced abstinence extinction test. After 30 days of forced abstinence (Day 30), we retested the same rats and injected either intra-BNST vehicle in n = 13 rats or intra-BNST R121919 in n = 15 rats in a mixed within-between subject design.

Experiment 2

After a week of recovery following surgery, we trained rats to self-administer fentanyl similar to *Experiment 1* for 5 days before dependence induction. Similar to *Experiment 1*, n = 14 animals were trained in 1.5 μg/kg/infusion. The terminal levels for responding were not significantly different in this cohort as well (see section "**Supplementary Results**") so we pooled the data together.

Dependence phase and ongoing self-administration: After 5 days of self-administration, we gave rats twice daily injections (morning and evening; every 12 ± 2 h) of either saline (non-dependent) or ascending doses of morphine (dependent). The morphine dosing regimen was 10, 20, 30, 40, 50, 60, and 70 mg/kg subcutaneous injections for 7 days (Cooper et al., 2008). We recorded rats' baseline weight at the end of 5 days of self-administration and also recorded their weights before morphine injections twice daily. We averaged the twice daily weights (Days 1–7) and also recorded weights once daily during self-administration post-dependence (Days 8–17). We calculated change in body weight by subtracting their daily weights from their baseline weight. On the 7th day of dependence phase, we injected the dependent rats with 70 mg/kg morphine only in the morning and saline in the evening before resumption of daily fentanyl self-administration training the next day. We trained rats to self-administer fentanyl for 10 more days while they were maintained on once daily saline or morphine (40 mg/kg s.c) injection after the end of each self-administration session. In an attempt to reduce the confound of differences in fentanyl consumption and in cue exposure for dependent and non-dependent rats, we established infusion maximum (I_{max}), the maximum number of infusions per session, during the post-dependence ongoing self-administration sessions. Once the I_{max} was met, the program ended, and rats were immediately removed from the self-administration chambers. The infusion maximum for the post-dependence self-administration sessions were I_{max} 35 for self-administration sessions 6 and 7, I_{max} 45 for sessions 8 and 9, I_{max} 55 for sessions 10 and 11, I_{max} 65 for sessions 12 and 13, and I_{max} 75 for sessions 14 and 15. The last post-dependence self-administration session coincided with last homecage morphine or saline injection. For data analysis, we assigned a value of 180 (the total length of the session) as the latency to reach I_{max} for rats that did not reach the I_{max}.

Dependence measures: 24 h after session 15 of post-dependence self-administration, we video recorded rats for 20 min in an operant chamber devoid of levers and cues. We video scored the number of wet-dog shakes; a single wet-dog shake is defined as a rapid bout of alternating head and body shaking lasting less than 2 s, which is a reliable measure of opioid withdrawal symptom (Lee et al., 1989). Two experimenters were blind to the dependence assignment and independently scored a subset (32%) of the same videos to ensure consistency and accuracy. Each individual's video scoring was significantly correlated ($r^2 = 0.92$, $p < 0.001$).

Acute and Protracted extinction tests: Because the dependence procedures we used result in acute withdrawal symptoms (Cooper et al., 2008), we performed the acute test 5 days after the last post-dependence self-administration session/last homecage morphine or saline injection. For the *Acute Withdrawal (Day 5 test)*, we gave one subset of rats intra-BNST vehicle injections (n = 14) or intra-BNST R121919 injections (n = 14) prior to extinction test as described above. We compared their extinction responding using a between-subject design. A subset of these rats was then tested in remifentanil demand threshold procedure outlined below. For the *Protracted withdrawal (Day 5 vs Day 30 test)*, we gave a different subset of rats (n = 16) intra-BNST vehicle injections on Day 5 and retested half with intra-BNST vehicle injections (n = 8) and half with intra-BNST R121919 injections (n = 8) prior to extinction test as described above.

Demand thresholding training and testing: Following their Day 5 extinction test, we trained the rats (n = 12) in the

acute withdrawal group from *Experiment 2* to self-administer a short-acting μ-opioid receptor agonist, remifentanil, in a within-session demand thresholding procedure. The drug infusion was paired with the light/tone compound cue on an FR1 schedule. Across the demand thresholding, the duration of each remifentanil infusion and cue was decreased every 15 min on a quarter log scale. Each session tested 10 doses of remifentanil (20, 11.2, 6.4, 3.6, 2, 1.1, 0.64, 0.36, 0.2, 0.1 μg/kg/infusion). The demand thresholding procedure lasted 150 min. A red house light was on for the entire duration of the session, except during the length of an infusion. To establish a baseline, we trained rats in six daily demand thresholding sessions before starting the test sessions. After baseline sessions, we gave within-subject subcutaneous injections of R121919 (10 mg/kg, 2 mL/kg/infusion) or vehicle 1 h before two counterbalanced demand thresholding test sessions (data not shown). Following subcutaneous injection, we injected (n = 10 rats) R121919 or vehicle intra-BNST, using the same dose and volume as described previously, 15 min before two counterbalanced demand thresholding sessions.

RESULTS

Experiment 1: Incubation of Fentanyl Craving

Self-administration training: We trained rats 3 h/day for 10 days in fentanyl self-administration. Experimental timeline is shown in **Figure 1A** and infusions + cues earned data in **Figure 1B**. We observed main effect of Session (1–10) on infusions + cues earned ($F_{9,243} = 6.83$, $p < 0.001$), demonstrating rats consumed more fentanyl and were exposed to more cue pairings as the sessions progressed (**Figure 1B**). Lever press data are shown in **Figure 1C**, with rats increasing their Active Lever presses over sessions and discriminating the fentanyl-paired Active Lever from the Inactive Lever, with main effects of Lever ($F_{1,27} = 19.26$, $p < 0.001$) and Session ($F_{9,243} = 6.87$, $p < 0.001$), and a Lever × Session interaction ($F_{9,243} = 4.88$, $p < 0.001$).

Incubation testing: To examine the involvement of BNST CRFR1 signaling in incubation of opioid craving, we gave all rats intra-BNST vehicle injections on Day 1, while on Day 30 we gave approximately half the rats intra-BNST vehicle and the other half intra-BNST R121919. During tests, there are no infusions earned, only cues earned on the same schedule of reinforcement as acquired during self-administration training. Cues earned data are shown in **Figure 1D**. We observed a main effect of Withdrawal Day ($F_{1,27} = 15.51$, $p = 0.001$) indicating rats earned more cues on Day 30 relative to Day 1, consistent with an incubation effect. We also observed a Withdrawal Day × Day 30 Treatment interaction ($F_{1,27} = 9.50$, $p = 0.005$). *Post hoc* analysis of cues earned revealed that intra-BNST vehicle treated rats earned more cues on Day 30 compared to Day 1 ($t_{12} = -4.93$, $p < 0.001$) while intra-BNST R121919 treated rats did not ($t_{14} = -0.62$, $p > 0.05$), suggesting incubation is prevented by treatment with the CRFR1 antagonist in the BNST.

Lever press data are shown in **Figure 1E**. We observed main effects of Lever ($F_{1,26} = 35.46$, $p < 0.001$) and Withdrawal Day ($F_{1,26} = 7.29$, $p = 0.012$) suggesting that while overall rats discriminated the Active Lever from Inactive Lever, they pressed more on withdrawal Day 30 for both Active and Inactive levers compared to Day 1. There was also a Withdrawal Day × Day 30 Treatment interaction ($F_{1,26} = 5.21$, $p = 0.031$) but no other main effects or other interactions (F's < 1.4, p's > 0.05). Because we only gave treatment on Withdrawal Day 30, we analyzed the timecourse of lever responding (and cues earned; see section "**Supplementary Material**") on Day 30 using within-subject factors of Lever (Active and Inactive) and Bin (30, 60, and 90 min) and between-subject factor of Day 30 Treatment (vehicle, R121919) (**Figure 1F**). This revealed main effects of Lever ($F_{1,26} = 46.21$, $p < 0.001$), Bin ($F_{2,52} = 10.52$, $p = 0.002$) and a Lever × Bin × Treatment interaction ($F_{2,52} = 5.40$, $p = 0.007$), indicating the effect of treatment across bins, varies by lever. An analysis of Active Lever data revealed main effects of Bin ($F_{2,52} = 6.92$, $p = 0.002$) and Day 30 Treatment ($F_{1,26} = 4.30$, $p = 0.049$) but the interaction did not reach significance ($F_{2,52} = 2.49$, $p = 0.093$). These data suggest, relative to vehicle, intra-BNST R121919 treatment attenuates Active Lever pressing across the entire Day 30 test session. While the Bin × Day 30 treatment interaction was only trending toward significance, qualitatively, it is notable that the greatest difference between R121919 and vehicle treatment is observed in the final bin of the Day 30 test session. We confirmed active responding timecourse did not differ for prospective Day 30 treatment groups by examining the time course of Active Lever responding on Day 1 test (**Supplementary Figure 1A**), which showed main effect of Bin ($F_{2,52} = 13.03$, $p < 0.001$) but no other main effects or interactions (F's < 0.26, p's > 0.05). While analysis of Day 30 Inactive Lever data showed a Bin × Treatment interaction ($F_{2,52} = 4.65$, $p = 0.014$), the difference in first bin responding did not reach significance between treatment groups (*post hoc t* test $p > 0.05$). Altogether these data suggest the effect of intra-BNST R121919 was specific to Active Lever responding on Day 30 and was not due to differences between groups on Day 1.

Experiment 2: Incubation of Fentanyl Craving in Opioid Dependence

Experimental timeline is shown in **Figure 2A**. Briefly, we trained rats 3 h/day for 5 days in fentanyl self-administration before giving twice daily morphine/saline injections for 7 days. We then resumed daily self-administration training for 10 more days with once daily morphine/saline injection.

Dependence measures: To investigate the effectiveness of the morphine regimen to induce dependence in our rats, we compared somatic signs of opioid withdrawal between opioid-dependent and non-dependent rats. We examined change in body weight across training and dependence (**Figure 2B**) and wet dog shakes 24 h after their last self-administration training and morphine/saline injections (**Figure 2C**). While non-dependent rats showed weight gain, dependent rats lost weight across training during the dependence phase (**Figure 2B**). Repeated measures ANOVA on body weight including within-subject factor of Day and between-subject factor of Dependence yielded

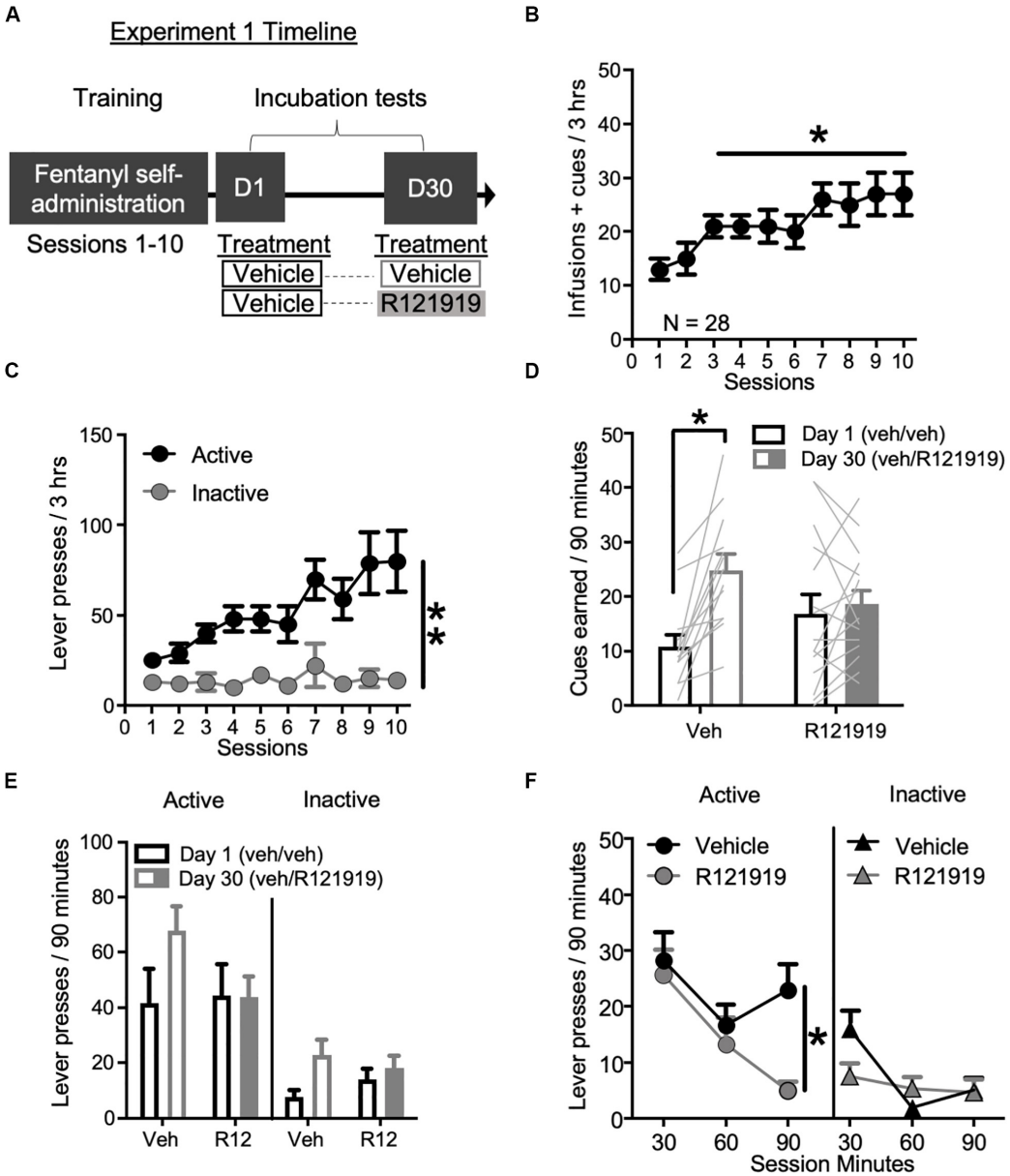

FIGURE 1 | Experiment 1: BNST CRFR1 antagonist effect on incubation of fentanyl craving. **(A)** Experimental timeline: we trained rats to self-administer fentanyl for 3 h/day for 10 days. On forced abstinence Day 1 and Day 30, we measured lever responding under extinction conditions. **(B)** Fentanyl infusions + cues earned across the 10 daily self-administration sessions. **(C)** Rats discriminated Active from Inactive Lever during self-administration training. **(D,E)** Incubation test data showing cues earned on FR1, 20 s TO schedule **(D)** and Active/Inactive Lever pressing **(E)** on Day 1 and Day 30 of forced abstinence for vehicle (open bars) treated and R121919 treated (filled bars) conditions. **(F)** Time course of Active and Inactive Lever presses on Day 30 test for both treatment groups. $^{*}p < 0.05$, $^{**}p < 0.01$, different from Session 1 in **(B)**, Lever main effect in **(C)**, different from Day 1 in **(D)**, and Treatment main effect in **(F)**. R12 = R121919. $n = 13$ Veh, $n = 15$ R12. Data are mean ± SEM.

main effects of Day ($F_{16,464} = 23.56$, $p < 0.001$) and Dependence ($F_{1,29} = 51.43$, $p < 0.001$) as well as a Session × Dependence interaction ($F_{16,464} = 82.66$, $p < 0.001$). In addition, dependent rats also displayed more wet-dog shakes, 24 h after the last morphine injection, compared to non-dependent rats (**Figure 2C**; independent samples t-test $t_{32} = 3.93$, $p < 0.001$).

Self-administration training: We analyzed the training data using mixed ANOVAs including within-subject factor of Session (either Sessions 1–5; before dependence phase, or Sessions 6–15; during dependence phase) and between-subject factor of Dependence (dependent, non-dependent). The self-administration infusion + cues earned data is shown in **Figure 2D**; we observed a main effect of Session for both pre-dependence sessions 1–5 ($F_{4,176} = 12.20$, $p < 0.001$) and during dependence phase sessions 6–15 ($F_{9,396} = 14.01$, $p < 0.001$) but no Session × Dependence interactions for either Sessions

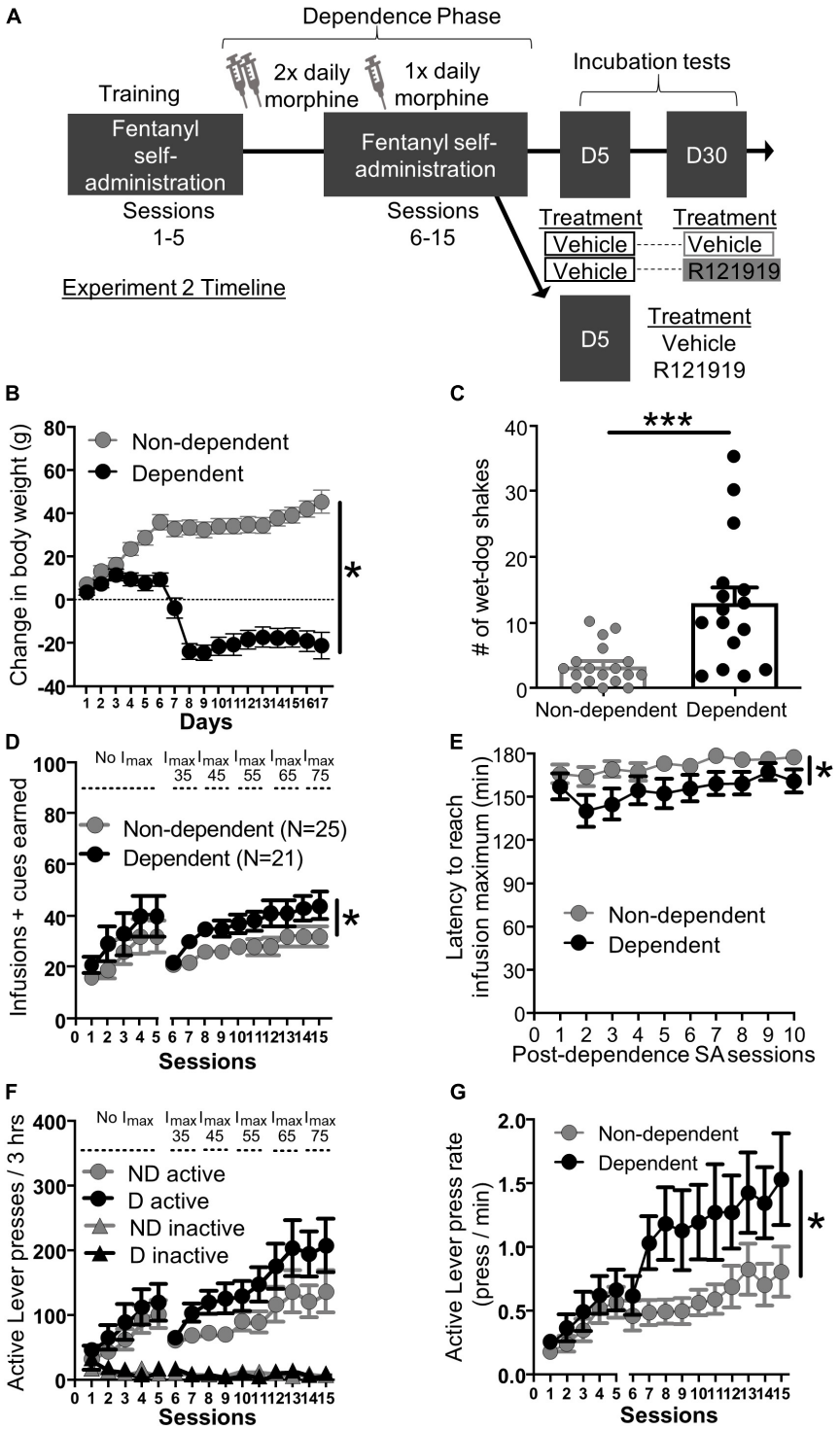

FIGURE 2 | Experiment 2: Fentanyl self-administration training in opioid dependent versus non-dependent rats. **(A)** Experimental timeline: We trained rats to self-administer fentanyl for 5 days before inducing opioid dependence. Rats resumed self-administration for 10 more days while dependence was maintained. On forced abstinence Day 5 and Day 30, we measured lever responding under extinction conditions. **(B)** Change in body weight across days for opioid-dependent and non-dependent rats after start of dependence phase. **(C)** Number of wet-dog shakes for opioid-dependent and non-dependent rats. **(D)** Fentanyl infusions + cues earned across the 15 3 h self-administration sessions, sessions 1–5: before dependence and no infusion maximum criteria, 6–15: during dependence with infusion maximum criteria. **(E)** Latency to reach infusion maximum criteria in minutes. **(F)** Active/Inactive Lever pressing for dependent and non-dependent rats during training. **(G)** Active Lever press rate. $^{*}p < 0.05$, main effect of dependence, $^{***}p < 0.001$, different from non-dependent. SA, Self-administration; ND, non-dependent; D, dependent. Data are mean ± SEM.

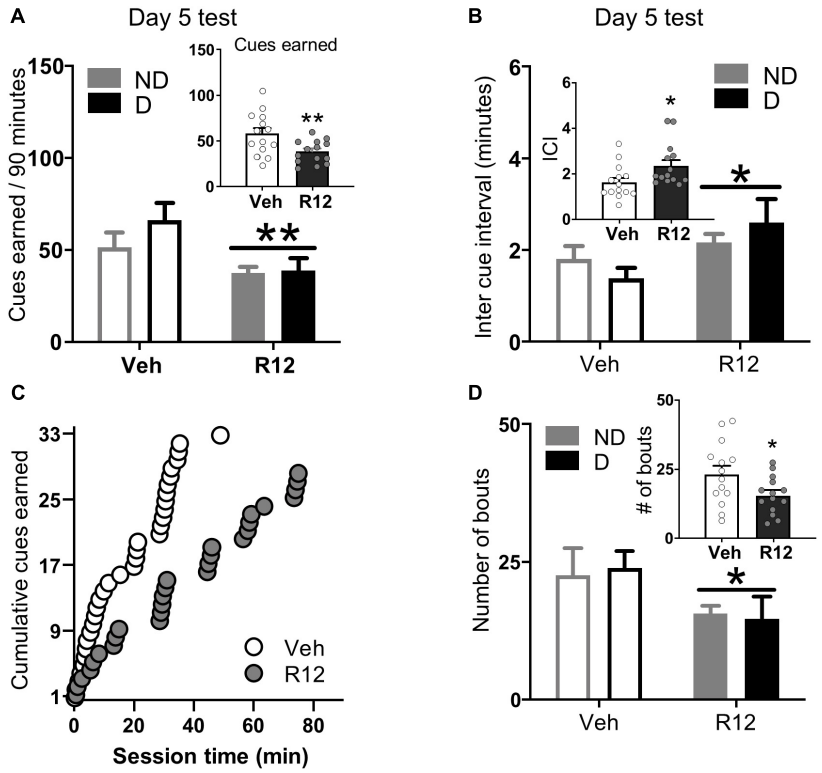

FIGURE 3 | Experiment 2: BNST CRFR1 antagonist effect on fentanyl seeking during acute withdrawal (Day 5) test. **(A)** Day 5 incubation test data showing cues earned on FR1, 20 s to schedule. **(B)** Inter cue interval (ICI), the time between presses that result in cues. **(C)** Cumulative cues earned pattern of a representative pair of vehicle ($\bar{X}$ = 1.52) and R121919 ($\bar{X}$ = 2.75) injected rat. **(D)** Number of bouts. A bout is defined as two or more presses for which the interval between successive presses did not exceed 20 s. *$p < 0.05$, **$p < 0.01$, main effect of treatment. All the inset graphs indicate mean ± SEM when collapsed across dependence. ND, non-dependent; D, dependent. Data are mean ± SEM.

1–5 and 6–15 ($F_{4,176}$ = 0.19, $p > 0.05$ and $F_{9,396}$ = 1.34, $p > 0.05$ respectively). As described in section "Materials and Methods," we set an infusion maximum (I_{max}) for self-administration Sessions 6 through 10 in order to limit differences in fentanyl consumption/cue exposure between dependent and non-dependent rats. Despite this, we still observed a main effect of Dependence on infusions + cues earned during dependence phase Sessions 6–15 ($F_{1,44}$ = 4.80, p = 0.034) but not pre-dependence phase (Sessions 1–5: $F_{1,44}$ = 1.10, $p > 0.05$). Dependent rats reached I_{max} faster than non-dependent rats (**Figure 2E**). Mixed ANOVA on latency to reach I_{max} data including within-subject factor of Session and between-subject factor of Dependence revealed main effect of Session ($F_{9,396}$ = 3.73, $p < 0.001$) and Dependence ($F_{1,44}$ = 5.03, p = 0.030) but no interaction ($F_{9,396}$ = 0.81, $p > 0.05$). Similar to infusions + cues earned, all rats increased their Active Lever responding over sessions and discriminated fentanyl-paired Active Lever from Inactive Lever (**Figure 2F**). For pre-dependence phase we ran a repeated measures ANOVA including within-subject factors of Lever (Active and Inactive) and Sessions (1–5) and between-subject factor of Dependence. The ANOVA yielded main effects of Lever ($F_{1,44}$ = 28.00, $p < 0.001$), Session ($F_{4,176}$ = 13.23, $p < 0.001$), and a Lever × Session interaction ($F_{4,45}$ = 13.00, $p < 0.001$), but no other interactions (F's < 0.42, p's > 0.05). For the dependence phase self-administration sessions 6–15 we ran a repeated measures ANOVA with Lever (Active and Inactive) and Session (6–15) as within-subject factors and Dependence as between-subject factors. This resulted in a main effect of Session ($F_{9,396}$ = 11.62, $p < 0.001$), Lever ($F_{1,44}$ = 62.52, $p < 0.001$), and a significant Lever × Session interaction ($F_{9,396}$ = 11.82, $p < 0.001$) but no other interactions (F's < 2.20, p's > 0.05). While there was no main effect of Dependence on lever presses during dependence phase self-administration sessions (Sessions 6–15: $F_{1,44}$ = 2.69, $p > 0.05$), dependent rats pressed the fentanyl-paired Active Lever more vigorously compared to non-dependent rats (**Figure 2G**). Active Lever press rate $\left(\frac{Active\ Lever\ presses}{Latency\ to\ reach\ I_{max} - Latency\ to\ first\ press}\right)$ from Sessions 1–5 to 6–15 was analyzed using mixed ANOVA with Session as a within-subject factor and Dependence as a between-subject factor. We found a main effect of Session (1–5: $F_{4,176}$ = 18.30, $p < 0.001$, 6–15: $F_{9,396}$ = 4.01, $p < 0.001$), and a main effect of Dependence on Sessions 6–15 ($F_{1,44}$ = 5.29, p = 0.026), but not on Session 1–5 ($F_{1,44}$ = 0.62, $p > 0.05$) and no Session × Dependence interactions during either phase (F's < 0.7, p's > 0.5).

Testing: After the dependence phase of self-administration, we split the rats into two groups: (1) a protracted withdrawal day treatment group tested both on Day 5 (vehicle/vehicle) and Day 30 (vehicle/R121919) to examine the effects of CRFR1 antagonism on fentanyl seeking during incubation and (2) an acute withdrawal day treatment group tested only on Day 5 (vehicle/R121919) to examine the effects of CRFR1 antagonism on fentanyl seeking during acute withdrawal.

Protracted withdrawal (Day 5 vs Day 30 test): All (dependent and non-dependent) rats received intra-BNST vehicle injections on Day 5, while half from each group received intra-BNST vehicle and the other half intra-BNST R121919 on Day 30. To our surprise, we did not observe the expected time-dependent increase, or incubation, for cues earned between Day 5 and Day 30. Cues earned data are shown in **Supplementary Figure 2A**. Instead, we observed the opposite, a reduction in number of cues earned on Day 30 relative to Day 5 across dependence and treatment groups. The ANOVA yielded a main effect of withdrawal day ($F_{1,12} = 8.80$, $p = 0.012$) indicating reduction in cues earned between Day 5 and day 30. This could be driven, in part by treatment effects on day 30, but because the number of subjects per group was low, we were unable to detect differences between Dependence groups or notable effects of Day 30 Treatment on the low levels of responding present on Day 30 (Dependence, Day 30 Treatment main effects and interactions all F's < 3.12, p's > 0.05). Despite this, we observed large effect sizes for cues earned between Day 5 and Day 30 tests (Cohen's $d \geq 1.28$) for rats treated with intra-BNST R121219 on Day 30, but only small effect sizes for rats treated with intra-BNST vehicle on Day 30 (Cohen's d 0–0.45), see section "**Supplementary Results**" for more detail. Together, this suggests that despite reduced responding on Day 30, CRFR1 antagonist likely acts to decrease cues earned during protracted withdrawal independent of dependence condition.

Acute Withdrawal (Day 5 test): To understand the role of BNST CRFR1 signaling for driving fentanyl seeking after acute withdrawal, we gave approximately half of the rats from each dependence group intra-BNST vehicle and the other half intra-BNST R121919 on Day 5. Cues earned data for acute withdrawal Day 5 are shown in **Figure 3A**. Rats injected with intra-BNST R121919 earned fewer cues on Day 5 test compared to vehicle injected rats (**Figure 3A**). The ANOVA on cues earned resulted in a main effect of Day 5 Treatment ($F_{1,24} = 8.36$, $p = 0.008$), but no main effect of Dependence ($F_{1,24} = 1.28$, $p > 0.05$) or a Day 5 Treatment × Dependence interaction ($F_{1,24} = 0.89$, $p > 0.05$). When we analyzed the pattern of pressing resulting in cue delivery, we found that more time elapsed between cue presentations for rats injected with intra-BNST R121919 compared to the intra-BNST vehicle injected rats (**Figure 3B**). An ANOVA on average inter cue interval (ICI) data revealed a main effect of Day 5 Treatment ($F_{1,24} = 6.18$, $p = 0.020$) but no main effect of Dependence or a Day 5 Treatment × Dependence interaction (F's < 2.40, $p > 0.05$). **Figure 3C** shows cumulative cues earned during a representative session from an intra-BNST vehicle and an R121919 injected rat. Qualitatively, this exemplifies the longer time elapsed between reinforced presses in R121919 relative to vehicle treated rats.

We performed an analysis of lever press bouts to determine whether the CRFR1 antagonist affected the way in which rats engaged in drug seeking during the test session. Compared to vehicle treated rats, intra-BNST R121919 treatment decreased the number of press bouts (**Figure 3D**). The ANOVA on number of press bouts resulted in a main effect of Day 5 Treatment ($F_{1,24} = 4.70$, $p = 0.040$), but no other main effects or interactions F's < 0.10, p's > 0.05. The ANOVA on inter-bout-interval data resulted in a marginally significant main effect of Day 5 Treatment ($F_{1,24} = 4.01$, $p = 0.054$; **Supplementary Figure 3C**) but no other main effects or interactions (F's < 1.94, p's > 0.05). R121919 treatment did not affect bout size or bout duration (**Supplementary Figures 3A,B**; F's < 1.77, p's > 0.5). The overall lever press, press vigor, and time out response data followed similar patterns (**Supplementary Material** and **Supplementary Figures 3D–F**). Altogether, these data show BNST CRFR1 antagonism limits rats from re-engaging in opioid seeking during test, resulting in less fentanyl-associated conditioned reinforcement.

Opioid demand: Finally, to confirm intra-BNST R121919 did not affect opioid consumption or ability to lever press we trained the acute withdrawal rats to self-administer short acting opioid, remifentanil in opioid demand threshold procedure. We compared remifentanil consumption at low cost (Q_0), demand elasticity (α), total remifentanil consumption, total Active Lever presses, and Active Lever presses in the last bin when the rats were injected with intra-BNST vehicle versus R121919. We found no significant difference in any of these measures between vehicle treated or R121919 treated conditions suggesting that intra-BNST R121919 injection doesn't preclude animals from lever pressing at high levels (∼400 presses/session) or consuming opioid drugs of abuse (see section "**Supplementary Results**" and **Supplementary Figure 4**).

Histological verification: Cannula placements for Experiments 1 and 2 are shown in **Figure 4A**. A majority of the placements were observed on the anterior to posterior axis between Bregma and −0.24 to Bregma, as summarized in **Figure 4A** and in representative image **Figure 4B**.

DISCUSSION

Here, we found BNST CRFR1 receptor antagonist reduced incubation of fentanyl craving after a month of forced abstinence. While control rats display incubated cue-reinforced fentanyl seeking after protracted withdrawal, rats injected with the CRFR1 antagonist R121919 did not display this time dependent increase in cue-reinforced opioid seeking. Next, we sought to determine whether opioid dependence enhanced incubation and determine the role of BNST CRFR1 signaling in opioid seeking. Again, R121919 attenuated cue-reinforced opioid seeking after an acute period of withdrawal in both dependent and non-dependent rats. Closer analysis revealed CRFR1 receptor antagonist acts to prevent re-engagement with the cue-reinforced lever pressing after periods of disengagement during test. Together, these results suggest a critical role of BNST CRFR1 receptors in cue reinforced opioid seeking after

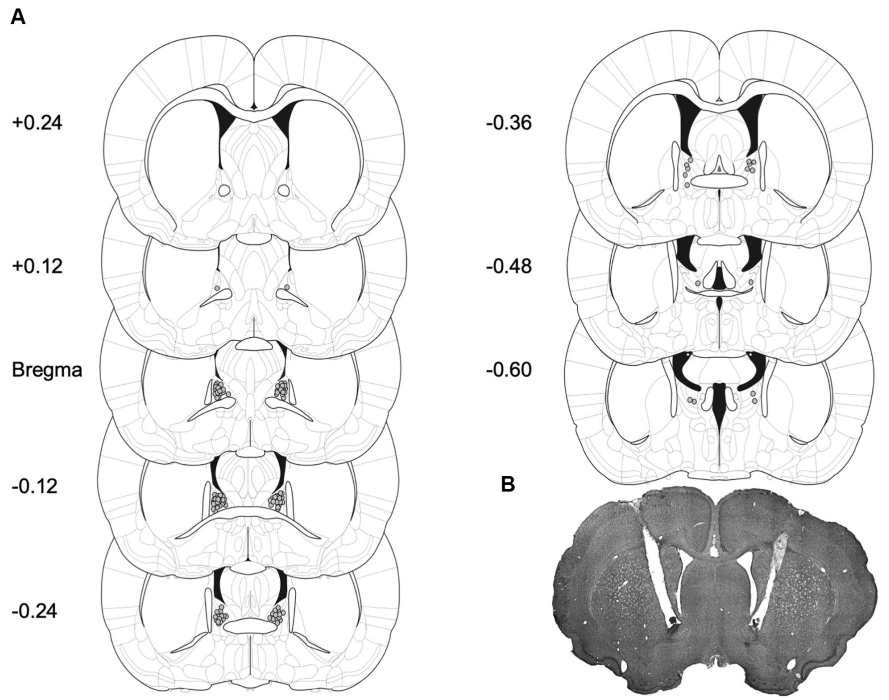

FIGURE 4 | Placement for injector tips in rats with BNST cannulae implants. **(A)** Gray dots represent sites of R121919 infusions on coronal sections as a distance from Bregma (mm). Drawings are adapted from Paxinos and Watson (2007). **(B)** A representative brain section depicting cannulae placement in the BNST.

periods of forced abstinence and extend previous findings on the important role of CRFR system in opioid addiction (Contarino and Papaleo, 2005; Papaleo et al., 2007; Greenwell et al., 2009; Logrip et al., 2011; Roberto et al., 2017; Reiner et al., 2019).

Methodological Considerations

In the present study, we used the same schedule of reinforcement for training and test, maintaining the 20 s timeout period between cue-reinforcement at test. Maintaining the same reinforcement schedule during testing enabled us to examine patterns of drug seeking based on previously learned cue reinforcement contingencies in the absence of the drug, instead of newly acquired cue reinforcement contingencies. Further, it provided us with a deeper understanding of the role of BNST CRFR1 receptors, as R121919 treatment increased the interval between reinforced presses and reduced the amount of fentanyl-associated conditioned reinforcement during test.

Here, we trained rats daily for 3 h to self-administer fentanyl, different from many incubation studies that train rats for 6 h or more (Reiner et al., 2019; Grimm, 2020). Even with limited training, we observed escalation of drug intake in both experiments (**Figures 1**, **2**). Moreover, we used a mixed within/between-subject design to compare responding on Day 1 versus Day 30 (within factor) in control and treated (between factor) rats, as opposed to a completely between subject design. We observed both incubation and treatment effects, demonstrating incubation of craving studies are accessible using limited access self-administration and mixed experimental design.

Because we did not infuse R121919 intra-BNST on Day 1 we cannot rule out the possibility that R121919 may have affected responding for cues prior to incubation. We don't expect this is the case because we found no difference in responding between vehicle and R121919 treatment in the last bin of demand sessions when drug dose is extremely low and non-reinforcing (**Supplementary Figure 4E**). Notably, in this last bin of remifentanil demand, any prior infusions have quickly cleared from the system and similar to Day 1 tests, cues alone are what reinforces responding during this time bin. While this is not an optimal representation of Day 1 responding, we tentatively conclude that in a non-incubated state, intra-BNST infusion of R121919 doesn't affect rats' lever pressing for cues. Future experiments with Day 1 infusion of intra-BNST R121919 are necessary to confirm this conjecture.

An unexpected finding using the mixed within/between design in Experiment 2 was that rats responded less on Day 30 compared to Day 5. This could be due to several factors. First, rats responded at high levels during the Day 5 test (more than two times higher than Experiment 1 Day 1 test), leading to more extinction experience on Day 5. This may have resulted in enhanced extinction learning that interfered with incubation and reduced responding on Day 30. Another possibility is that by Day 5, incubation of fentanyl craving might have had already occurred resulting in the high level responding we observed in the first test. Indeed, there is evidence for incubation of opioid craving as early as 6 days (Shalev

et al., 2001). Incubation data classically follow an inverted U-shaped curve, with low responding on day 1, maximal responding occurring between 6 and 30 days that returns to day 1 levels after 2 months of withdrawal from heroin (Shalev et al., 2001; Pickens et al., 2011). Finally, in experiment 2, we incorporated a dependence phase and extended self-administration experience well beyond the typical ten days of opioid experience. These factors could have influenced the level of responding we observed on Day 30, which could, in part, be due to testing on the backside of the incubation curve. Lack of incubation for discrete cues has been observed for other reinforcers, including alcohol seeking after abstinence (Jupp et al., 2011) and for context induced incubation of methamphetamine seeking (Adhikary et al., 2017). While we failed to observe incubation for discrete fentanyl associated cues in Experiment 2, the evidence for incubation on Experiment 1 suggests that the lack of incubation in Experiment 2 is a procedural issue and not lack of incubation of opioid craving which is well established pre-clinically (Reiner et al., 2019).

Relative to control manipulations, we observed substantially lower levels of cue-reinforced responding with BNST CRFR1 antagonism on Day 30, suggesting a role for this system in driving opioid seeking after protracted withdrawal. Despite this, future studies are needed to confirm whether BNST CRFR1 signaling is involved in protracted withdrawal after opioid dependence. An important consideration for future studies is to strike a balance between testing early enough in acute withdrawal to capture the front end of the incubation curve, while still avoiding the most severe opioid withdrawal symptoms that could interfere with lever pressing.

BNST CRFR1 Receptors in Relapse and Dependence

Several studies have implicated CRF action in the BNST in drug addiction related behaviors. Intra-BNST CRF antagonists block stress-induced reinstatement of morphine conditioned place preference (Wang et al., 2006) and cocaine reinstatement following foot shock (Erb and Stewart, 1999), whereas intra-BNST CRF infusions promote stress induced cocaine reinstatement (Erb and Stewart, 1999; Mantsch et al., 2016). The CeA is one source for CRF in the BNST that is heavily implicated in addiction related behaviors (Mantsch et al., 2016). A study using pharmacological inactivation of the CRF pathway asymmetrically inactivating the CeA and antagonizing BNST CRF receptors showed that this pathway drives footshock induced reinstatement of cocaine seeking (Erb et al., 2001). More recently, optogenetic silencing of the CeA → BNST CRF circuitry reduced alcohol seeking only in alcohol-dependent rats (de Guglielmo et al., 2019). This study went on to demonstrate that BNST CRFR1 is the primary site of action for this effect. While there is evidence that BNST neurons can synthesize CRF locally within the BNST (Shepard et al., 2006), it is not clear whether local action of BNST synthesized CRF plays a role in addiction related behaviors. We predict that the effect of BNST CRFR1 antagonism on cue reinforced fentanyl seeking observed here is likely due to blocking the action of CRF released from CeA terminals. Future opioid incubation studies using projection specific manipulations to target CeA → BNST CRF transmission could test this prediction.

The BNST CRFR1 system is previously implicated in opioid withdrawal (Nakagawa et al., 2005; Greenwell et al., 2009; Luster et al., 2020). In the present study, we investigated the role of BNST CRFR1 in driving the time dependent increase in cue induced opioid seeking, attempting to understand how opioid dependence and acute withdrawal states influence subsequent self-administration and incubation of opioid craving. During self-administration we observed that dependent rats consumed more fentanyl and were exposed to more fentanyl-associated cues than non-dependent rats. Despite this, we did not see a difference in opioid seeking between dependent and non-dependent rats during acute or protracted withdrawal tests. Further, the CRFR1 receptor antagonist, R121919, in the BNST did not differentially affect the behavior of dependent and non-dependent rats during these tests. Systemic injections of R121919 dose dependently decrease heroin seeking in heroin-dependent rats (Greenwell et al., 2009). In that study rats were injected systemically with R121919 while still having access to opioids. In contrast, in our study we injected rats with R121919 intra-BNST when rats had no access to fentanyl but only fentanyl associated cues. R121919 attenuated cue-reinforced presses both in the protracted withdrawal incubation test and in the acute withdrawal test for both dependent and non-dependent rats. Notably, our tests were conducted in rats with a history of dependence, tested after the dependence phase had ended, which may have limited our ability to detect differences in treatment effects between dependence groups. When we trained rats in a within session economic demand paradigm to self-administer remifentanil, we found no difference between intra-BNST vehicle or R121919 treated conditions for remifentanil consumption at low price, total consumption, or in total Active Lever presses. This suggests that BNST CRFR1 receptors are uniquely involved in cue-reinforced opioid seeking after forced abstinence, but not in ongoing opioid consumption.

Further, intra-BNST R121919 increased the interval between reinforced presses, suggesting the antagonist promotes disengagement from opioid seeking. There could be several possibilities by which R121919 could be limiting engagement in cue-reinforced opioid seeking. Mechanistically, CRF and CRFR1 receptor expression is altered in the amygdala and extended amygdala after chronic drug use and withdrawal (Maj et al., 2003; Sommer et al., 2008; Roberto et al., 2017). We speculate that R121919 could be attenuating the enhanced transmission at BNST CRFR1 receptors to promote disengagement from opioid seeking. Psychologically, CRF action promotes aversive states that motivate drug-seeking behaviors (Roberto et al., 2017). If we had observed stronger effects of CRFR1 antagonism in dependent rats we may have speculated that R121919 alleviated conditioned aversive states, however, we did not see evidence for this. Instead we expect intra-BNST R121919 acts to diminish the incentive properties of fentanyl associated cues to promote disengagement from opioid seeking. Future experiments are

needed to disentangle these psychological constructs and identify the precise underlying mechanisms.

Limitations and Conclusion

Extrahypothalamic CRFR1 system antagonism has been shown to exacerbate somatic signs of spontaneous opioid withdrawal (Papaleo et al., 2007) while it attenuates somatic signs of naltrexone-induced precipitated opioid withdrawal (Iredale et al., 2000). Here, we did not determine if intra-BNST R121919 exacerbates or attenuates somatic signs of opioid withdrawal. This could be of interest in future studies. In addition, we used only male rats in this study which is an important limitation as it has been shown that a subpopulation of females is more sensitive to CRF-induced reinstatement of cocaine seeking compared to males (Buffalari et al., 2012). While there is limited evidence for sex differences in incubation of fentanyl seeking (Reiner et al., 2020), evidence for BNST-mediated sex differences in opioid withdrawal (Luster et al., 2020) encourage future studies investigating sex differences in BNST CRFR1 action. We used a single dose of R121919 (1 μg/hemisphere) based on a prior study (Pomrenze et al., 2019) to test the role of BNST CRFR1 receptors in fentanyl craving. There is evidence for CRFR1 antagonist reducing frustration stress-induced binge like palatable food consumption with dose as low as 25 ng in the BNST (Micioni et al., 2014). Future studies with multiple doses of R121919 are needed to test if lower doses of the antagonist are sufficient to reduce incubation of opioid craving. Our study bolsters previous findings on the importance of CRFR1 in addiction related behaviors. We provide evidence of the importance of BNST CRFR1 signaling for driving cue-reinforced opioid seeking after periods of forced abstinence and extend our understanding of this system in driving specific facets of opioid incubation.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Maryland Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

DC and UG conceived the project, designed the experiments, interpreted the data, and wrote the manuscript. UG and DM acquired the data and analyzed the data. All authors contributed to manuscript revision, read, 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/fnbeh.2020.00153/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Selective Knockout of the *Vesicular Monoamine Transporter 2* (*Vmat2*) Gene in Calbindin2/Calretinin-Positive Neurons Results in Profound Changes in Behavior and Response to Drugs of Abuse

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The vesicular monoamine transporter 2 (VMAT2) has a range of functions in the central nervous system, from sequestering toxins to providing conditions for the quantal release of monoaminergic neurotransmitters. Monoamine signaling regulates diverse functions from arousal to mood, movement, and motivation, and dysregulation of VMAT2 function is implicated in various neuropsychiatric diseases. While all monoamine-releasing neurons express the *Vmat2* gene, only a subset is positive for the calcium-binding protein Calbindin 2 (Calb2; aka Calretinin, 29 kDa Calbindin). We recently showed that about half of the dopamine neurons in the mouse midbrain are positive for Calb2 and that Calb2 is an early developmental marker of midbrain dopamine cells. Calb2-positive neurons have also been identified in other monoaminergic areas, yet the role of Calb2-positive monoaminergic neurons is poorly understood. To selectively address the impact of Calb2-positive monoaminergic neurons in behavioral regulation, we took advantage of the Cre-LoxP system to create a new conditional knockout (cKO) mouse line in which *Vmat2* expression is deleted selectively in Calb2-Cre-positive neurons. In this $Vmat2^{lox/lox;Calb2-Cre}$ cKO mouse line, gene targeting of *Vmat2* was observed in several distinct monoaminergic areas. By comparing control and cKO mice in a series of behavioral tests, specific dissimilarities were identified. In particular, cKO mice were smaller than control mice and showed heightened sensitivity to the stereotypy-inducing effects of amphetamine and slight reductions in preference toward sucrose and ethanol, as well as a blunted response in the elevated plus maze test. These data uncover new knowledge about the role of genetically defined subtypes of neurons in the brain's monoaminergic systems.

Keywords: amphetamine, dopamine, locus coeruleus, raphe nuclei, serotonin, substantia nigra, ventral tegmental area, vesicular monoamine transporter (VMAT2)

INTRODUCTION

The monoamine systems of the brain are crucial for normal brain function and their dysfunction is highly correlated with neuropsychiatric and neurological disorders (Ng et al., 2015). Monoamine neurotransmitters, defined by the presence of a single amino group, include the catecholamines—dopamine, noradrenaline, and adrenaline—as well as serotonin and histamine. Though their syntheses differ, these systems share the protein responsible for packaging the neurotransmitter into synaptic vesicles, the vesicular monoamine transporter 2 (VMAT2). A member of the solute carrier (SLC) superfamily of transporter proteins, it is encoded by the *Vmat2* gene, also known as *Slc18a2*.

Several of the brain's main monoamine systems are located in the midbrain and medulla oblongata, including the midbrain dopamine system comprising the ventral tegmental area (VTA), substantia nigra pars compacta (SNc) and the retrorubral field (RRF), the dorsal raphe (DR) consisting of both dopaminergic and serotonergic neurons, and the locus coeruleus (LC) which is the main brain center for noradrenergic neurons. Common for all these systems is their discrete localization in distinct nuclei, as opposed to glutamatergic and GABAergic neurons that are present throughout the brain. In contrast to the defined presence of cell bodies within brain nuclei, the projections of monoamine neurons are vast and reach large parts of the brain. Through their intricate interactions with many brain areas, monoaminergic neurons exert a strong impact on physiology and behavior. For example, monoamine signaling is associated with motor regulation (Schultz et al., 1989), arousal (Haas et al., 2008; Sara and Bouret, 2012), emotional behaviors (Cools et al., 2008; Likhtik and Johansen, 2019), learning (Keiflin and Janak, 2015) and motivation (Fields et al., 2007; Cools, 2008; Salamone and Correa, 2012). Following their important roles, compromised monoaminergic function is linked to several neurological and neuropsychiatric diseases such as Parkinson's Disease, substance use disorder, depression, and schizophrenia, as well as brain dopamine-serotonin vesicular transport disease (Christiansen et al., 2007; Gutiérrez et al., 2007; Rilstone et al., 2013; Padmakumar et al., 2019). By removing monoamines from the cytosol, parallel to ensuring vesicular packaging of monoamines, VMAT2 also protects neurons from oxidative stress-related damage (Guillot and Miller, 2009; Lohr et al., 2016). Further, drugs such as reserpine and amphetamine that affect monoamine packaging in synaptic vesicles by acting directly on VMAT2 have profound acute and prolonged effects on behavior by influence on both motor and cognitive functions (Schuldiner et al., 1995; Sulzer et al., 2005).

Experimental studies in transgenic mice exploring the role of VMAT2 have observed various deficits following perturbation of its function, including behavioral changes related to locomotion, anxiety, feeding, and response to drugs. These studies have targeted VMAT2 genetically either by systemic knockout/knockdown strategies (VMAT2 heterozygotes or complete knockouts; Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997; Mooslehner et al., 2001; Fukui et al., 2007), or by the use of conditional knockout (cKO) strategies based on neurotransmitter phenotype (defined by uptake transporters or synthesis enzymes; Narboux-Nême et al., 2011, 2013; Ohara et al., 2013; Isingrini et al., 2016a,b). Recently, the selective expression of receptors or transcription factors have been used to direct selective targeting of the *Vmat2* gene (Xu et al., 2017; Bimpisidis et al., 2019). Limiting the intervention to selected VMAT2-positive neuronal populations, leaving the remainder of monoamine signaling cells with unaltered VMAT2 function, allows for probing of the functional role of specific neurons and their circuits. The impact of these more selective alterations can in turn be used to study discrete behavioral phenotypes related to monoamine dysfunction such as stress, depression, and movement disorders.

The calcium-binding protein Calbindin 2 (Calb2, also known as Calretinin and 29 kDa Calbindin) is present in a range of structures in the brain—including, but not limited to, the cortex, hypothalamus, midbrain, pons, and medulla—and is implicated in several functions including developmental processes and neuroprotection (Barinka and Druga, 2010; Schwaller, 2014; Fairless et al., 2019). In the midbrain dopamine system of rodents and primates, some, but far from all, dopamine neurons of the VTA and SNc are positive for *Calb2* gene expression (Rogers, 1992; Isaacs and Jacobowitz, 1994; Mouatt-Prigent et al., 1994; Fortin and Parent, 1996; Liang et al., 1996; McRitchie et al., 1996; Nemoto et al., 1999; Poulin et al., 2014; Viereckel et al., 2016; Mongia et al., 2019).

Calb2-positive dopamine neurons thus form a subtype of dopamine neurons, joined by their expression of the *Calb2* gene. Following up on data obtained in a microarray analysis of the mouse midbrain, by comparing expression in the VTA with the SNc using histological methods we have previously shown that Calb2 mRNA is prominent in both the VTA and the SNc, with the strongest signals detected in lateral VTA, rostral linear nucleus (RLi) and SNc, and somewhat weaker in the medially positioned interfascicular nucleus (IF; Viereckel et al., 2016). To further address this Calb2-population, we recently used fluorescent *in situ* hybridization to allow co-detection and could show that about 50% of the dopamine neurons in the mouse VTA are positive for Calb2 mRNA. Calb2-positive dopamine neurons were identified throughout all VTA subareas: the IF, parainterfascicular nucleus (PIF), paranigral nucleus (PN), parabrachial pigmented nucleus (PBP), and rostral VTA (VTAR; Bimpisidis et al., 2019). This finding is coherent with previous immunohistochemical observations (Liang et al., 1996). Calb2 mRNA was also detected in scattered dopamine neurons throughout the SNc. In addition to dopamine neurons, Calb2 mRNA was identified in glutamate and GABA neurons within the VTA and SNc. Calb2-positive neurons thus show a heterogeneous neurotransmitter phenotype in the VTA and SNc (Bimpisidis et al., 2019). By implementing a protocol for optogenetic stimulation in the VTA coupled with behavioral testing in a place preference paradigm (Bimpisidis et al., 2020), no significant response was obtained when Calb2-Cre mice were tested as opposed to when stimulating other subtypes of

VTA dopamine neurons (Bimpisidis et al., 2019). This finding was surprising, given the amount of Calb2-positive neurons in the VTA area, but may be explained by a low amount of extra-VTA projections, tentatively suggesting that Calb2-positive VTA neurons primarily interact with other VTA neurons (Bimpisidis et al., 2019). This was the first study probing the functional role of Calb2 cells in the VTA which highlighted the need for specific and varied experimental approaches to address the functional role of Calb2-positive neuronal populations.

Further following up on the identification of Calb2-positive neurons in the ventral midbrain of the adult mouse, we recently addressed the embryonal development of this same brain area. We found that Calb2 mRNA can be detected already at embryonal day 14.5 (E14.5), shortly after the neurons acquire their dopamine phenotype (Dumas and Wallén-Mackenzie, 2019). Calb2 was not detected at E11.5, however, suggesting that the onset of Calb2 expression is between E11.5 and E14.5. Beyond the midbrain dopamine system, Calb2 has also been observed in other monoamine areas, including several raphe nuclei, and in hypothalamic dopaminergic cells; in contrast, Calb2 has been demonstrated as absent in the noradrenergic LC (Arai et al., 1991; Résibois and Rogers, 1992). However, little is known about the role of Calb2-positive monoaminergic neuronal populations in behavioral regulation.

In this study, we aimed to anatomically and functionally probe the Calb2 subtype of monoaminergic neurons. We implemented histological and gene-targeting approaches followed through with behavioral assessments and pharmacological challenges. Upon confirmed deletion of *Vmat2* gene expression selectively in Calb2-Cre-positive neurons, behavioral analysis of this new $Vmat2^{lox/lox;Calb2-Cre}$ line of *Vmat2* cKO mice revealed significantly altered behaviors in terms of locomotion, anxiety, and responses to sucrose, ethanol, and amphetamine. The results demonstrate that the Calb2-positive subtype of monoaminergic neurons is crucial for normal behavior.

MATERIALS AND METHODS

Mice

All experiments were conducted according to Swedish (Animal Welfare Act SFS 1998:56) and European Union legislation (Convention ETS 123 and Directive 2010/63/EU) and following Uppsala Ethical Committee for Laboratory Animal Research. The mice were group-housed (2–5 mice per cage) in a temperature- and humidity-controlled animal husbandry room, on a 12:12 light:dark cycle (lights on at 06:00 AM), and provided with food and water *ad libitum*. Heterozygous Calb2-Cre transgenic mice (The Jackson Laboratory, $Calb2^{tm1(cre)Zjh}$/J) were bred with $Vmat2^{lox/lox}$ mice, which have exon 2 of the *Vmat2* gene flanked by LoxP sites (Narboux-Nême et al., 2011). This strategy allowed for generation of (cKO; $Vmat2^{lox/lox;Calb2-Cre-tg/wt}$) and Cre-negative controls (Ctrl; $Vmat2^{lox/lox;Calb2-Cre-wt/wt}$; **Figure 1A**). Genotyping was performed by PCR with the following primer sequences: Calb2-Cre 5′-ACG AGT GAT GAG GTT CGC AAG A-3′; 5′-ACC GAC GAT GAA GCA TGT TTA G-3′; Vmat2lox 5′-GAC TCA GGG CAG CAC AAA TCT CC-3′; 5′-GAA ACA TGA AGG ACA ACT GGG ACC C-3′. Control and cKO adult mice (>8 weeks) of both sexes were used for the experiments.

Histological Analysis

In Situ Hybridization (ISH) and Fluorescent *In Situ* Hybridization (FISH)

Brains were dissected from deeply anesthetized cKO and control mice at 17 days of age, and snap-frozen in isopentane (2-Methylbutane) on dry ice. Sections were cut on a cryostat and slides were prepared for *in situ* hybridization.

Experiments were performed as previously reported (Bimpisidis et al., 2019). Briefly, radioactive oligoprobes (for ISH; **Figure 1B**) and riboprobes (for FISH) or combined FISH/brightfield ISH; **Figure 1C**) were used. The following oligoprobe sequences were used: Calb2: NM_00786.1; bases 33–61, 946–979; and 1299–1332. Th: NM_009377.1; bases 774–807, 272–305, 1621–1655. Vmat2exon1 (wildtype probe): NM_172523.3; bases18–51 and 83–116. Vmat2exon2 (KO probe): NM_172523.3; bases 201–237 and 240–276. The following riboprobe sequences were used: Calb2: NM_007586.1; bases 80–793. Vmat2 Probe1: Vmat2: NM_0130331.1 (rat); bases 701–1439 (corresponds to exon 6–15 of mouse sequence NM_172523.3). Vmat2 Probe2: NM_172523.3; bases142–274 i.e., the whole exon 2. The latter riboprobes targeting different exons of the Vmat2 mRNA termed Vmat2 Probe1 and Vmat2 Probe2 were used for detection of wildtype and recombined Vmat2 DNA as described below.

Validation of Recombination of Floxed Vmat2 Exon 2 Driven by the Calb2-Cre Transgene

A three-probe *in situ* hybridization approach was employed to visualize monoaminergic Calb2-positive cells (**Figure 1C**). In addition to labeling with a fluorescent probe for Calb2 mRNA, two probes were used to visualize Vmat2 mRNA as previously reported (Bimpisidis et al., 2019). Vmat2 mRNA derived from exon 6–15 was detected by Probe1 (green fluorescent), whereas Probe2 (purple/black) detected mRNA from exon 2. In the case of *Vmat2* wildtype cells, both probes bind to Vmat2 mRNA giving a combination of green and purple/black labeling. In those cells in cKO mice in which recombination has taken place, only Probe1 (green) can bind as exon 2 has been excised. Thus, knockout (KO) cells with abrogated Vmat2 are visualized by a green signal and a lack of purple/black signal.

Quantification of Vmat2 Knockout Cells

Serial sections from two mice of each genotype were analyzed for the occurrence of the recombined *Vmat2* allele (binding of only Probe1, *Vmat2* KO cells) *vs.* control (binding of both Probe1 and Probe2, *Vmat2* wildtype cells). Manual counting of cells positive for Probe1 *vs.* Probe1 + Probe2 was performed with Probe1 used as a reference for anatomical boundaries and outline of distinct cell soma.

Immunohistochemistry

Deeply anesthetized adult cKO and control mice were transcardially perfused with room-temperature PBS followed by ice-cold 4% formaldehyde. Brains were dissected and

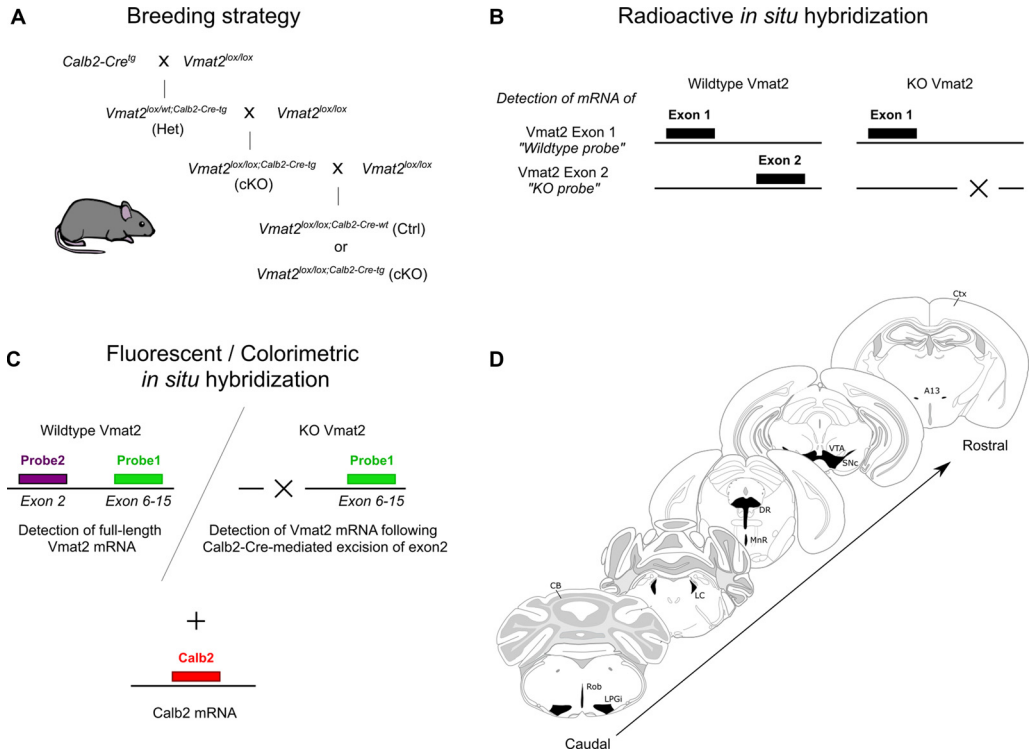

FIGURE 1 | Illustrations of the experimental approach. Schematic of breeding strategy to produce Calb2-directed VMAT2 conditional knockouts (cKO) using a Cre-LoxP system **(A)**. Radioisotope-labeled *in situ* hybridization **(B)**, and three-probe fluorescent/colorimetric *in situ* hybridization **(C)** method to verify *Vmat2* recombination and Calb2 co-localization. The span of sections analyzed with illustrated monoaminergic areas **(D)**. CB, cerebellum; Ctx, cortex; DR, dorsal raphe; LC, locus coeruleus; LPGi, lateral paragigantocellular nucleus; MnR, median raphe; Rob, raphe obscurus; SNc, substantia nigra compacta; VTA, ventral tegmental area.

post-fixed overnight, transferred to PBS, and sectioned at 60 μm using a vibratome. Free-floating sections were processed for immunohistochemistry according to standard protocols. After a series of washes with PBS and PBS containing 0.1% Triton-X, they were incubated for 1 h in an appropriate blocking solution (5% serum in 0.1% PBS-T) at room temperature (RT). Incubation of primary antibodies diluted in 0.1% PBS-T with 5% serum took place overnight at 4°C [rabbit anti-TH 1:1,000, Millipore, #MAB318; rabbit anti-TPH2 1:1,000 Novus Biologicals #NB100–74555]. Sections were subsequently washed in 0.1% PBS-T and incubated with a biotinylated goat anti-rabbit antibody (ABC kit; Vector laboratories #PK-4001) in 0.1% PBS-T for 1.5 h at RT. After subsequent washing steps, DAB (Vector Laboratories) was used as a chromogen. The sections were mounted on glass slides, incubated in cresyl violet, dehydrated in a series of ascending ethanol solutions, and coverslipped using DPX (Sigma–Aldrich, 06522). Images were captured using a NanoZoomer S60 scanner and processed using the Ndp2.view software (Hamamatsu) or ImageJ.

Behavioral Analysis

Baseline Locomotion

Mice were placed in polycarbonate boxes (Makrolon), containing 1.5-cm bedding, covered with a transparent, perforated plexiglass lid (cKO n = 21, control n = 17). Spontaneous horizontal activity and habituation to a novel environment were recorded for 60 min by the EthovisionXT (Noldus) tracking software. Data are expressed as distance moved during the recording period.

Sucrose Preference Test

Mice were housed individually and were presented with two drinking bottles (cKO n = 9, control n = 6). After 48 h of habituation to the experimental setup, the mice were presented with one bottle containing tap water and one sucrose solution (1, 3, and 10%). Each bottle was weighed and replaced every 24 h. Each sucrose concentration was tested twice (i.e., two consecutive days) and the position of the bottles was alternated to exclude side biases. Preference for the sucrose bottle was calculated as the percentage of volume consumed from the sucrose bottle compared with total liquid consumption (from both sucrose and water bottle).

Ethanol Preference Test

The experimental setup was similar to the one described for the sucrose preference test (cKO n = 16, control n = 16). After habituation to two water bottles, the mice were introduced to one bottle of tap water and one containing a solution of increasing concentration of ethanol (3, 6, and 10%). The bottles were weighed and changed every 24 h, with alternating positions, and each concentration was tested four times (i.e., four consecutive days of each concentration). Preference for the ethanol bottle

was calculated as the percentage of volume consumed from the ethanol bottle compared with total liquid consumption (from both ethanol and water bottle).

Elevated Plus Maze

Animals were placed on an elevated plus maze with two open and two closed arms for 5 min without prior habituation to the apparatus (cKO n = 21, control n = 22). Mice were positioned close to and facing away from the center on one of the open arms. During the recording time, EthovisionXT (Noldus) tracking software was used to monitor their behavior, including time spent in each compartment and transitions between them.

Rotarod

Motor coordination assessment took place on a Rotarod apparatus (Panlab) under an incremental fixed speed schedule and a session with accelerating rotation speed. On consecutive days, mice were trained at rotation speeds of 4, 8, and 16 RPM (cKO n = 17, control n = 16). Latency to fall at each speed was noted during three trials, separated by 30 min, with three attempts per trial. A trial was ended when all three attempts were made, or after a maximum of 120 s was achieved. For the session with accelerating rotation speed, latency to fall was noted during three trials, separated by 30 min, with a rotation speed increasing from 4 to 40 RPM over 5 min (cKO n = 8, control n = 8). Statistical analysis was performed on the maximum value achieved during each trial.

Amphetamine Sensitization

The behavior of the mice upon injections of amphetamine or saline was monitored in boxes as described above for baseline locomotion. Mice received intraperitoneal injections of saline (day 1, "Saline") followed by 3 mg/kg amphetamine (days 2–5, "Amph 1–4"), and a final injection of the same dose of amphetamine on day 17 ("Challenge"; cKO n = 13, control n = 10). Horizontal activity was recorded 30 min before and 90 min following injection, using EthovisionXT (Noldus).

Statistical Analysis

Appropriate statistical tests were performed in GraphPad PRISM version 8. For comparisons of two factors (such as genotype and time), repeated measure two-way ANOVA was used, with *post hoc* tests for testing within factor means. Simple averages between groups were tested using Student's t-test or Mann–Whitney test.

RESULTS

The presence of VMAT2 is restricted to monoamine-releasing cells. To delete the ability for monoamine vesicular packaging selectively in Calb2-positive cells, a Cre-LoxP strategy was utilized in which expression of Cre recombinase is driven by promotor sequences for the *Calb2* gene (**Figure 1A**). By crossing the Calb2-Cre-line with a floxed allele of *Vmat2*, in which exon 2 of the *Vmat2* gene is surrounded by LoxP sites (Narboux-Nême et al., 2011), we generated a new cKO mouse line in which the floxed sequence containing exon 2 of the *Vmat2* gene is excised specifically in Calb2-Cre cells. Throughout the experiments, PCR-verified cKO mice positive for Calb2-Cre and homozygous for the floxed allele ($Vmat2^{lox/lox;Calb2-Cre-tg}$; cKO) were compared with littermates homozygous for the floxed allele but negative for Calb2-Cre ($Vmat2^{lox/lox;Calb2-Cre-wt}$; Ctrl).

Histological Evaluation of Calb2-Cre-Driven Targeting of the Vmat2 Allele

Using two different *in situ* hybridization approaches (**Figures 1B,C**), we confirmed the expression of *Vmat2* and its targeted deletion. Multiple brain sections in series were analyzed encompassing major monoaminergic systems in the fore-, mid-, and hindbrain, including the hypothalamus, VTA, SNc, RRF/caudal linear nucleus (RRF/CLi), LC, and raphe nuclei (**Figure 1D**).

First, radioisotope-labeled *in situ* hybridization was performed to grossly detect mRNAs. Calb2 was confirmed in the VTA and SNc, and also identified with a vast presence in subcortical areas, midbrain, medulla, and cerebellum (**Figure 2A**). Two different *Vmat2* probes were used to address Vmat2 expression and allow detection of the Vmat2 KO allele: one "wildtype probe" detecting the wildtype *Vmat2* allele (exon 1) and one "KO probe" detecting the *Vmat2* exon2 KO allele (normal in wildtype cells and excised in KO cells; **Figure 1B**). As expected, in control mice, both the wildtype and KO probes were detected at similar intensity across all analyzed monoaminergic areas (**Figures 2A,B**, **Supplementary Figure 1**). In contrast, a weaker signal was observed from the *Vmat2* KO probe in monoaminergic areas of the cKO mice compared to controls. With this method, the lower detection was particularly evident in the VTA and SNc (**Figure 2B**). Detection levels of Calb2 mRNA were generally similar between genotypes (**Supplementary Figure 1**). Tyrosine hydroxylase (Th) mRNA was analyzed to address the integrity of catecholaminergic neurons. Similar Th mRNA levels in control and cKO mice were found throughout all catecholaminergic areas including the VTA, SNc, RRF/CLi, and LC (**Figure 2B**; **Supplementary Figure 1**).

With the confirmed detection of Vmat2 exon2-deletion in the midbrain VTA and SNc neurons, a more careful analysis was performed to ascertain the histological phenotype of cKO brains. For this purpose, fluorescent *in situ* hybridization (FISH) was implemented to allow the co-detection of several mRNAs. First, we sought to address the presence of Calb2 mRNA to identify areas in which the *Vmat2* gene might be deleted (summarized in **Table 1**, **Figures 3**, **4**). In the midbrain, in addition to the VTA and SNc (**Figure 3**, **Table 1**), Calb2 mRNA was identified in the RLi, RRF and CLi (**Figures 2B**, **3**), and several raphe nuclei, including the dorsal, lateral, and ventral dorsal raphe (DRD, DRL, DRV; **Figure 4A**), median raphe (MnR), raphe Magnus/raphe interpositus (RMg/RIP), interfascicular nucleus (DRI), and raphe obscurus (Rob; **Figure 4B**). Caudally, Calb2 mRNA was found in the LC (**Figure 4C**), the lateral paragigantocellular nucleus (LPGi), and in distinct adrenergic subareas (C1–C3) of the medulla oblongata (**Table 1**). Further, rostrally, low levels of Calb2 mRNA, or its

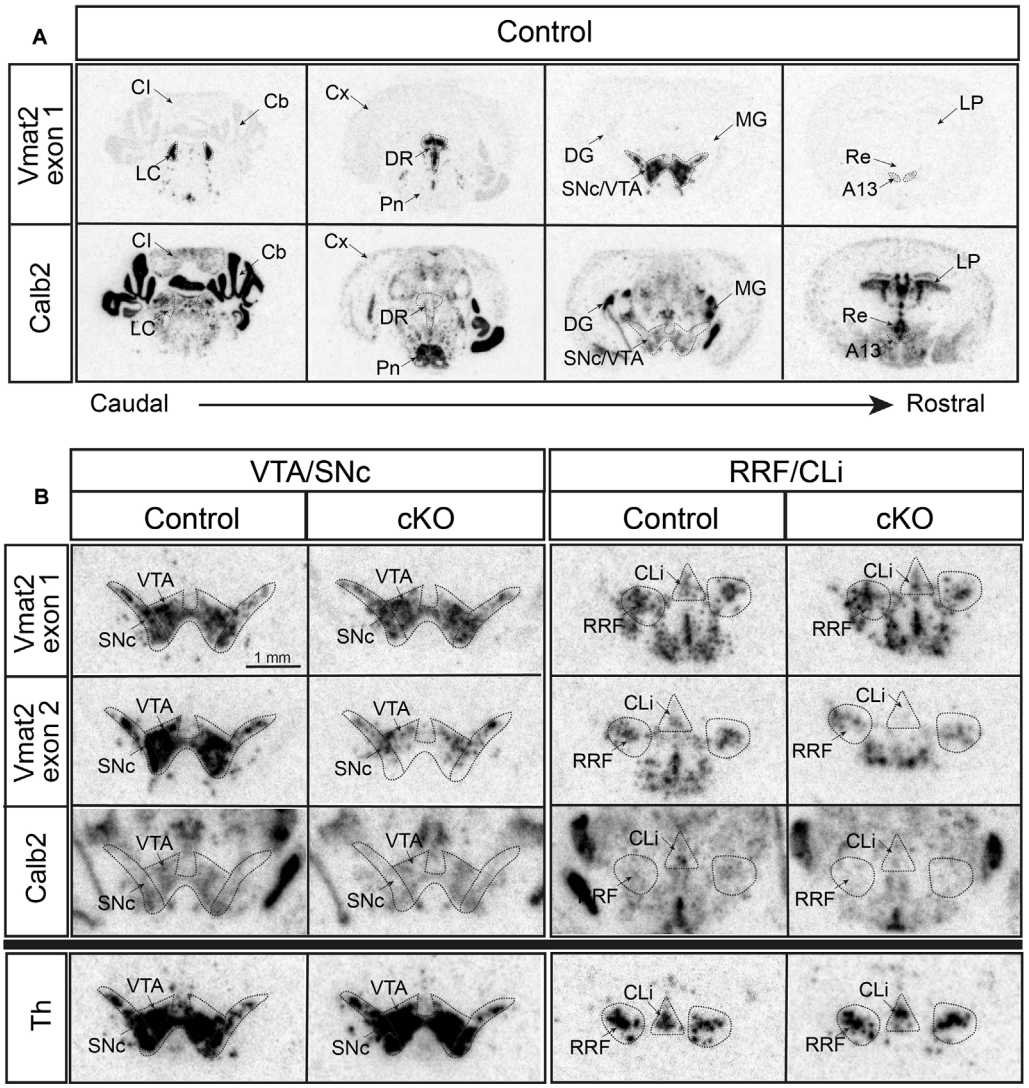

FIGURE 2 | Histological validation of cKO of VMAT2 in Calb2-driven Cre-producing cells by radioisotope-labeled *in situ* hybridization. Detection of Vmat2 exon 1 (using "wildtype" probe) and Calb2 mRNA in various monoaminergic areas of the brain in control mice **(A)**. Detection of Vmat2 exon 1 (using "wildtype" probe), Vmat2 exon 2 (using "KO" probe), Calb2, and Th mRNA in midbrain dopaminergic areas for control and cKO mice **(B)**. Cb, cerebellum; Cl, inferior colliculus; Cli, caudal linear nucleus; Cx, cortex; DG, dentate gyrus; DR, dorsal raphe; IF, interfascicular nucleus; LC, locus coeruleus; LP, lateral posterior thalamic nucleus; MG, medial geniculate nucleus; PBP, parabrachial nucleus; PIF, parainterfascicular nucleus; Pn, pontine nuclei; Re, reuniens nucleus; RLi, rostral linear nucleus; RRF, retrorubral field; SNc, substantia nigra compacta; Th, tyrosine hydroxylase; VTA, ventral tegmental area.

absence, were detected in the mammillary and premammillary nuclei, arcuate nucleus, and anterior hypothalamus (AH; **Table 1**, **Figure 4D**) as well as hypothalamic dopamine neurons of area A13 (**Table 1**).

Calb2/Vmat2 co-localization analysis next showed that Calb2 mRNA colocalized with Vmat2 mRNA throughout these areas: VTA, SNc, RRF, RLi, CLi, DR (mostly DRV), MnR, DRI, RMg/RIP, Rob, LC, LPGi, C1–3, and arcuate nucleus. No Calb2/Vmat2 co-localization was seen in hypothalamic nuclei, mammillary, and premammillary nuclei. Also, the dorsal aspect of the DR, DRD, was devoid of Calb2/Vmat2 colocalization. In areas where Calb2 and Vmat2 mRNAs did colocalize, the level varied between areas (**Figures 3**, **4**, and **Table 1**).

To assess the extent of Calb2-Cre-driven recombination of the floxed *Vmat2* allele, all areas identified as positive for Calb2/Vmat2 in control mice were next analyzed for the presence of Vmat2 KO cells in the cKO mice. Here, to identify the precise location and extent of recombination patterns with high anatomical resolution, we employed a three-probe *in situ* hybridization approach. Labeling of Calb2 mRNA (red) was combined with two different Vmat2 probes to allow the detection of both wildtype and KO Vmat2 mRNA (**Figure 1C**). Cells with wildtype Vmat2 mRNA in both control and cKO mice were visualized by the binding of both a fluorescent probe (green; Probe1, binding to exon 6–15) and a colorimetric probe [purple/black; Probe2, binding to exon 2 (the floxed

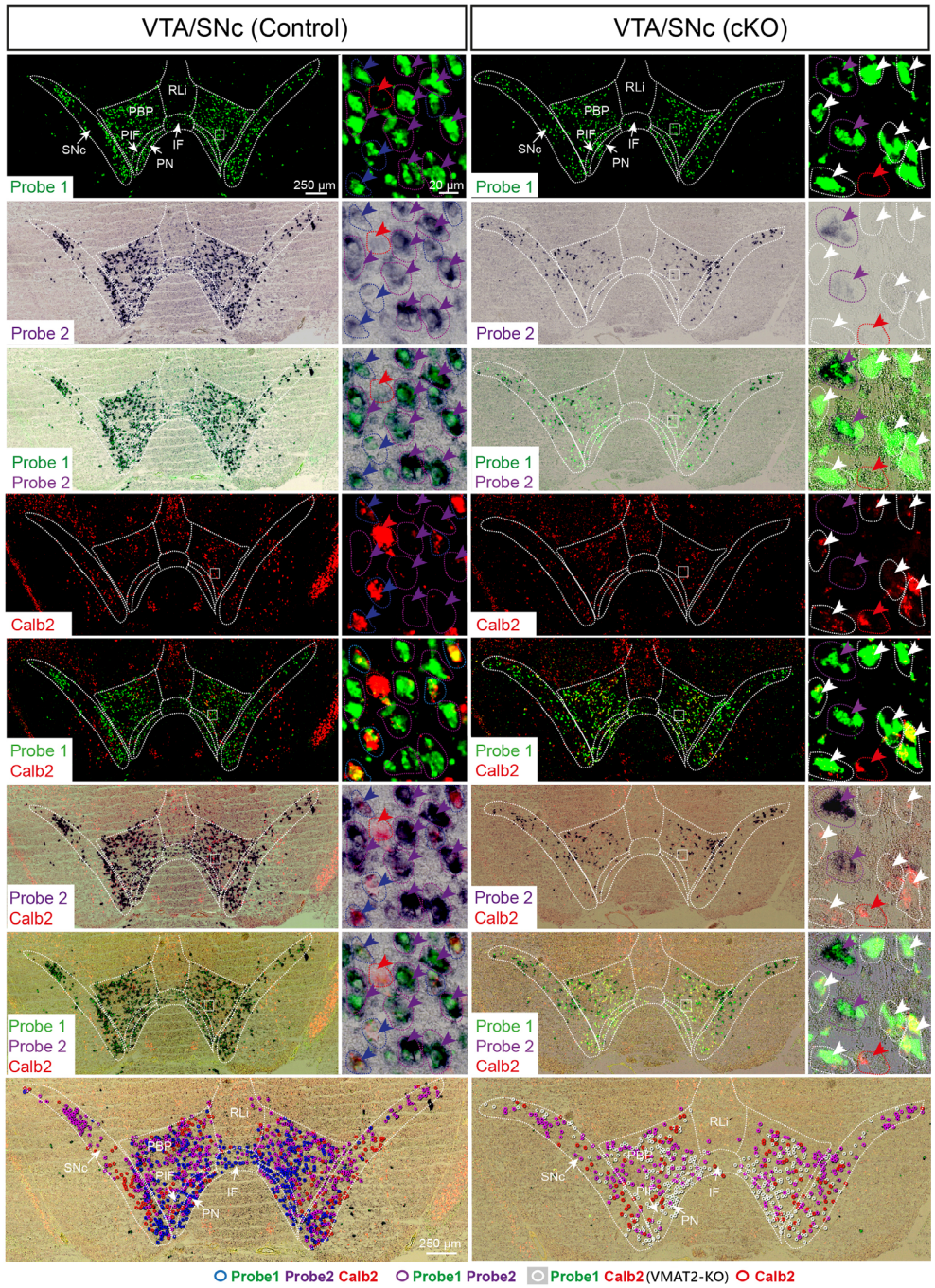

FIGURE 3 | Three-probe *in situ* hybridization in midbrain dopaminergic areas. Wildtype cells are identified by binding of probes to mRNA of exons 6–15 (Probe1; green) as well as exon 2 (Probe2; purple/black), whereas green signal alone identifies knockout (KO) cells in which recombination has led to the removal of exon 2. Additionally, co-localization with Calb2 (red) was studied. Arrow colors correspond to the legend provided at the bottom of the figure, where different combinations of expression patterns are symbolized and superimposed on the image to show their distribution (bottom row). Blue circles symbolize cells with wildtype Vmat mRNA co-localizing with Calb2 mRNA; purple circles symbolize cells with wildtype Vmat2 negative for Calb2; white circles symbolize VMAT2-KO cells; red circles symbolize non-monoaminergic Calb2-positive cells. IF, interfascicular nucleus; PBP, parabrachial nucleus; PIF, parainterfascicular nucleus; PN, paranigral nucleus; RLi, rostral linear nucleus; SNc, substantia nigra compacta; VTA, ventral tegmental area.

exon)]. In contrast, in cells in which Calb2-Cre-mediated recombination of the floxed *Vmat2* exon 2 has taken place, probe 2 fails to bind. Vmat2 KO cells could thereby be detected by their clear green fluorescence due to the binding of only Probe 1. This strategy allowed Vmat2 KO cells to be readily visualized.

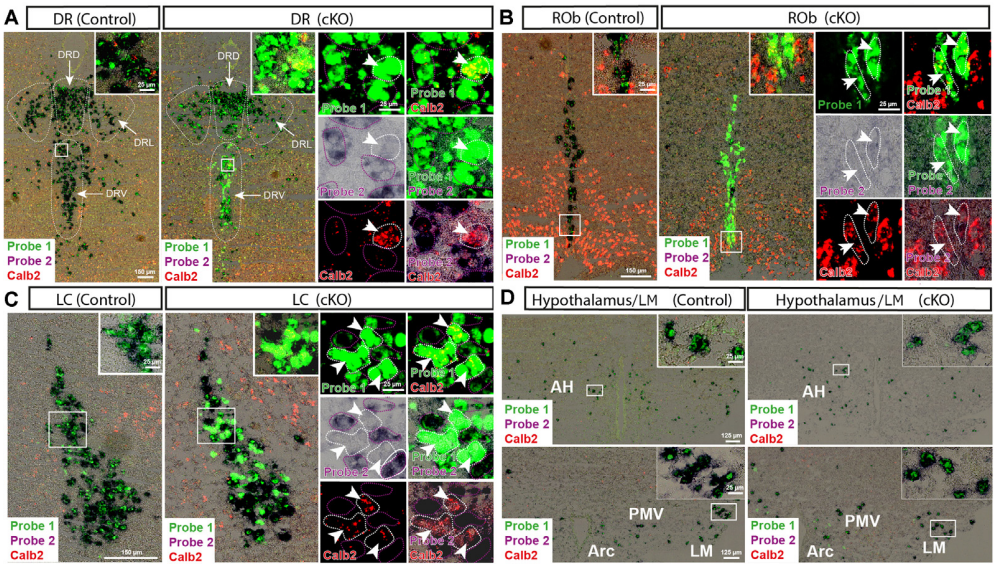

FIGURE 4 | Detection of Vmat2 deletion and Calb2 co-localization in additional areas in the brain. Insets show high magnification of signals from Vmat2 Probe1 (green), Probe2 (purple/black), and Calb2 (red) in the ventral and lateral portions of the dorsal raphe (DRV; DRL; **A**), nucleus raphe obscurus (ROb; **B**), LC **(C)**, and hypothalamic areas **(D)**. White arrows indicate KO cells positive for Vmat2 Probe1 and Calb2, but negative for Vmat2 Probe2. AH, anterior hypothalamus; Arc, arcuate nucleus; DR, dorsal raphe; DRL, lateral portion of dorsal raphe; DRD, dorsal portion of dorsal raphe; LM, lateral mammillary nucleus; PMV, ventral premammillary nucleus.

TABLE 1 | Extent of recombined (knockout) cells and Calb2/Vmat2 distribution in the brain of *VMAT2*$^{lox/lox;Calb2-Cre-tg}$ (cKO) mice.

	Region		Vmat2 KO cells	Calb2/Vmat2 coloc	Calb2
Hypothalamus	A13 dopamine cells		0	0	
	Anterior hypothalamus (AH)		0	0	
	Arcuate nucleus		+	+	Weak or no signal
Premammillary nucleus	PMV		0	0	
Mammillary nucleus	LM		0	0	
Dopaminergic cells (A8–A9–A10)	VTA (A10)	IF/PN/PIF	++/+++	++/+++	
		PBP	+/++	+/++	
	SNc (A9)	Ventral SNc	+++	+++	
		Medial and lateral SNc	+	+	
	RRF (A8)		++/+++	++/+++	
Linear Nuclei	RLi		+	+	
	CLi		++/+++	++/+++	
Raphe nuclei	DR	DRD	0	0	Yes
		DRL	+	+	
		DRV	++/+++	++	
	MnR		++/+++	++	
	DRI		++/+++	++/+++	
	RMg/RIP		++/+++	++/+++	
	Rob		+++	++	
Noradrenergic cells	LC	Rostro-ventral part	+	0/+	
		Rostro-dorsal part and caudal	++	+	
Adrenergic cells	LPGi		++/+++	++/+++	
	C1–C2–C3		+	+	

Symbols express range of percent accordingly: 0, + (0–30%), ++ (30–60%), +++ (60–90%), ++++ (90–100%). CLi, caudal linear nucleus; DRD, dorsal part of dorsal raphe; DRI, interfascicular dorsal raphe; DRL, lateral part of dorsal raphe; DRV, ventral part of dorsal raphe; IF, interfascicular nucleus; LC, locus coeruleus; LM, lateral mammillary nucleus; LPGi, lateral paragigantocellular nucleus; MnR, median raphe; PBP, parabrachial nucleus; PIF, parainterfascicular nucleus; PMV, ventral premammillary nucleus; PN, paranigral nucleus; Rob, raphe obscurus; RLi, rostral linear nucleus; RMg/RIP, raphe magnus/raphe interpositus; RRF, retrorubral field; SNc, substantia nigra compacta; VTA, ventral tegmental area.

Consistent with the observed sites of Calb2/Vmat2 co-localization, Vmat2 KO cells were detected in cKO mice in all brain areas listed above as positive for Calb2/Vmat2 in control mice: VTA, SNc, RRF, RLi, CLi, DR (mostly DRV), MnR, DRI, RMg/RIP, Rob, LC, LPGi, C1–3, as well as the arcuate nucleus (**Table 1**, **Figures 3**, **4**). The level of recombination (i.e., proportion of Vmat2-positive cells positive for only Probe1) was similar to the level of co-localization in control mice (**Table 1**, and described further below). In contrast, consistent with their lack of

Calb2/Vmat2 co-localization, Vmat2 mRNA in hypothalamic nuclei, mammillary and premammillary nuclei as well as DRD was unaffected and appeared similar in control and cKO mice (**Table 1**, **Figures 4A,D**).

Careful analysis showed a high occurrence of Vmat2 deletion in midbrain dopamine cells in cKO mice. Following the results of our previous study (Bimpisidis et al., 2019), approximately half of the VTA/SNc cells were also positive for Calb2 mRNA (Probe1+/Probe2+/Calb2+), and these were positive for the Vmat2 KO mRNA in cKO mice (Probe1+/Probe2−/Calb2+). Rare KO cells did not show detectable levels of Calb2 (Probe1+/Probe2−/Calb2−), a finding which might reflect developmental *Calb2* expression not detected in the adult but which contributed with Calb2-Cre activity. Within the VTA, KO cells were present in all subregions, the IF, PIF, PN, PBP, and VTAR, following the previously described distribution of Calb2 mRNA (**Figure 3**). Extensive *Vmat2* recombination was detected in the ventral part of the SNc, corresponding to the higher levels of Calb2 mRNA here than in dorsolateral SNc (**Figures 2B**, **3**, also Bimpisidis et al., 2019). Further, a few Vmat2 KO cells were found in the RLi, and a moderate to an extensive amount of KO cells was detected in both the RRF and CLi (**Table 1**). Moderate to extensive *Vmat2* recombination was observed in the raphe nuclei, including DR (with most in DRV), MnR, DRI, RMg/RIP, Rob (**Figure 4**, **Table 1**). The LPGi also contained moderate to extensive proportions of Vmat2 KO cells, and a few knockout cells were observed in the adrenergic cells of the medulla, as well as the arcuate nucleus (**Table 1**).

Overall, Calb2-Cre-driven targeting of the *Vmat2* gene was confirmed in ample midbrain and hindbrain monoaminergic areas in a pattern that largely follows the endogenous expression of Calb2 mRNA. The patterns and the level of recombination were following the patterns and amount of cells co-expressing the *Calb2* and *Vmat2* genes, thus verifying the efficiency of the cKO strategy.

Finally, immunohistochemistry for TH and TPH2 (as markers for catecholaminergic and serotonergic neurons, respectively) did not reveal any gross anatomical differences in the mesostriatal system, LC, or in the DR (**Figure 5**). Together with the stable presence of Th and Calb2 mRNA (above), this finding confirmed consistent cytoarchitecture despite the prominent loss of Vmat2 mRNA.

Changes in Weight and Basal Locomotion, but Not in Motor Coordination

Having confirmed histologically that recombination of the floxed Vmat2 exon2 takes place in Calb2 neurons, we examined if there are any phenotypic differences between cKO mice and their control littermates. Knockout mice displayed attenuated growth, increasing at relatively the same rate after 4 weeks of age (best-fit slope Ctrl 1.01 g/week, cKO 1.03 g/week), albeit consistently maintaining a weight of approximately 7 g less than control animals of the same age (**Figure 6A**; nonlinear regression, $p < 0.0001$ $F_{(2,370)} = 252.1$; *y*-intercept Ctrl 12.4 g, cKO 4.9 g). In the cohort of cKO mice, less than 20% did not survive past weaning age, with mortality occurring at varying postnatal time points. No premature deaths were recorded among control animals.

To evaluate basal locomotion, mice of both genotypes were placed in an open field and horizontal activity was recorded for 60 min. During this time, control animals gradually habituated, as shown by a decrease in horizontal movement. cKO mice, on the other hand, did not show habituation to the novel environment during the testing period, maintaining their initial level of movement throughout the testing period (**Figure 6B**; two-way RM ANOVA, the effect of genotype $p < 0.001$ $F_{(1,36)} = 13.00$; Sidak's *post hoc* comparison Ctrl 10 *vs.* 60 min: $p < 0.0001$, cKO 10 *vs.* 60 min: $p = 0.777$). Furthermore, cKO mice spent significantly less time in the center of the arena than controls (23% *vs.* 36%, cKO *vs.* Ctrl; Student's *t*-test, $p < 0.01$; **Figure 6C**).

As targeted deletion of *Vmat2* had been verified in dopamine areas controlling movement, including the SNc, mice were tested on a rotarod to assess motor coordination. cKO and control mice did not differ in their capacity to maintain their motor coordination as measured by latency to fall off during fixed speed training sessions nor at accelerating rotation speeds. For the fixed speeds, there was a negative effect on latency as a function of rotation speed (two-way RM ANOVA, the effect of speed $p < 0.001$), but no difference between genotypes (effect of genotype $p > 0.5$). For the accelerating speed test, no difference in latency to fall was observed between genotypes (Ctrl: 155.6 ± 13.4 s, cKO: 128.2 ± 16.0 s; Student's *t*-test, $p > 0.2$; **Figure 6D**).

Confounding Findings in the Elevated Plus Maze

The lack of habituation and less time spent in the center of the open field arena shown by the cKO mice prompted an examination of their response in the elevated plus maze to assess anxiety-related phenotypes. The control group spent more time in the closed arms compared to the open arms and center, as expected for this test. Surprisingly, cKO mice spent significantly more time in the open arms than controls (cKO 42%, controls 10%), with a respective decrease in time spent in closed arms (**Figure 6E**; two-way RM ANOVA, the effect of genotype $p < 0.001$; Sidak's multiple comparison Ctrl *vs.* cKO open arms $p < 0.001$, closed arms $p < 0.01$). cKO mice made significantly fewer entries into the open arms (Mann–Whitney, $p < 0.001$ $U = 74$; **Figure 6F**), and fewer total transitions (Mann–Whitney, $p < 0.001$ $U = 69.5$; **Figure 6G**). Finally, cKO mice had a higher latency to the first entry of the closed arms (Student's *t*-test, $p < 0.001$; **Figure 6H**), as well as lower overall locomotion (Student's *t*-test, $p < 0.001$; **Figure 6I**). A composite heatmap indicating the frequency of position is presented in **Figure 6J**.

Blunted Sucrose and Ethanol Preference

To study potential differences in reward processing between genotypes, we performed sucrose and ethanol preference assays. When presented with a choice between water or a sucrose solution of 1, 3, or 10%, both control and cKO mice preferred increasing concentrations of sucrose, although

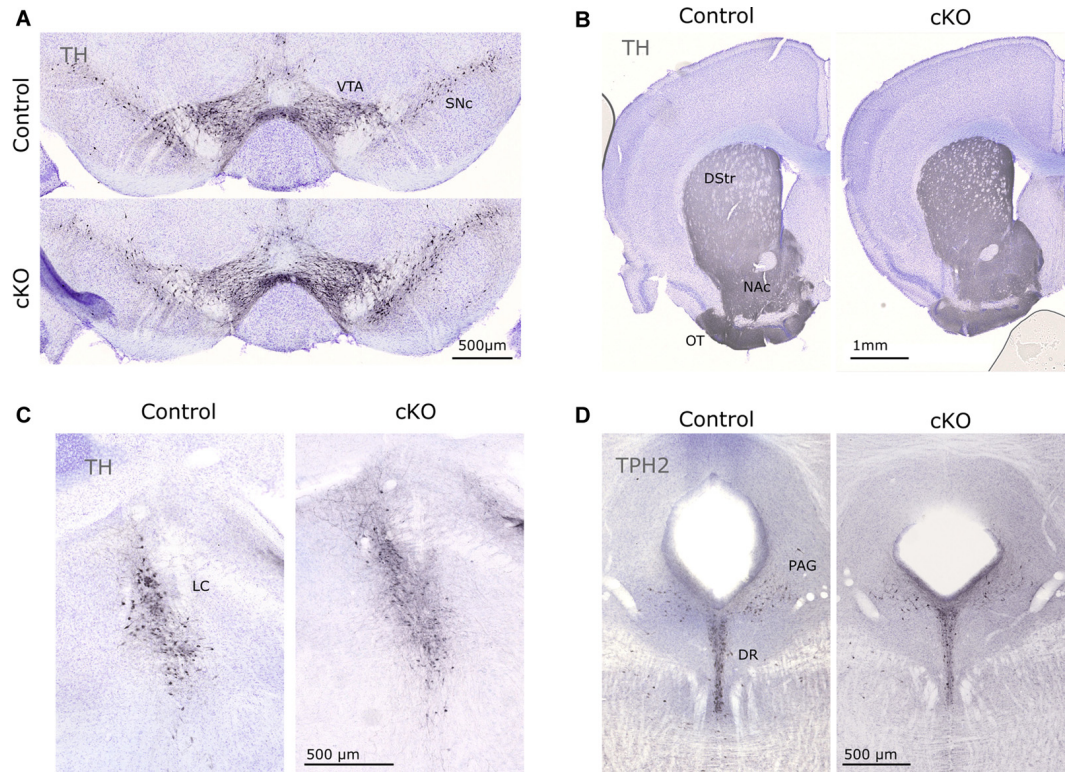

FIGURE 5 | Immunohistochemical evaluation of control and cKO brain tissue. TH was used to visualize cell bodies of dopaminergic cells in the VTA and SNc **(A)** and projections in the striatum **(B)**, as well as catecholaminergic neurons in the LC **(C)**. TPH2 identifies serotonergic cell bodies in the DR **(D)**. DR, dorsal raphe; DStr, dorsal striatum; LC, locus coeruleus; NAc, nucleus accumbens; OT, olfactory tubercle; PAG, periaqueductal gray; TH, tyrosine hydroxylase; TPH2, tryptophan hydroxylase 2.

there was a significantly lower preference score for 10% compared to controls (**Figure 6K**; two-way RM ANOVA, the effect of genotype $p < 0.05$, Sidak's *post hoc* test Ctrl *vs.* cKO 10% $p < 0.05$). When mice were tested in the ethanol preference assay for the concentration of 3, 6, and 10%, a three-way ANOVA revealed a significant effect of genotype but only a slight effect of sex (effect of genotype $p < 0.001$, the effect of sex $p = 0.049$), and this factor was thus consolidated before further analysis. From the subsequent analysis, a lower preference score for cKO mice at 6 and 10% compared to controls was observed (**Figure 6L**; two-way RM ANOVA, the effect of genotype $p = 0.001$, Sidak's multiple comparison Ctrl *vs.* cKO 3% $p = 0.5$, 6% $p < 0.001$, 10% $p < 0.01$). cKO mice did not prefer ethanol over water at any concentration, scoring below 50% preference at all measuring points (**Figure 6L**).

Decreased Locomotion on Repeated Doses of Amphetamine

Amphetamine-like psychostimulants exert their physiological effects by acting, among other targets, through VMAT2. We applied an amphetamine sensitization paradigm to investigate putative differences between cKO and control mice. After receiving a saline injection on the first day, mice received daily injections of 3 mg/kg amphetamine for 4 days, followed by an interim of approximately 2 weeks, at which point the same dose was administered ("Challenge"; **Figure 7A**). Each session consisted of 30 min baseline recording before and 90 min of recording after i.p. injection. The dose of 3 mg/kg was selected based on previous analyses in several different VMAT2 cKO mouse lines by Isingrini et al. (2016b), which had shown that DAT-Cre mice heterozygous for *Vmat2* showed locomotor hypersensitivity to acute delivery of this particular dose. In the present study, control mice consistently increased their horizontal activity following each amphetamine administration, indicating sensitization over time (**Figure 7B**; Amph1 196.6 ± 20.3 m, Challenge 463.8 ± 39.4 m; two-way RM ANOVA, the effect of session $p < 0.01$, Sidak's *post hoc* test Ctrl Amph1 *vs.* Challenge $p < 0.001$). In contrast, cKO mice did not increase their response over time but rather decreased their horizontal activity (**Figure 7B**; Amph1 190.8 ± 24.0 m, Challenge 87.8 ± 16.8 m, Sidak's *post hoc* test cKO Amph1 *vs.* Challenge $p < 0.05$). Whereas the two groups moved a similar distance during the first amphetamine session (Ctrl 196.6 ± 20.3 m; cKO 190.8 ± 24.0 m; Sidak's *post hoc* test Ctrl *vs.* cKO Amph1 $p > 0.9$), cKO mice moved significantly less during the fourth amphetamine session (Ctrl 325.1 ± 32.1 m; cKO 157.3 ± 12.7 m). Finally, during the challenge, cKO mice moved nearly only one-fifth of the distance covered by controls (Ctrl 463.8 ± 39.4 m; cKO

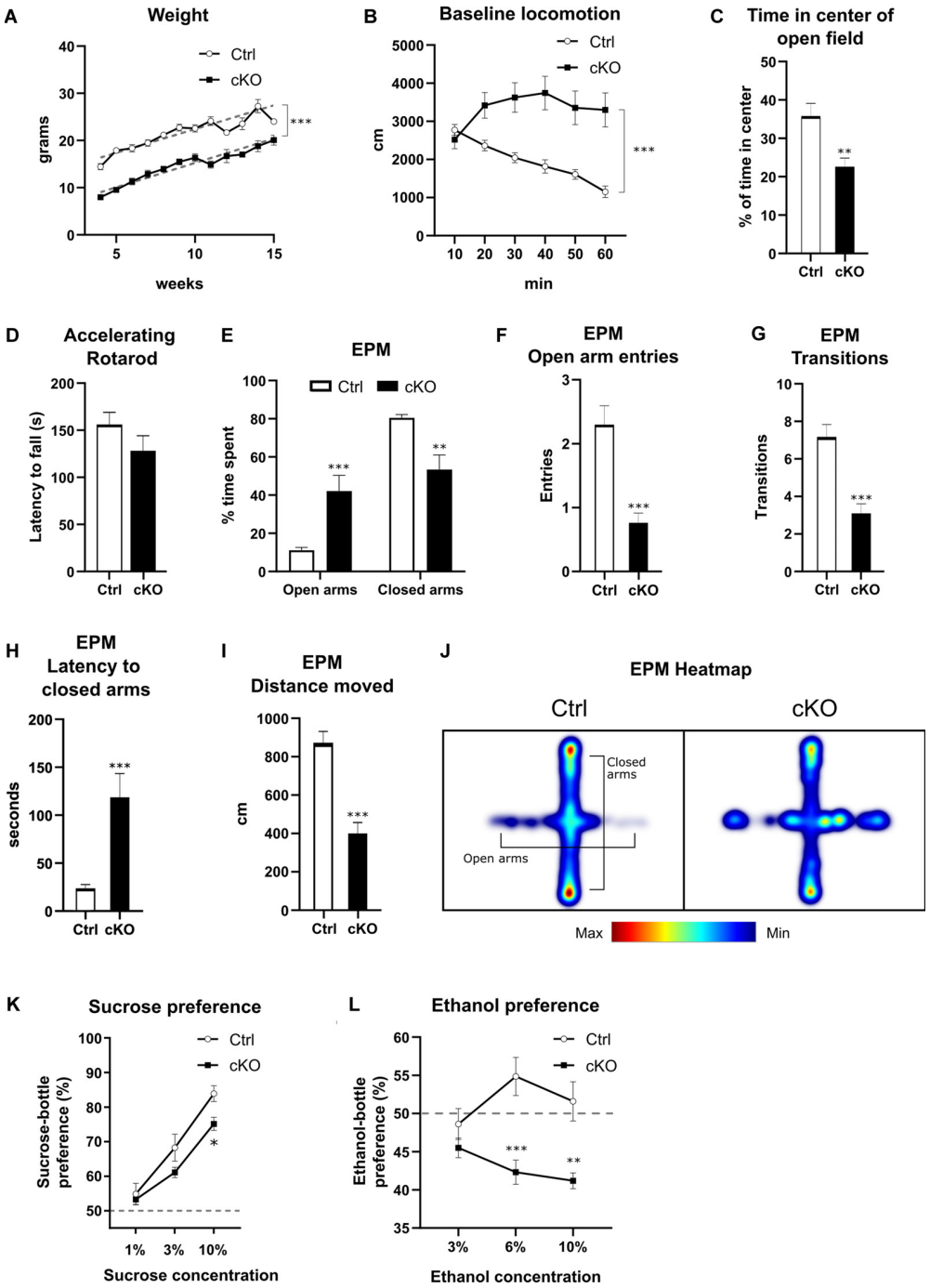

FIGURE 6 | Behavioral characterization of cKO mice and littermate controls in various aspects of development and behavior. Weight curves **(A)** with lines of best fit (gray dashed lines). Horizontal locomotion during 60 min in a novel open field environment **(B)**, analyzed for time spent in the center of the arena **(C)**. Latency to fall from a rotarod at accelerating speeds **(D)**. Various parameters measured in the elevated plus maze **(E–I)**: the proportion of time spent in open *vs.* closed arms **(E)**, number of entries into open arms **(F)**, overall transitions in all compartments **(G)**, latency to first enter closed arms **(H)**, and distance moved during the test **(I)**. Cumulative frequency of position on the apparatus visualized in a composite heat-map **(J)**. Preference for a solution of either sucrose **(K)** or ethanol **(L)** over water. Data presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

87.8 ± 16.8 m; two-way RM ANOVA, the effect of genotype $p < 0.0001$, Sidak's *post hoc* test Ctrl *vs.* cKO Amph4 $p < 0.01$, Challenge $p < 0.0001$).

A closer investigation of the timeline of each session revealed numerous differences between the two genotype groups (**Figure 7C**). During the 30-min pre-injection period,

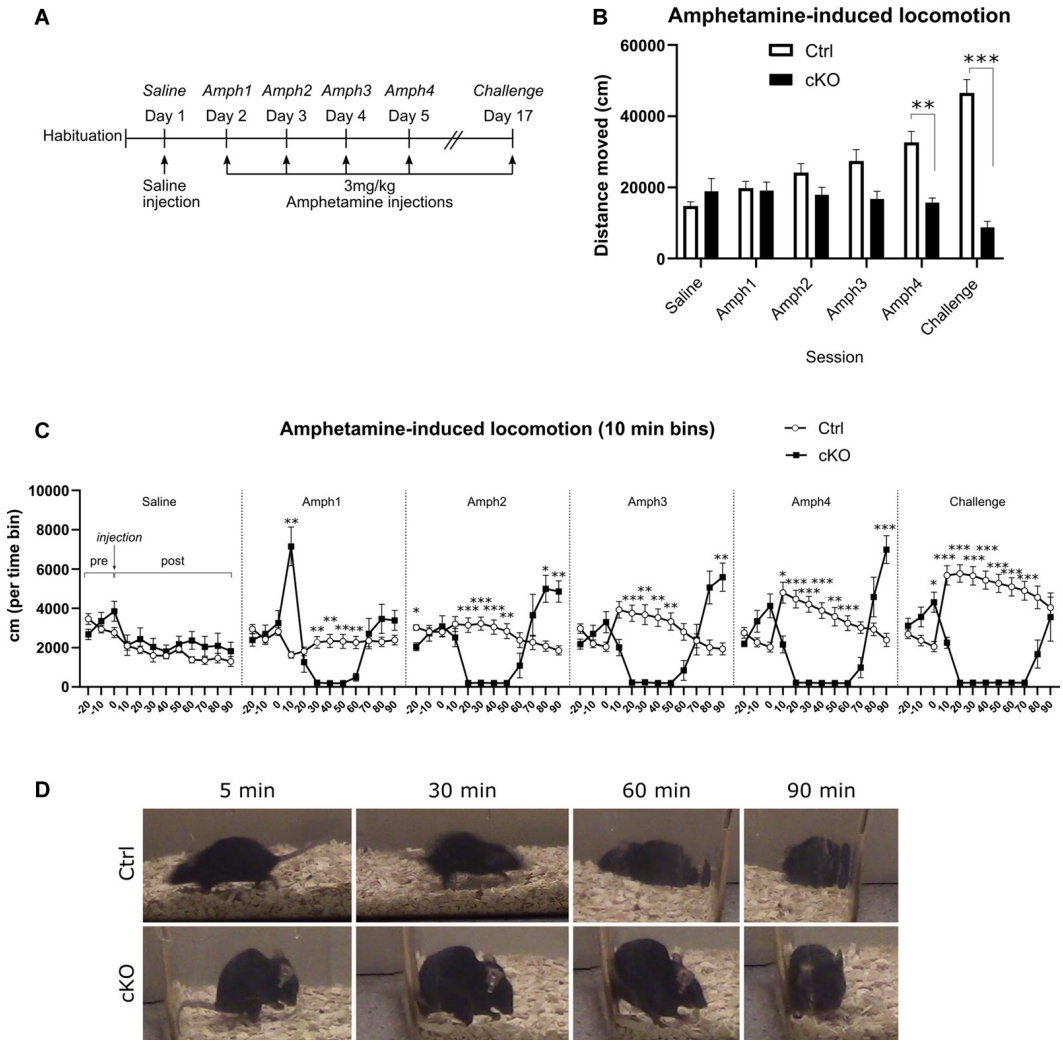

FIGURE 7 | Amphetamine-induced locomotion. A sensitization paradigm **(A)** was applied to investigate the behavioral responses to amphetamine. Horizontal locomotion during 90 min following saline or amphetamine injections **(B)**. Discrete 10-min segments of each session **(C)**, including 30 min before injection ("pre") and 90 min following injection ("post"). An example of the stereotypies exhibited at several time-points after injection of amphetamine **(D)**. Data presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and similarly to the basal locomotion recordings, cKO mice did not habituate to the environment as controls; rather, they tended to increase their horizontal activity during the 30 min leading up to injection (**Figure 7C**, "pre"-part of each session). Ten-min time-bin analysis after amphetamine injection unveiled a contrasting pattern of response between genotypes. Control mice responded to each successive injection of amphetamine by increasing initial surges of activity and gradually returning to pre-injection levels. On the other hand, after amphetamine injections, cKO mice displayed very low levels of horizontal activity, and these low-activity periods became longer during each successive session, followed by a return to, and overshoot of, the baseline activity (**Figure 7C**; two-way RM ANOVA, the effect of genotype $p < 0.0001$; Tukey's *post hoc* test. Each time point is compared between genotypes. $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$. Detailed statistics in **Supplementary Table 1**). During visual inspection of each session as well as a separate detailed analysis of one randomly selected case of each genotype, it became apparent that the observed immobility was the result of engagement in stereotypies, manifested mostly as gnawing of bedding (**Figure 7D**, bottom panels).

DISCUSSION

Given the importance of monoaminergic signaling in mental health and disease, and the heterogeneity of cells that compose these systems, dissecting the circuitry and physiological relevance of specific neuronal sets is paramount to provide possible mechanisms for various neuropsychiatric conditions. Here we have pursued genetic deletion of *Vmat2* selectively in Calb2-positive monoaminergic neurons by creating a new transgenic

strain based on Cre-LoxP technology to address the impact of these neurons on behavioral regulation. We find varying degrees of co-localization of Calb2 and Vmat2 mRNA in fore-, mid-, and hindbrain areas: VTA, SNc, RRF, RLi, CLi, DR (mostly DRV), MnR, DRI, RMg/RIP, Rob, LC, LPGi, C1–3, and also in the arcuate nucleus. Calb2-directed Cre activity resulted in the broad but specific deletion of *Vmat2* in these same areas, which resulted in distinct phenotypic changes. Smaller in size than controls, cKO mice displayed heightened basal locomotion, anxiety-related behaviors, reduced preference for sucrose, and apparent aversion to increasing concentrations of ethanol, as well as a dramatic disengagement in locomotion following amphetamine administration. The anxiety-related behavior was not clear-cut based on the contrasting observations made in the open field and the plus maze, suggesting that further studies might be needed to fully understand this phenotype.

Taken together, the aberrant behavioral displays of the cKO mice give evidence for the substantial impact of Calb2-positive monoaminergic cells and highlight the need for specific and varied approaches to probe subpopulations. In a recently published study, the Calb2-Cre population in the VTA—representing approximately 50% of TH+ cells—was compared to another discrete VTA population defined by the transcription factor NeuroD6, which accounts for only 12% of the TH+ population in the medial aspect of the VTA (Bimpisidis et al., 2019). The Calb2 identity was further characterized as occurring in 50% of Dat (dopamine transporter)-positive cells, 7% of glutamatergic cells (Vglut2-positive), and 20% of GABAergic cells (defined by Viaat, the vesicular inhibitory amino acid transporter). Projection patterns differed for the Calb2 and NeuroD6 populations of the VTA, with fewer extra-VTA targets for the Calb2 population, and a prominent pathway from the VTA to nucleus accumbens in NeuroD6(NEX)-Cre mice. Further, optogenetic stimulation in the VTA indeed produced approach behavior for the NEX-Cre mice in a real-time place preference paradigm, while no such response was observed when Calb2-Cre mice were optogenetically stimulated in the VTA. When *Vmat2* was gene-targeted in NEX-Cre cells, using the same floxed *Vmat2* allele as here, $Vmat2^{lox/lox;NEX-Cre-tg}$ cKO mice, similar to control mice, displayed a preference for sucrose or ethanol over water, as well as conditioned place preference induced by amphetamine or cocaine. However, in an amphetamine sensitization paradigm, $VMAT2^{Vmat2lox/lox;NEX-Cre-tg}$ cKO mice showed locomotor hypersensitivity compared to controls (Bimpisidis et al., 2019; Wallén-Mackenzie, 2019). This is in stark contrast to the behavior observed in $VMAT2^{lox/lox;Calb2-Cre-tg}$ cKO mice studied here, following the same sensitization protocol. Thus, by comparing the phenotypes of mice in which VMAT2 has been deleted in the Calb2 *vs.* the NeuroD6 monoaminergic populations, it is clear that altering monoaminergic signaling in these two populations gives rise to very different behavioral effects. Granted, this likely stems in part from the difference in the distribution of these two genetic subpopulations—whereas NeuroD6/Vmat2 cells are present only in the VTA, Calb2/Vmat2 cells are more widely distributed in the brain, as clearly outlined in the present study.

Histological Findings

The two-probe *in situ* hybridization approach for the detection of Vmat2 mRNA allows unambiguous identification of recombined cells. Binding of Probe1 and Probe2 indicates wildtype cells, whereas binding exclusively of Probe1 identifies cells that have undergone Cre-mediated recombination, resulting in *Vmat2* deletion. As opposed to identifying a mere "loss" of signal in KO cells, this strategy has the advantage that recombination is readily visualized and makes it possible to pinpoint the location and distribution of KO cells. Combined with a fluorescent probe for Calb2 mRNA, we were able to establish the monoaminergic identity of Calb2 cells, and show that recombination of the *Vmat2* allele was directed specifically to these cells. The data solidly confirm recombination of the floxed *Vmat2* allele in several monoaminergic populations throughout the fore-, mid- and hindbrain, more specifically in the VTA, SNc, RRF, RLi, CLi, DR (mostly DRV), MnR, DRI, RMg/RIP, Rob, LC, LPGi, C1–3, and also in the arcuate nucleus. In contrast, Vmat2 mRNA in other hypothalamic and mammillary nuclei was left unmodified. The $Vmat2^{lox/lox;Calb2-Cre-tg}$ cKO is thus a broad KO by targeting multiple monoaminergic systems, but specific in the sense that it is directed to Calb2-positive subpopulations within these monoaminergic systems.

Within the midbrain dopaminergic system—the VTA and SNc—our findings corroborate the approximate distributions of monoaminergic Calb2 neurons found in the rat (Rogers, 1992; Isaacs and Jacobowitz, 1994; Nemoto et al., 1999) and mouse (Liang et al., 1996; Bimpisidis et al., 2019). Among the hypothalamic dopaminergic cell groups, the limited number of Calb2/Vmat2 co-localizing cells in the arcuate nucleus concur with the literature on the rat brain (Rogers, 1992). The absence of Calb2/Vmat2 co-localization in anterior hypothalamic areas as well as the premammillary and lateral mammillary nuclei, and A13 dopaminergic groups, suggest that the Calb2-positive populations identified previously in the rat brain (Résibois and Rogers, 1992) are not monoaminergic. Importantly, beyond the dopaminergic systems, recombination was observed in various serotonergic and noradrenergic nuclei. In these areas, Calb2 mRNA was detected at varying levels, and recombination was to a high degree localized to cells expressing detectable levels of Calb2.

In the raphe nuclei, neurons with Calb2-immunoreactivity have been observed in some serotonergic subareas (Arai et al., 1991; Résibois and Rogers, 1992; Charara and Parent, 1998), although their neurotransmitter phenotype has not been established. Here, within areas such as the DR, there was clear segregation of KO cells between subareas. Surprisingly, no recombined cells were present in the DRD—corresponding to the serotonergic/dopaminergic A10dc part of the DR (Stratford and Wirtshafter, 1990)—which may have been expected given the observed colocalization of Calb2 and TH here (Rogers, 1992). On the other hand, both the DRL and, more prominently, DRV, contained numerous KO cells. Beyond neurotransmitter phenotype and projection patterns, the DR

contains molecularly diverse cells (Huang et al., 2019), and further work would be needed to determine the cellular profile that Calb2/Vmat2 neurons correspond to. Additional serotonergic raphe nuclei with KO cells included the IF, raphe magnus, median raphe, and raphe obscurus. These results give evidence to the heterogeneity of the monoaminergic phenotype of Calb2 cells, which represent subpopulations within parts of the raphe.

With regards to other catecholaminergic cells, although Calb2 protein was not detected in the LC of rats (Résibois and Rogers, 1992; Rogers, 1992), it has been observed in other species (Bhagwandin et al., 2013), and mRNA is detectable in the mouse according to the Allen Brain Atlas (Lein et al., 2007). In the present study, Calb2 and Vmat2 mRNAs co-localize, and there is evident Calb2-Cre-mediated *Vmat2* recombination in the LC, the major source of noradrenaline in the brain, thus giving new evidence to Calb2 as a subpopulation marker of this nucleus. Furthermore, the adrenergic lateral paragigantocellular nucleus displayed numerous knockout cells. The results clearly show that Calb2-driven Cre expression may be used as a genetic tool to target select parts of serotonergic and catecholaminergic populations in the brain.

Conditional Knockout Phenotype

Several of the knockout mouse lines generated to genetically target *Vmat2* preclude behavioral studies, as these transgenic models are so severely affected during development that offspring do not survive into adulthood (Takahashi et al., 1997; Wang et al., 1997; Isingrini et al., 2016b). Here, mice positive for Calb2-Cre and homozygous for *Vmat2lox* ($VMAT2^{lox/lox;Calb2-Cre-tg}$; cKO mice) were characterized by lower weight compared to littermates. This is consistent with several studies investigating deficiencies in monoaminergic signaling (Takahashi et al., 1997; Mooslehner et al., 2001; Narboux-Nême et al., 2013; Isingrini et al., 2016b), where serotonin seems to be critical for a postnatal growth spurt, and intact dopamine signaling necessary for survival and growth past this age. In the present study, while the growth of cKO mice is stunted, the majority survive into adulthood, with more than 80% surviving past weaning age. The observed phenotype is likely the result of a combinatorial effect of changes in dopamine and serotonin signaling, such that most mice survive, albeit never fully recovering a normal body weight. Additionally, noradrenaline signaling is crucial to postnatal survival (Ohara et al., 2013), for which the normal function of VMAT2 in Calb2 noradrenergic neurons does not appear to be crucial. Developmental changes in monoamine signaling induce neuroadaptations, and thus the structures affected by genetic targeting may have altered activity, contributing to aberrant behavior or metabolism. Among the affected areas here are the raphe obscurus, related to breathing (DePuy et al., 2011) and feeding (Wu et al., 2012); the arcuate nucleus, related to several aspects of homeostasis; the LC, related to arousal, sympathetic regulation, and responses to stress as well as cognitive functions (reviewed by Chandler, 2016); as well as the VTA and SNc, related to motivation, reward-related behavior, and movement (Schultz, 1998; Salamone and Correa, 2012; Howe and Dombeck, 2016). Consequently, to address the impact of these cellular changes on behavior, multiple tests were performed to characterize the cKO phenotype.

Conflicting Aspects of Anxiety and Stress-Related Parameters

When placed in a novel environment, cKO mice displayed impaired habituation, suggesting an anxious-like phenotype. Less time spent in the center of the arena than controls supports this finding, arguing against the increased exploratory drive. The elevated plus maze is an established method to study anxiety in rodents, recently validated in a virtual-reality test adapted to humans (Biedermann et al., 2017), where avoidance of open arms measured as time spent is a correlate of anxiety. When tested in the elevated plus maze, cKO mice spent more time in the open arms compared to controls, indicating an anxiolytic phenotype. However, there were important observations during this test that affect this interpretation. cKO mice demonstrated high latency to first entering the closed arms, lower number of entries made into the open arms, and less total movement during the test. The immobility of cKO mice could be interpreted as increased anxiety (Pellow et al., 1985; Walf and Frye, 2007), corroborating the behavior seen in the novel open field test. The results are similar to those seen for a serotonin system-specific knockout of VMAT2 ($VMAT2^{lox/lox;SERT-Cre}$, Narboux-Nême et al., 2011), suggesting this observation could be a serotonergic effect. A more recent study using the elevated plus maze showed no change in heterozygous $VMAT2^{lox/wtx;SERT-Cre}$ mice compared to controls (Isingrini et al., 2016b). Thus, our results indicate that the observed behavioral change stems from a dysfunction in a specific set of neurons rather than a global decrease in serotonin. However, beyond this hypothesis, a firmer validation would require more advanced experimental tools.

For constitutive VMAT2 heterozygous animals, a change in depressive-like phenotype as measured by a decreased preference for sucrose solution has been reported, without concurrent changes in anxiety (Fukui et al., 2007). Similarly, in the present study, cKO mice show a somewhat blunted preference for the sucrose solution—indicative of anhedonia—but show equivocal anxiety-related responses. The possibility of increased freezing responses related to stress specifically in the elevated plus maze remains. Thus, further tests are required to disentangle anxiety, stress, and/or motor-related phenotypes in the cKO mice.

Reduced Ethanol Intake

Monoamine signaling has been linked to several neuropsychiatric conditions, and alterations in these systems may contribute to predisposition to addictions including alcoholism, for which certain variations in the promoter region of the human VMAT2 are indicated as protective factors (Lin et al., 2005). VMAT2 deficient heterozygotes are hypersensitive to the locomotor effects of ethanol (Wang et al., 1997) and show a decreased preference toward ethanol in a two-bottle test (males) as well as diminished conditioned place preference (Savelieva et al., 2006). In the present study, cKO mice did not display a preference for any concentration of ethanol. Rather, a preference score below 50% at all points measured with a

tendency to decrease with increasing ethanol concentration suggests a possible aversion. However, to clearly demonstrate aversion, more specific behavioral tests would be needed, such as conditioned taste aversion. It is unknown for instance if the behavior seen here is a consequence of an aversion to the pharmacological effects or taste of the ethanol. Further, it is unknown whether this effect is caused by a global decrease of neurotransmitter or *via* a functional change specifically in the Calb2/Vmat2 cells. Of note, however, is that the phenomenon is presented in both sexes, rather than isolated to males as for VMAT2 heterozygotes (Savelieva et al., 2006). Cellular recordings of VTA neurons in response to ethanol show a preferential response in medial regions (Mrejeru et al., 2015), and several other regional differences in sensitivity have been indicated. Thus, this population of Calb2 VTA neurons is a potential tool to investigate reward-related responses to ethanol. Finally, the CLi, which here showed extensive recombination of the *Vmat2* gene, has been implicated as a key structure mediating alcohol preference (Dudek and Hyytiä, 2016) and nicotine self-infusion (Ikemoto et al., 2006), further warranting inquiry in the particular role of Calb2-positive monoaminergic neurons in this region. Thus, although direct optogenetic stimulation of Calb2-Cre neurons in the VTA did not result in increased approach behavior in an optogenetic real-time place preference paradigm (Bimpisidis et al., 2019), the present results do tentatively indicate a potential role of these cells in reward-processing.

Motor Activity and Response to Amphetamine

While some results indicate that constitutive *Vmat2* heterozygote mice show no change in basal locomotion (Takahashi et al., 1997), others have shown a decrease in spontaneous activity (Fukui et al., 2007), appears to be specifically related to a reduction of VMAT2 in dopamine transporter (DAT)-Cre-positive cells (Isingrini et al., 2016b). Here, $VMAT2^{lox/lox;Calb2-Cre-tg}$ animals display heightened basal locomotor activity, exemplified by a lack of habituation. During the rotarod task, no difference in motor coordination was detected at different fixed speeds, similar to observations of constitutive VMAT2 heterozygotes (Takahashi et al., 1997).

Different aspects of monoamine signaling have been identified as contributing to the locomotor response to drugs. For instance, altering dopamine signaling is sufficient to alter the motor response to acute amphetamine, while the same relationship was observed between the serotonin system and cocaine (Isingrini et al., 2016b). Here, there is most likely involvement of several of the transmitter systems, although to a more restricted extent than complete knockout of any one system. The decrease in locomotor activity on repeated injection of amphetamine may be explained by increased sensitivity to and engagement in stereotypical behaviors, similar to previous observations in *Vmat2* heterozygotes (Wang et al., 1997) and so-called VMAT-LO mice, which have a hypomorphic *Vmat2* allele resulting in a 95% reduction of VMAT2 (Mooslehner et al., 2001). A possible mechanism is a developmental compensatory oversensitivity to dopamine, which exacerbates the behavioral response to the extra dopamine released on amphetamine stimulation. Importantly, this has previously been linked to neurochemical changes in the direct and indirect output of striatal areas (Mooslehner et al., 2001), and lesions of the nigrostriatal pathway (Fibiger et al., 1973) and the patch compartment of the striatum (Murray et al., 2015) attenuate stereotypy induced by amphetamine and cocaine respectively. As previously observed for VMAT2 heterozygous mice (Wang et al., 1997), the response to the first amphetamine session here was, in fact, greater for cKO mice than controls, while lower in subsequent sessions. This is in keeping with increased engagement in stereotypes following chronic administration of amphetamine (Segal and Mandell, 1974), supporting the hypothesis of neuroadaptive changes. As observed in previous studies (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997; Isingrini et al., 2016b, 2017), dopamine seems to be produced at a normal rate in cells lacking VMAT2 but metabolized more quickly, overall decreasing cellular content. Thus, although amphetamine elicits the non-physiological release of dopamine by reversal of dopamine transporter from cells lacking VMAT2, it is likely that sensitization of output areas plays a greater role in explaining the response. Additionally, given the sites of KO cells including the LC, the behavioral output seen here likely stems from effects exerted by other signaling systems, notably that of noradrenaline signaling, which has considerable interactions with dopaminergic signaling with relevance to the response to stimulants (Ferrucci et al., 2019), and has been implicated in sensitivity to stereotypies (Weinshenker et al., 2002).

Further Directions

Among the areas with an extensive knockout of VMAT2 were the raphe magnus and lateral paragigantocellular nuclei. *Via* distinct projections to the spinal cord, these may have different modulatory effects on nociceptive signaling (Condés-Lara et al., 2012; Gautier et al., 2017). Although it is known that monoamine signaling affects pain signaling, it is hitherto unknown whether VMAT2 deficiency is of any consequence for pain sensitivity. Other brain regions implicated in pain control include the LC (reviewed by Taylor and Westlund, 2017), further warranting investigation of the role of altered VMAT2 function given the histological findings presented here.

In addition to specific tests as presented here, phenotyping of transgenic mouse lines can be performed by home-cage monitoring, which is gaining increasing attention in research methodology. This would enable collecting data on such parameters as wake/sleep patterns, feeding behaviors, and social interactions, allowing further characterization of the knockout line (Richardson, 2015; Balzani et al., 2018; Pernold et al., 2019). Considering the major roles played by monoaminergic neurons, this type of combined analysis in the home-cage environment would likely enhance the understanding of the phenotype obtained when deleting VMAT2 selectively from the Calb2-Cre population of neurons.

Limitations

When assessing the effects of a cKO of any gene, it is relevant to point out that developmental neuroadaptations may

have taken place. This is important not least in the current context since both *Calb2* and *Vmat2* genes, i.e., both the driver and the effector, are induced early on in embryonal development (Dumas and Wallén-Mackenzie, 2019). Thus, it is not possible to conclude as to when the herein reported phenotypes are established, other than that they are present in the adult mouse.

Further, while the present study aimed at characterizing the distribution of Calb2+/Vmat2+ populations in the brain, and also address their significance in behavior by genetic targeting of the *Vmat2* gene selectively in the Calb2-positive subtype of monoaminergic neurons, no analysis of neurotransmitter release or the expected down-regulation of VMAT2 protein levels were performed. This is a caveat of the present study. Other studies have indeed reported these types of analyses (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997; Mooslehner et al., 2001; Narboux-Nême et al., 2011; Isingrini et al., 2016b) while the present study lacked this focus and hence, certain mechanistic conclusions cannot be drawn.

For many of the parameters studied, crosstalk on molecular and cellular levels between the different monoamine systems exist—for instance, locomotor response to cocaine has been suggested to be exaggerated due to potentiation of the 5HT1A receptor (Szumlinski et al., 2004), and indeed diverging results were found depending on the involvement of one or several monoamine systems (Isingrini et al., 2016b). At the network level, the transmission of neurotransmitters has cross-modulatory effects, such as described for dopamine and noradrenaline (Smith and Greene, 2012; Ferrucci et al., 2019). Considering the above-mentioned limitations of the present study, pharmacological interventions or the site-specific introduction of shRNA targeting *Vmat2* in adult Calb2-Cre mice may clarify the involvement of each region and corresponding transmitter system, as well as avoid developmental effects of a constitutive knockout.

Conclusion

Taken together, the present findings contribute to the observations that disrupted monoaminergic signaling in neurons defined and joined by their expression of the *Calb2* gene plays important roles in diverse facets of behavior, and provides new evidence for the presence and distribution of Calb2 mRNA in monoaminergic brain regions. Site-specific and adult interventions targeting selected Calb2-positive monoaminergic populations would allow delineation of the precise role of these 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 Uppsala Ethical Committee for Laboratory Animal Research (Uppsala djurförsöksetiska nämnd Uppsala Tingsrätt Box 1113 751 41 Uppsala).

AUTHOR CONTRIBUTIONS

ÅW-M conceived the study and was in charge of overall planning. NK, ZB, SD, and ÅW-M designed and performed research, and analyzed data. ÅW-M, NK, and ZB wrote the manuscript. 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/fnbeh.2020.578443/full#supplementary-material.

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Conflict of Interest: SD is the owner of Oramacell.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Drosophila para^{bss} Flies as a Screening Model for Traditional Medicine: Anticonvulsant Effects of *Annona senegalensis*

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Epilepsy is among the most common serious neurological disorders and affects around 50 million people worldwide, 80% of which live in developing countries. Despite the introduction of several new Anti-Epileptic Drugs (AEDs) in the last two decades, one third of treated patients have seizures refractory to pharmacotherapy. This highlights the need to develop new treatments with drugs targeting alternative seizure-induction mechanisms. Traditional medicine (TM) is used for the treatment of epilepsy in many developing countries and could constitute an affordable and accessible alternative to AEDs, but a lack of pre-clinical and clinical testing has so far prevented its wider acceptance worldwide. In this study we used *Drosophila melanogaster paralytic bangsensitive* (*para*^{bss}) mutants as a model for epileptic seizure screening and tested, for the first time, the anti-seizure effect of a non-commercial AED. We evaluated the effect of the African custard-apple, *Annona senegalensis,* which is commonly used as a TM for the treatment of epilepsy in rural Africa, and compared it with the classical AED phenytoin. Our results showed that a stem bark extract from *A. senegalensis* was significantly more effective than a leaf extract and similar to phenytoin in the prevention and control of seizure-like behavior. These results support that *Drosophila* constitutes a robust animal model for the screening of TM with potential value for the treatment of intractable epilepsy.

Keywords: *Annona senegalensis*, bang sensitive, *Drosophila melanogaster*, epilepsy, *para*^{bss}, phenytoin, seizure, eas^{2F}

INTRODUCTION

Epilepsy is among the most common serious neurological disorders and affects around 50 million people worldwide, with 80% of them living in developing countries (1–4). Epilepsy can cause frequent seizures, which are brief episodes of involuntary shaking involving part of or the entire body, and are sometimes accompanied by a loss of consciousness. Seizures can vary in intensity from brief lapses of attention or muscle jerks, to severe and prolonged convulsions. They can also vary in frequency, from less than one per year to several per day.

These episodes are triggered by hyper-excitation and/or abnormal synchronization of activity across neuronal circuits (5). The causes of epilepsy are attributed to acquired vs. genetic factors. Acquired epilepsy, such as those resulting from trauma, stroke, neoplasm, infection, congenital malformations, birth anoxia and autoimmune, represent slightly more than a quarter of the cases. Epilepsy with complex inheritance, ranging from mono-gene mutations to the presence of modifiers and susceptibility alleles has emerged as the main causes of idiopathic epilepsy (6). Mutations in channels, that determine cellular excitability, have been associated with a wide range of epilepsies (5, 7). Amongst them is the sodium channel SNC1A, the human ortholog of the *para*^{bss} gene of *Drosophila melanogaster*, with over 600 different mutations found in patients (8). Links to other ionic channels (e.g., voltage-gated sodium channel a2 gene subunit SCN2A), receptors (e.g., the N-methyl-D-aspartate-type glutamate receptor NR2A subunit GRIN2A), synaptic proteins (e.g., PRRT2 synaptic release) and brain development pathways (e.g., mTOR) have also been proposed. Anti-epileptic drugs (AEDs) mechanism of action aims to control neuronal hyper-excitation by modifying ion channels functioning (8). Recent studies in both developed and developing countries have shown that up to 70% of epilepsy patients can be successfully treated (i.e., their seizures completely controlled) with AEDs (9, 10). But these drugs produce undesired secondary effects and in 30% of patients they are ineffective, highlighting the need to develop new alternative treatments (9, 10) aimed at alternative cellular mechanisms acting to stabilize neuronal activity.

In many developing countries, particularly in Africa and Asia, phenobarbital is the most commonly first-line prescribed AED as recommended by the World Health Organization (WHO) (7, 11–13), and this is likely because the other proven AEDs phenytoin, carbamazepine, and valproate are up to 5, 15, and 20 times more expensive, respectively (14–16). Despite the fact that access to medicines can cost as little as US$5 per year, three quarters of patients with epilepsy have no access to treatment (1). The availability and accessibility of Traditional Medicine (TM) means that it plays an important role in meeting the demands of primary health care. This was recognized by the 2008 WHO Congress on Traditional Medicine in Beijing (17), which resolved to promote the wider role of TM in worldwide health care and declared that TM should be "further developed based on research and innovation" (17).

The wild African custard-apple, *Annona senegalensis* (Magnoliales: Annonaceae) is commonly found in savannas throughout tropical Africa. Also known as *soursop*, *dorgot* (Wolof) and *sunkungo* (Mandinka), a decoction of its leaves and roots is used by rural African communities as a TM for the treatment of seizures, suggesting that the preparation has anticonvulsant and/or sedative properties (18). Studies have shown that extractions from root or stem have mild anticonvulsant properties on chemically induced seizures in rodents, supporting a possible role of this TM in the treatment of epilepsy (19–24). These experiments are very promising, but the screening of drugs in rodents is too costly to test the majority of ethnobiologically important candidates. Furthermore, follow-up studies investigating the pharmacological properties of novel drug candidates are intricate and even more expensive. There is therefore a need to develop a reliable, cost-effective and high throughput method for the screening of TMs with apparent anti-seizure properties that could be implemented prior to testing in animal models and clinical trials.

Adult *Drosophila* flies represent a genetically accessible and behaviorally tractable model for the study of seizures (25–27). Its strength resides in the high evolutionary conservation of most molecules controlling neural function (28). In particular, ion channels and synaptic transmission machinery proteins are largely comparable (29). *Drosophila* and humans also share several similarities in seizure phenotype thereof: (i) all individuals have a seizure threshold; (ii) seizure susceptibility can be modulated by genetic mutations; (iii) seizure activity threshold is increased by a previous electroconvulsive shock treatment; (iv) seizure activity spreads through the central nervous system (CNS) along particular pathways; (v) there is a spatial segregation of seizure activity into particular regions of the CNS; and (vi) seizure phenotypes in flies can be ameliorated by several AEDs used in humans including sodium valproate, phenytoin, gabapentin, and potassium bromide (25).

Furthermore, because *Drosophila* presents little gene redundancy, it offers a unique opportunity to study human mutated genes in an animal model (30, 31). Using CRISPR/Cas9 (32) it is easy to generate knock-in mutants where the endogenous copy of the gene is replaced by a mutated version of the human homolog, and these flies can then be used for drug testing to reveal behavioral, physiological and molecular effects (30, 31).

In this study we evaluated, for the first time, the effect of a non-commercially available drug for the treatment of seizure in *Drosophila* adult flies. We tested the hypothesis that *A. senegalensis* leaf and stem bark extracts affect the seizure patterns present in *para*^{bss} mutant adult flies (33, 34). We showed that *A. senegalensis* stem bark extract was significantly more effective than a leaf extract and similar to phenytoin in the prevention and control of seizure-like behavior. These results support that *Drosophila* is a robust and sensitive animal model that can be used to screen pharmacologically untested compounds which, combined with centuries of transmitted knowledge about anti-convulsant TMs, could expedite the development of the most effective TMs into novel AEDs for patients irresponsive to classical treatments.

MATERIALS AND METHODS

Identification and Extract Preparation of *Annona senegalensis*

Annona senegalensis leaves and root barks were collected from "Boroboro," 5 Km from Lira Municipality along Soroti road, Northern Uganda. Geographic coordinates are 2.190341, 32.929115 (2011'25.2"N 32055'44.8"E). Plant was identified/deposited at Department of Biology Mbarara University of Science and Technology, Uganda and given a voucher No. Moses Odur 002.

The leaves and bark removed from stem were dried and subjected to an aqueous extraction method at the Department of Pharmacology laboratory (School of Health Sciences, Kampala International University Western Campus, Ishaka-Bushenyi, Uganda). Specifically, dry leaves and stem were grounded using a blender to obtain 200 g of powder which was mixed with 1 l of distilled water in a sterile conical flask. The mixture was placed on a shaker for 72 h and then sieved to remove debris. The remaining liquid was filtered by gravity using Whatman no. 1 filter paper. The filtrate was incubated at 35°C for 1 week to evaporate the water and obtain a dry powder (35). The powder extract, approximately 10 g, was stored at 4°C until the behavioral experiments were performed.

A phytochemical analysis was performed on the aqueous extracts of the leaf and stem bark at the Kampala International University Biochemistry department lab (**Supplementary Tables 1, 2**).

Drug Preparation

Given the concentration of the active compound in the aqueous *A. senegalensis* extract or the effective dose was unknown, the initial assessments were done using a high concentration (26.66 mg.ml^{-1}). Animals treated with this extract solution appeared healthy and showed less seizure-like behavior than controls. We decided to expand the analysis to lower extract concentrations. *A. senegalensis* leaf and bark stem extracts and phenytoin were administered by mixing them with standard cornmeal food, which consists of 420 g of cornmeal; 450 g of dextrose; 90 g of yeast; 42 g of agar; 140 ml of 10% Nipagin in 95% EtOH; 22 ml of propionic acid and 6.4 l of water. Drug and extract solutions were prepared fresh before each experiment. Experimental doses are expressed as mg of compound per ml of food. For 13.33 and 26.66 mg.ml^{-1}, the powder leaf or bark stem extract was dissolved in distilled water in a beaker at room temperature. Warm food (<60°C) was then added and mixed before aliquoting approximately 1.5 ml per testing vial. For the low concentration doses of 0.26, 1.33, and 2.67 mg.ml^{-1} a stock solution of 2.67 mg.ml^{-1} was used.

For phenytoin, one 100 mg tablet (Tophen; Agog Pharma Ltd, India) was suspended in 1 ml of distilled water, and 50, 250 µl or 500 µl of drug suspension were then mixed with 5 ml of food resulting in the low and high concentrations, respectively: phenytoin 0.909, 4.76, and 9.09 mg.ml^{-1}.

Flies Treatment and Behavioral Analysis

Flies of the bang sensitive family, specifically *bang senseless* (*bss1*) mutants of the paralytic gene *para*^{bss1} and easily shocked (*eas*^{2F}) were obtained from Prof Richard Baines laboratory (University of Manchester, UK), were used (26). Oregon R (OrR) is a wildtype strain that served as a control.

Young adult flies (6–9 days) were anesthetized by cold exposure in a freezer (−20°C), separated on a cold plate into males and females and assigned to vials with 10 animals each. The flies were then randomly assigned to the different treatments vials, (standard cornmeal food with or without drug/extract). Each treatment was tested in at least 3 different days with flies from different cultures.

Flies were allowed to feed for 24 h prior to behavioral manipulations. On the day of behavioral assessment, flies were gently transferred into empty vials and immediately mechanically stimulated by placement on a bench-top vortex at maximum speed for 10 s (36, 37). Number of flies per vial on their backs, paralyzed, shaking or standing were recorded at 30 s intervals for 20 min. Paralyzed and shaking behaviors were recorded as seizures, whereas fully recovered flies were defined as showing standing behavior. Number of replicates and number of flies tested is detailed in **Figure 1** legend and in **Table 1**. A few flies died before testing (sticking to the food during transfer or the 24 h drug treatment) and we had counted 1 or 2 extra flies in a few tubes. The average number of female flies tested for each treatment compared with the control was not different apart for female leaf 0.27 mg.ml^{-1} and 1.33 mg.ml^{-1} [ANOVA $F_{(13,\ 101)} = 4.20$, $p < 0.0001$, $\eta^2 = 0.35$; Dunnett's multiple comparisons test $p = 0.008$ and $p= 0.01$, respectively]. No differences were registered in males [$F_{(13,95)} = 1.65$, $p = 0.09$, $\eta^2 = 0.18$]. This supports that survival of flies was not affected by drug or extract treatment condition. We aimed to have 8 tube replicas for each extract or drug concentration but labeling errors between male and female flies produced variability on the final sample size.

Mean recovery time was calculated as the mean of the time it took for each individual that showed a bang sensitive phenotype to complete recovery. Flies that never seized were not included and animals that had not completely recovered at the end of the experiments were included as 1,200 s.

Sequencing of *paralytic* Gene

Male flies were given a vortex shock to induce seizures as describe above. Seizing males were separated from non-seizing males. The DNA of individual *para*^{bss} and OrR flies was extracted using microLysis plus (Clent-Life Science), part of gene amplified using F1: TGTCAAGTGTTTATGTCTCGAGC and R1: CAGATGTTGAACAGGGCCG and sequenced using F2: TCCAGGAGCTTTAGTCGCC and R1 primers that allow to amplify the region that encodes the amino acid in 1699 which is mutated in *para*^{bss} mutants (38).

Gut Content Quantification

Groups of 10 flies were fed with the extract solutions plus 0.5% (w/v) bromophenol blue salt (B5525, Sigma) for 24 h, as in the behavioral experiments. Sub-groups of 3 flies where homogenized in 30 µl of PBS (1x) with a plastic pestle in a 1.5 ml Eppendorf tube, centrifuged twice at 13,000 rpm for 2 min (39). Dye content of the second supernatant was quantified at 594 nm using a NanodropOne spectrophotometer (Thermo Scientific). The background absorbance was obtained processing groups of flies reared in the same manner but on dye-free food. The gut food content was calculated based on a standard curve done with serial dilutions of 0.5% (w/v) bromophenol blue in PBS.

Seizuring Data Normalization

Seizure data for each sex was normalized independently. Mean recovery of control flies (without extract/drug) at $t = 0$ (Rc_{t0}) was considered as 0% recovery, and used to normalize recovery for

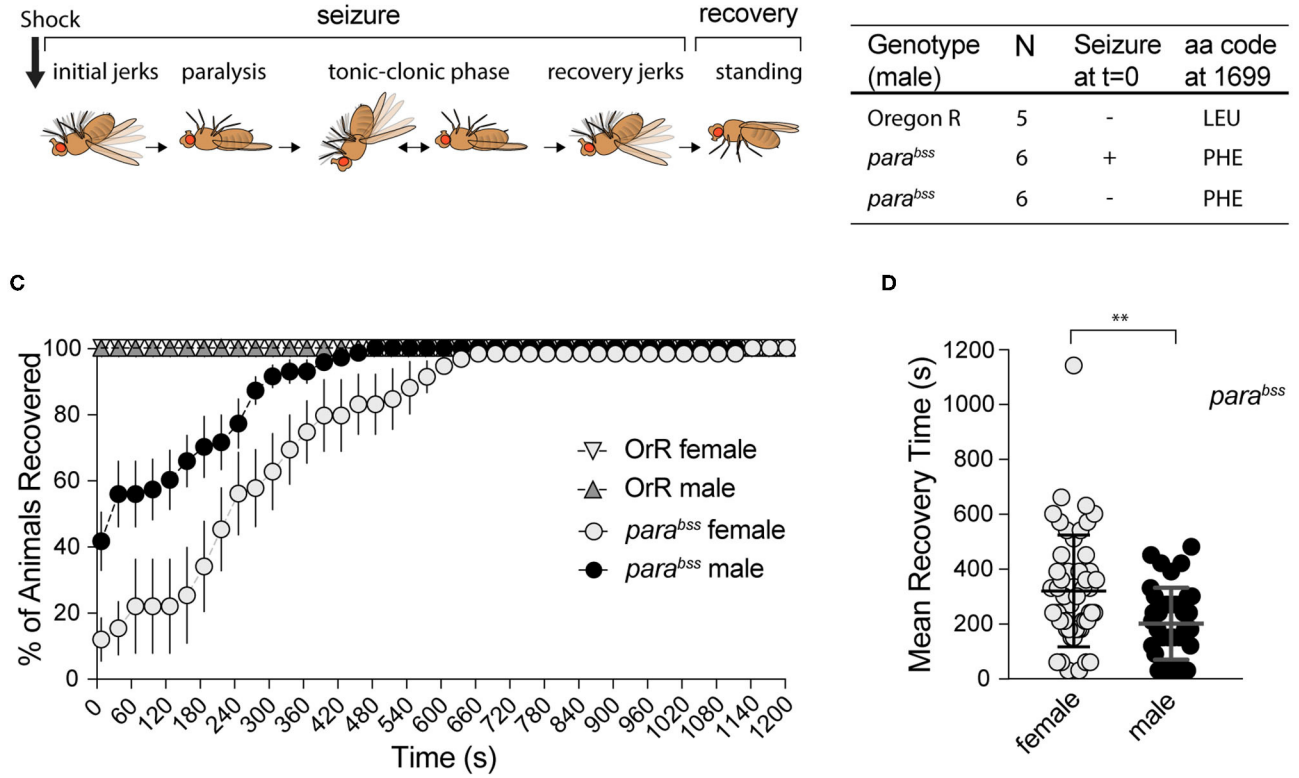

FIGURE 1 | Effect of mechanical shock on seizuring behavior for female and male adult flies of *para*^{bss} or OrR genetic background. **(A)** Schematic of the different phases of seizure after a mechanical shock [modified from Parker et al. (38)]. **(B)** Result of *para*^{bss} gene sequencing in seizuring and non-seizuring males to test for the presence of the PHE mutation in position 1699 of the protein. All flies have the mutation. **(C)** Mechanical stimulation did not induce seizuring in OrR flies. *para*^{bss} flies showed high proportion of seizuring at $t = 0$, the percentage of flies recovered increased as a function of time. The graph shows the mean % of flies recovered ± SEM [*para*^{bss} females $n = 6$ (60 flies); *para*^{bss} males $n = 7$(70 flies); OrR females $n = 3$ (30 flies); OrR males $n = 3$(30 flies)]. **(D)** Mean recovery time comparing males and females *para*^{bss} ($_{Mann-Whitney}U = 667$, $n_{females} = 51$, $n_{males} = 41$, $P = 0.0034$ two-tailed).

each experimental condition at $t = 0$ (Rti_{t0}). Therefore, recovery normalized was calculated as $R_{norm} = (Rti_{t0}–Rc_{t0})/(100–Rc_{t0}) \times 100$. Normalized values were used to calculate mean and SEM.

Statistical Analysis

All behavioral data was expressed as the mean % of flies per experimental vial ± SEM. Mixed ANOVAs, with group (sex or treatment) as the between-subject factor and time as the within-subject factor, were used to analyse recovering behavior. If the ANOVA showed a significant effect of time, drug treatment or the interaction between time and drug treatment, we performed a Dunnett's *post hoc* test to compare between sexes and/or drug concentrations. An ANOVA and Dunnett's *post hoc* test comparing treatments was applied to evaluate differences at $t = 0$.

Because the distribution of MRT is not normal (as tested with D'Agostino & Pearson's normality test), a Kruskal-Wallis test followed by Dunn's multiple comparisons test to compare the MRT for each drug treatment to the control was done. A two-way ANOVA with *post hoc* Dunn's multiple comparisons test was performed to analyse the food intake experiment. The α level was set at 0.05 for all analyses.

Biosecurity Statement

The animal facility, flyroom, is a secure facility with access only granted to researchers who have had an induction to the animal facility covering health and safety and biosecurity arrangements. Access is through a swipe card system with approval only given on completion of the induction. To prevent animals escaping, the flyroom windows are sealed and the doors remain closed except when people are coming in and out and when moving equipment. In all cases there are at least three doors between the flyroom and the outside to further minimize the risk of animals escaping from the facility.

RESULTS

Behavioral Characterization of *para*^{bss} Mutants

In order to evaluate the effect of *A. senegalensis* extracts on seizures, we first characterized the seizure-like phenotype (from now on referred to as seizuring) of our *para*^{bss} mutant strain. *para*^{bss} is a bang-sensitive mutant, extremely sensitive to seizures that are characterized by 6 phases (**Figure 1A**). In response to

TABLE 1 | Prophylactic effect of the Drug and Extract Treatments in *para*^{bss} flies.

Treatment *para*^{bss} flies	Concentration (mg.ml-1)	% of recovery at $t = 0$ Females			% of recovery at $t = 0$ Males		
		(Mean ± SEM)	*p*	*n* (# flies)	(Mean ± SEM)	*p*	*n* (# flies)
Control	0	15.85 ± 4.71		12 (134)	53.95 ± 4.07		9 (89)
Phenytoin	0.91	66.13 ± 9.55	<0.0001	8 (71)	89.86 ± 3.28	<0.0001	8 (79)
	4.76	54.67 ± 10.68	0.01	5 (56)	91.67 ± 3.33	<0.0001	8 (76)
	9.09	56.13 ± 4.82	0.003	6 (59)	83.15 ± 5.12	0.001	6 (51)
A. senegalensis	0.27	34.88 ± 5.06	0.39	8 (68)	75.87 ± 3.88	0.01	8 (75)
Leaf extract	1.33	45.36 ± 4.52	0.015	9 (78)	78.7 ± 6.64	0.01	6 (57)
	2.66	45.39 ± 4.17	0.25	8 (79)	66.25 ± 5.98	0.31	8 (80)
	13.3	58.84 ± 7.70	0.04	8 (89)	71.94 ± 4.80	0.07	8 (77)
	26.6	51.34 ± 7.96	0.006	9 (89)	76.28 ± 6.65	0.01	8 (75)
A. senegalensis	0.27	64.20 ± 10.56	<0.0001	8 (72)	86.83 ± 5.53	<0.0001	8 (78)
Stem Bark extract	1.33	71.77 ± 5.97	<0.0001	7 (62)	89.89 ± 3.61	<0.0001	7 (65)
	2.66	45.39 ± 4.17	0.02	9 (88)	86.25 ± 3.75	<0.0001	8 (80)
	13.3	44.42 ± 7.55	<0.0001	9 (92)	74.21 ± 6.24	0.03	8 (82)
	26.6	49.09 ± 7.25	0.001	8 (78)	77.47 ± 4.97	0.005	9 (92)

The percentage of recovery of para^{bss} *flies was evaluated at t = 0, just at the end of the mechanical stimulation. ANOVAs showed effect of treatment in female [*$F_{(13,114)} = 4.48$, $p < 0.0001$*] and male flies [*$F_{(13,108)} = 4.74$, $p < 0.0001$*]. Dunnett's post hoc test comparing each treatment against the control for each sex is shown in column p. The n (# flies) column shows the number of replicates (n) and flies tested (in brackets) per condition.*

a mechanical shock, many flies become immediately paralyzed while others have a short period of shaking accompanied by leg twitching, abdominal muscle contractions, rapid wing flapping and proboscis extensions before paralysis. In a fraction of animals' paralysis is interrupted by muscle jerks, in what has been described as a tonic-clonic phase. Most flies recover within 10 min following the mechanical shock, some of them after a short period of recovery shaking. After 20 min all animals are capable of maintaining their standing posture and can walk or fly (38) (**Figure 1A**).

The seizure phenotype observed in *para*^{bss} is due to a gain of function mutation, L1699F, in the sole fly voltage gated sodium channel gene *paralytic* (38) (**Figure 1B**). This mutation alters the voltage dependency of channel inactivation, making neurons more excitable and increasing the risk of aberrant electrical activity and seizures (38). Mutations in the human ortholog, *SCN1A*, are associated with a wide spectrum of epilepsies with over 600 mutations registered in this sole locus (8).

To induce seizures, we performed a mechanical stimulation (10 s vortex) to *para*^{bss} and wildtype OrR adult flies. We evaluated behavioral patterns of female and male flies separately because *para*^{bss} mutation is located in the X chromosome.

Immediately after induction ($t = 0$), none of the 60 (10 per vial) wildtype OrR flies evaluated showed a seizuring phenotype, while 88 ± 5% of *para*^{bss} females and 59 ± 8% of males seizuring (Two-way ANOVA, $p = 0.023$; **Figure 1C**). As expected, after a few seconds paralyzed, flies started to recover. Some of them recovered their standing posture directly while others went through a tonic-clonic phase, showing strong wing flapping and leg shaking, before standing. Mean recovery time (MRT) for flies showing a seizure-like phenotype at the beginning of the experiment was longer for females (319 ± 28 s) than for males (201 ± 20 s; Mann-Whitney test: $U = 677$, $p = 0.0034$; **Figure 1D**).

A mixed ANOVA on percentage of animals recovered (**Figure 1C**) showed an effect of time [time: $F_{(40,440)} = 34.03$, $p < 0.001$, $\eta^2 = 0.76$] and sex [$F_{(1,11)} = 11.16$, $p = 0.007$, $\eta^2 = 0.50$], and an interaction [time X sex: $F_{(40,440)} = 3.17$, $p < 0.001$, $\eta^2 = 0.22$]. These results showed an unexpected sexual dimorphism in seizuring phenotype, with males being more resistant to the initiation of seizures and recovering faster than females if and when they showed the seizure phenotype. We confirmed that our stock still carries the mutation described in the literature, sequencing the paralytic gene in OrR wildtype flies and *para*^{bss} mutants. All 12 mutants, 6 of which did not seize at $t = 0$, had LEU mutated to PHE in position 1699. Therefore, despite these differences, the high penetrance of seizure-like behavior and the reproducibility of the phenotype confirm that *para*^{bss} flies are well suited to evaluate anti-seizure properties of candidate compounds as well as the dynamics of recovery from seizuring and indicate that male and female behavior need to be assessed separately.

Effect of *Annona senegalensis* Extracts: Female Flies

To evaluate the effect of *A. senegalensis* as an anticonvulsant, we compared the seizure-like behavior of *para*^{bss} flies kept in control food to animals treated with food mixed with aqueous extract from leaf or stem bark of *A. senegalensis*. As a positive control, a group of flies was treated with phenytoin, a commonly prescribed AED which has already been tested in flies (36) (**Figure 2**). Flies received an acute drug treatment consisting of feeding on control or treated food for 24 h before behavioral testing. Seizure-like behavior measured as the % of recovered animals across time

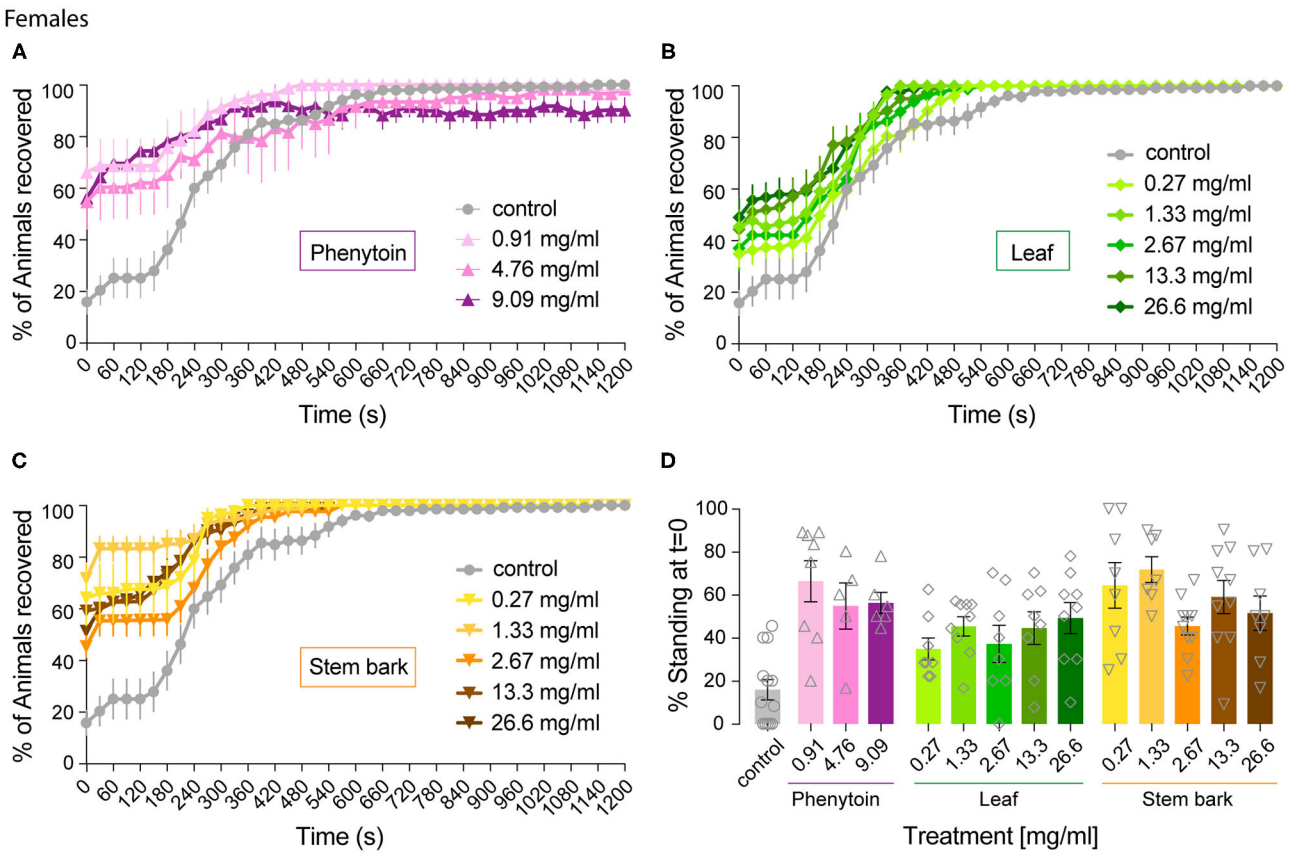

FIGURE 2 | Effect of phenytoin and *A. senegalensis* leaf and stem extracts on recovery from seizures of *para*^{bss} female flies. *para*^{bss} flies treated with control food showed a very low percentage of flies recovered from seizures after mechanical perturbation, which improved over time. **(A)** Treatment with phenytoin increased the percentage of recovered flies at three different doses. **(B)** Treatment with *A. senegalensis* leaf extracts increased the percentage of flies recovered. **(C)** *A. senegalensis* stem extract doses showed a greater effect than the leaf extract. **(D)** Dose response analysis at $t = 0$ for all treatments. Graphs show the mean % of flies seizuring ± SEM (for number of replicates, flies and significance values see **Table 1**).

was analyzed by mixed ANOVA comparing the effect of drug and time for female flies. An effect of time [time: $F_{(40,4040)} = 238.83$, $p < 0.0001$, $\eta^2 = 0.70$], drug [drug: $F_{(13,101)} = 4.53$, $p < 0.0001$, $\eta^2 = 0.37$] and an interaction [time X treatment: $F_{(520,4040)} = 3.29$, $p < 0.0001$, $\eta^2 = 0.30$] was found in females confirming that the treatments modified the time of recovery from seizures. We therefore analyzed the individual effect of each drug treatment on the seizure-like phenotype.

As expected, *para*^{bss} flies kept in control food were significantly affected by the mechanical stimulation, with 15.85 ± 9.55% of flies standing at $t = 0$ and a MRT of 284 ± 21 s (**Figure 3A**).

As a positive control we evaluated phenytoin, a drug known to modulate the gating of voltage gated sodium channels (40). Phenytoin has already been shown to diminish seizures in *para*^{bss} flies, but those tests used different concentrations to the range used in our experiments and males and females were analyzed together. In the present study, phenytoin significantly improved the seizure-like phenotype of *para*^{bss} females over time. A mixed ANOVA on % of animals recovered in control vs. the three phenytoin concentrations showed significant interaction effects of treatment across time [time X treatment: $F_{(120,1120)} = 5.07$, $p < 0.0001$, $\eta^2 = 0.35$] and time alone [time: $F_{(40,120)} = 39.94$, $p < 0.0001$, $\eta^2 = 0.59$], and an effect of treatment [$F_{(3,28)} = 3.69$, $p = 0.02$, $\eta^2 = 0.28$; **Figure 2A**]. A follow-on *post hoc* analysis showed a significant effect of phenytoin at 0.91 mg.ml^{-1} (Dunnett test: vs. control $p = 0.007$) but no effect at the higher doses, 4.76 and 9.09 mg.ml^{-1} ($p > 0.3$). At 4.76 and 9.09 mg.ml^{-1} we observed that the curves of % of recovery were below the curve of control after 480 s indicating that *para*^{bss} flies were recovering faster than the flies treated with the higher concentrations (**Figure 2A**). Indeed, phenytoin failed to shorten the MRT at 4.76 and 9.09 mg.ml^{-1} and the tendency was to increase it (**Figure 3A**), suggesting that the higher concentrations might have a small toxic effect on the flies. However, phenytoin had a clear prophylactic effect at $t = 0$. The three concentrations increased the percentage of recovered flies being the lower concentration the most effective (Dunnett test: control vs. 0.91 mg.ml^{-1} $p < 0.0001$; vs. 4.76 mg.ml^{-1} $p = 0.009$ and vs. 9.09 mg.ml^{-1} $p = 0.003$; **Figure 2D** and **Table 1**). At these concentrations, phenytoin protected against the initiation of seizure (**Figures 2A,D**, **3**).

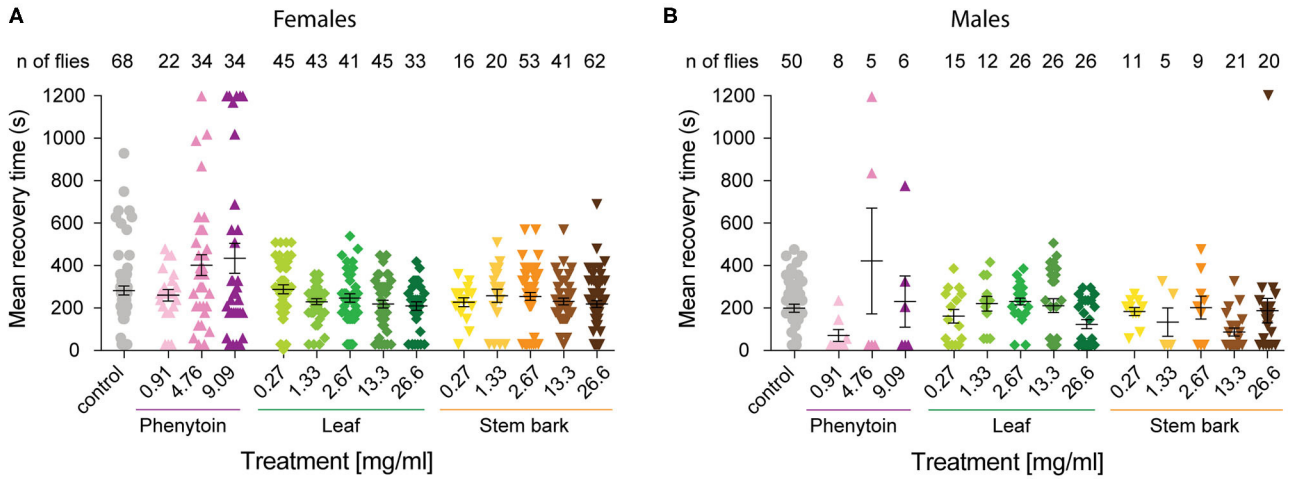

FIGURE 3 | Mean recovery time for *para*^{bss} flies treated with phenytoin and, leaf and stem bark *A. senegalensis* extracts. The time it took for each individual that showed a bang sensitive phenotype to complete recovery was calculated. Flies not seizuring were not included and if a fly was still seizuring at the end of the experiment its time was recorded as 1,200 s. Mean ± SEM is shown. **(A)** The MRT was significantly affected by the treatment in females (Kruskal-Wallis test $H_{(14)} = 30.21$, $p = 0.004$). There were no differences between the control and other treatments as tested with a Dunn's *post hoc* test. **(B)** The MRT was significantly affected by the treatment in males (Kruskal-Wallis test $H_{(14)} = 31.04$, $p = 0.003$). Dunn's *post hoc* test showed that the control was only different with the stem bark 13.3 mg.ml^{-1} treatment ($p = 0.02$). Number of males is lower because MRT measures the time to recovery of paralyzed flies, and fewer males than females where paralyzed at $t = 0$.

We then analyzed the effect of *A. senegalensis* leaf extract (**Figures 2B,D**). The five curves of animals treated with the leaf extract showed an improvement in the percentage of flies recovered from seizure over time. This observation was confirmed by a mixed ANOVA on the percentage of flies recovered, which showed significant effects of time [time: $F_{(40,1960)} = 189.37$, $p < 0.0001$, $\eta^2 = 0.79$], treatment [drug: $F_{(5,49)} = 6.56$, $p < 0.0001$, $\eta^2 = 0.40$] and their interaction [time X drug: $F_{(200,1960)} = 3.07$, $p < 0.0001$, $\eta^2 = 0.24$]. A follow-on Dunnett's *post hoc* analysis comparing each drug with the control showed no effect at 0.27 mg.ml^{-1} ($p = 0.31$) and a greater time-dependent recovery in animals treated with the leaf extract above 1.33 mg.ml^{-1} (control vs.: 1.33 mg.ml^{-1} $p < 0.0001$; 2.67 mg.ml^{-1} $p = 0.05$; 13.3 mg.ml^{-1} $p = 0.002$ and 26.6 mg.ml^{-1} $p = 0.001$). The quantification at $t = 0$ showed an improvement in the percentage of flies insensitive to seizure depending on the concentration with a significant prophylactic effect at 0.27, 1.33, 13.33, and 26.6 mg.ml^{-1} (**Figure 2D** and **Table 1**). To test if the leaf extract alleviates the seizure by reducing their duration, we quantified the MRT. There was no shortening of the MRT comparing the *para*^{bss} control to the five concentrations of leaf extract (Kruskal- Wallis with Dunn's *post hoc* test; **Figure 3A**). All together, these results indicate an anti-seizure effect of *A. senegalensis* leaf extract on *para*^{bss} female flies that is mainly attributed to a prophylactic effect of the drug.

Finally, we evaluated the anti-convulsant properties of *A. senegalensis* stem bark extract (**Figures 2C,D**). A mixed ANOVA on percentage of flies recovered showed significant effects of time [time: $F_{(40,1920)} = 105.02$, $p < 0.0001$, $\eta^2 = 0.69$], treatment [$F_{(5,48)} = 9.67$, $p < 0.0001$, $\eta^2 = 0.50$] and of their interaction [time X treatment: $F_{(200,1920)} = 5.05$, $p < 0.0001$, $\eta^2 = 0.35$]. A follow-on *post hoc* analysis comparing the different concentrations to the control showed that the stem bark extract treatment produced a very significant improvement of the % of recovery (Dunnett test: control vs. 0.27, 1.33, 13.33, and 26.6 mg.ml^{-1} $p < 0.0001$ and 2.6 mg.ml^{-1} $p = 0.006$). Furthermore, the five concentrations of the drug showed a significant increment of the % of flies recovered compared to control at $t = 0$ (**Figure 2D** and **Table 1**). However, this differences over time were not reflected in shorter MRTs (**Figure 3A**). Meaning that if a fly has started seizing it will recover on average at the same pace as a *para*^{bss} mutant which was not treated with the stem extract. As for the other drugs tested, the improvement in seizure-like behavior quantified in flies treated with the stem bark was mainly due to its prophylactic effect.

Effect of *Annona senegalensis* Extracts on Male Flies

We then evaluated the seizure phenotypes of males exposed to the same treatments as females. In males under control conditions, the percentage of flies recovered after mechanical stimulation is significantly higher than in females (200 ± 21 s; **Figure 1C**) and the MRT is significantly shorter (204 ± 19 s; **Figure 3B**). Significant drug effects were therefore expected mainly at the early time points of behavioral analysis. A mixed ANOVA on the percentage of male flies recovered after mechanical stimulation showed an effect of time [time: $F_{(40,3800)} = 96.55$, $p < 0.0001$, $\eta^2 = 0.50$], an interaction between time and treatment [time X treatment: $F_{(520,3800)} = 108.97$, $p < 0.0001$, $\eta^2 = 0.32$], and an effect of treatment alone [treatment: $F_{(13,95)} = 4.41$, $p < 0.0001$, $\eta^2 = 0.38$].

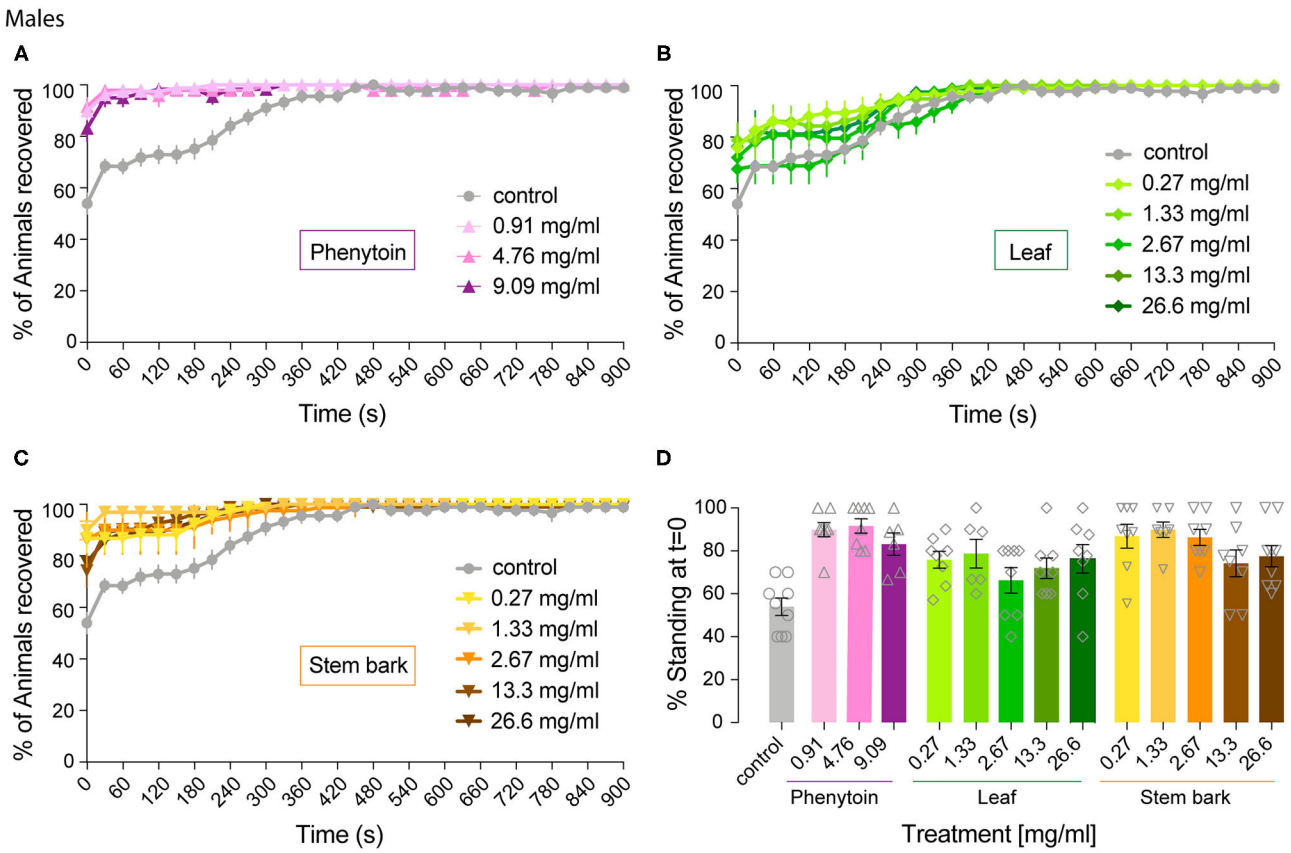

FIGURE 4 | Effect of phenytoin and *A. senegalensis* extracts on recovery from seizures of *para*^{bss} males. Mechanical stimulation of *para*^{bss} control male flies induced a seizure-like phenotype that was less penetrant than in females. The effects of phenytoin **(A)**, *A. senegalensis* leaf **(B)** or stem extract **(C)** are shown. **(D)** Dose response analysis at $t = 0$ for all treatments. Graphs show the mean % of flies seizuring ± SEM (for number of replicates, flies and significance see **Table 1**).

We then analyzed the behavioral effect for each drug treatment independently. A mixed ANOVA on percentage of flies recovered comparing phenytoin treated animals with controls showed an effect of time [time: $F_{(40,1080)} = 30.95$, $p < 0.0001$, $\eta^2 = 0.53$], an interaction between time and treatment [time X treatment: $F_{(120,1080)} = 12.40$, $p < 0.0001$, $\eta^2 = 0.58$] and of treatment [$F_{(3,27)} = 21.94$, $p < 0.0001$, $\eta^2 = 0.71$]. Further *post hoc* analysis showed that all three concentrations were significantly better than the control (Dunnett: control vs. 0.91, 4.76 and 9.09 mg.ml^{-1} of phenytoin $p < 0.001$; **Figure 4A**). Significant behavioral differences ($p < 0.001$) were found at $t = 0$ for the three concentrations tested (**Figures 4A,D** and **Table 1**). We did not observe differences in MRT across groups, probably due to a low number of flies seizuring at early time points. However, as observed in females, at 4.76 and 9.09 mg.ml^{-1} of phenytoin, a few flies had longer MRT then in controls, suggesting that the highest concentrations tested have a slight toxic effect on male flies as well (**Figure 3B**).

We then evaluated the effect of *A. senegalensis* leaf extract treatment. A mixed ANOVA on the percentage of flies recovered showed a significant effect of time [time: $F_{(40,1640)} = 68.50$, $p < 0.0001$, $\eta^2 = 0.63$], and a significant interaction between time and drug treatment [time X treatment: $F_{(200,1640)} = 1.46$, $p < 0.0001$, $\eta^2 = 0.15$], but no effect of drug alone [treatment: $F_{(3,29)} = 1.77$, $p = 0.14$, $\eta^2 = 0.18$; **Figure 4B**]. The lower effect of the leaf extract on males was further supported by a generalized more modest improvement at $t = 0$ (**Figure 4D** and **Table 1**). The MRT was not significantly improved for any concentration of the leaf extract (**Figure 3B**).

An effect of time, treatment and their interaction were present after treatment with stem bark extract of *A. senegalensis* in males [time: $F_{(40,1720)} = 48.57$, $p < 0.0001$, $\eta^2 = 0.53$; treatment: $F_{(5,43)} = 6.84$, $p < 0.0001$, $\eta^2 = 0.44$; time X treatment: $F_{(200,1720)} = 4.10$, $p < 0.0001$, $\eta^2 = 0.32$]. A Dunnett's *post hoc* analysis confirmed the improvement in the % of recovery of the flies treated with the stem bark extract compare to the untreated control, all concentrations being highly significant (control vs. 0.27, 1.33, and 26.6 mg.ml^{-1} $p < 0.0001$ and vs. 2.67 mg.ml^{-1} $p = 0.001$ and 13.3 mg.ml^{-1} $p = 0.002$; **Figure 4C**). As in phenytoin treated animals, stem bark extracts very significantly decreased the percentage of animals seizuring at $t = 0$ pointing to a prophylactic effect against the initiation of seizure-like behavior (**Figure 4D** and **Table 1**). Furthermore, the treatment with stem bark extract at 13.3 mg.ml^{-1} was the only treatment to significantly decrease

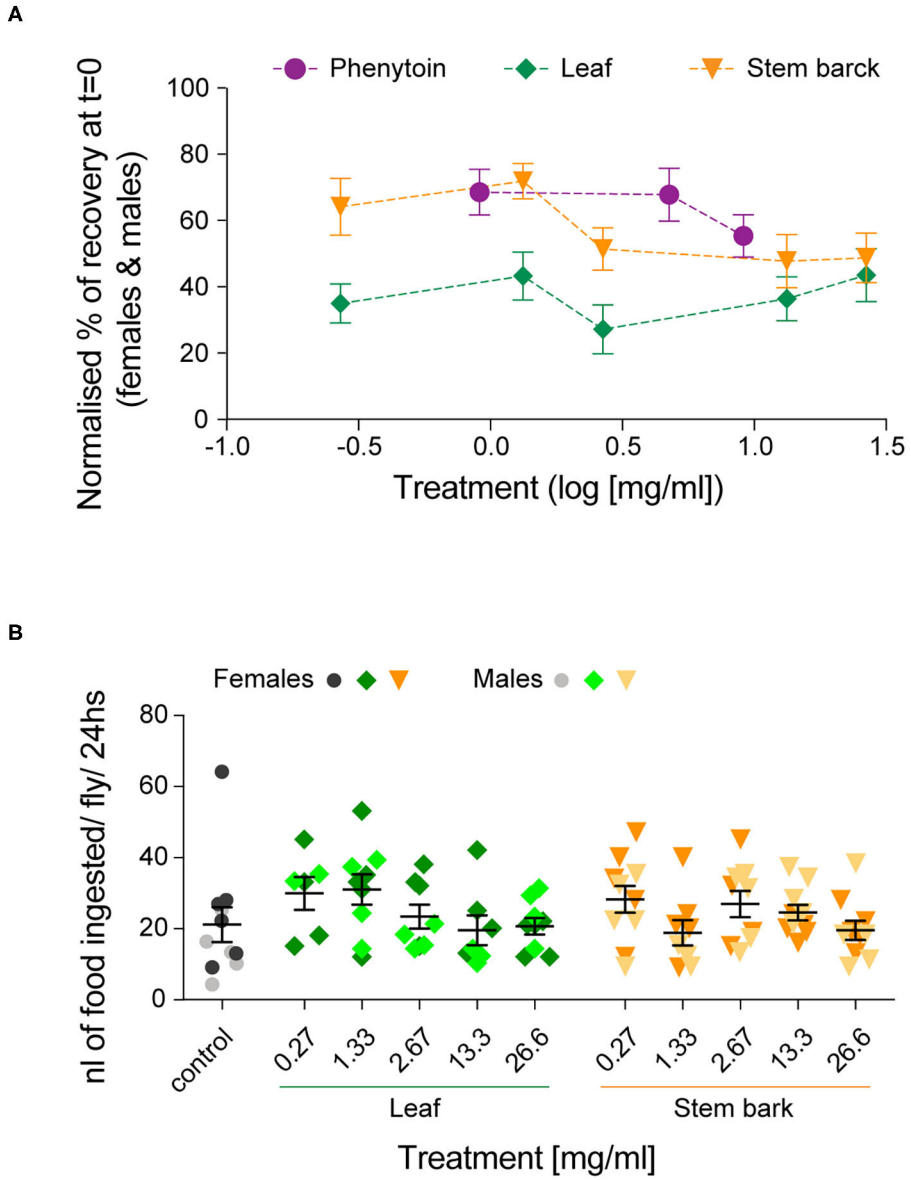

FIGURE 5 | Dose-dependent prophylactic effect of phenytoin and, leaf and stem bark *A. senegalensis* extracts. **(A)** Normalized % of recovery at $t = 0$ for females and males. The concentration is in $\log_{10}$ scale. An ANOVA comparing the effect of the three drugs treatment was significant [$F_{(2,199)} = 15.06$, $p < 0.0001$, $\eta^2 = 0.13$]. A *post hoc* Bonferroni multiple comparisons test was used to test for differences between the drugs (phenytoin vs. leaf $p < 0.0001$; vs. stem $p = 0.35$ and leaf vs. stem $p = 0.0001$). No significant differences between concentrations of each drug were found, apart when comparing with the control (Tukey's multiple comparisons test). **(B)** Amount of food ingested per fly and per 24 h. A two way ANOVA showed no effect of drug [$F_{(1,103)} = 0.03$, $p = 0.86$], no effect of concentration [$F_{(5,103)} = 1.63$, $p = 0.16$] and no interaction [$F_{(5,103)} = 1.26$, $p = 0.28$]. A *post hoc* Dunnett's multiple comparisons test comparing the control with each treatment did not yield any significant differences.

the MRT ($p = 0.02$; **Figure 3B**) supporting the effectiveness of the drug.

Altogether, these results show that recovery in both female and male flies was improved by treatments with *A. senegalensis* stem and leaf extract as well as with the commonly used AED phenytoin. To gain a better insight into the dose-response curve, we normalized the percentage of recovery. This allowed us to only evaluate the effect of the drug and extracts on flies seizuring at $t = 0$ (now at $t = 0$ there is $0 \pm$ SEM % recovery for all treatments; **Figure 5A**). In this condition the sexual dimorphism is no longer present, and we therefore pooled the data of both sexes. The three curves are in the plateau phase and no significant effect of concentration was found, apart for when comparing with the control. At saturated doses, the prophylactic effect of phenytoin is comparable to *A. senegalensis* stem bark administration ($p = 0.66$; **Figure 5A**). There is a slight but

non-significant decrease of phenytoin and stem bark effects, suggesting that higher concentrations of these compounds may have toxic or sedative effect. The leaf extract of *A. senegalensis* showed a lower protective effect than phenytoin ($p < 0.0001$) and stem bark ($p = 0.0002$), present at all concentrations (**Figure 5A**). Differences of prophylactic effects are not explained by differences in amount of food ingested since there were no significant differences in gut content between treatments (**Figure 5B**). The strong effect of *A. senegalensis* stem extract suggests the existence of an AED compound that could offer an alternative for the treatment of intractable epilepsy.

Effect of *Annona senegalensis* Extracts on *easily shocked* Mutants

To test the efficacity of *A. senegalensis* when the molecular cause of the hyperexcitability is other than mutations of an ionic channel, we decided to evaluate its effect on *easily shocked* mutants (eas^{2F}), which carry a mutation in an ethanolamine kinase which affects the phospholipid composition of neuronal membranes (41). This bang sensitive mutant is known to improve upon treatment with phenytoin but is partially or completely insensitive to other drugs like carbamazeprine, ethosuximide, and vigabactrin (36). When we tested the leaf and stem bark extract at 2.67 and 26.6 mg.ml^{-1} on eas^{2F} flies, we did not observe any improvement in their percentage of recovery (**Table 2**). Rather the mutants were more paralyzed and the only significant changes in the MRT were due to a delay in recovery (**Table 2**).

This experiment shows that despite the similarity in the prophylactic effect between phenytoin and *A. senegalensis* extract on $para^{bss}$, it is likely that their mode of action differs.

DISCUSSION

Approximately 30 AEDs are currently approved for treatment of patients affected by epilepsy, an aetiologically complex and variable disease. A non-negligible percentage of epileptic experience undesired secondary effects of these AEDs, and 30% of patients are insensitive to them altogether (9, 10), highlighting the necessity of discovering alternative compounds to incorporate in the design of novel therapies.

Almost all commonly prescribed AEDs were discovered by animal screenings (40) and their mechanisms of action were also studied in non-human species, emphasizing the importance of animal testing for drug discovery in epilepsy. Medicinal plant extracts have been historically used for the treatment of various diseases, including epilepsy. The knowledge gathered and transmitted for generations about the anti-seizure properties of herbal TM preparations constitute a rich source of information for the discovery of new drug candidates. However, an empirical testing of their efficacy as AED and the pharmacological characterization of the active compound is required. A few hundred drugs used in TM have been tested in epilepsy animal models but the lack of follow up clinical and pharmacological studies places constraints on the clinical recommendation of herbal TMs (42).

In the present study, we used a *Drosophila* seizure model to test the effect of *A. senegalensis* extracts as an anti-seizure treatment (25–27). We used a fly with a gain of function mutation in the unique voltage gated sodium channel of *Drosophila* genome, $para^{bss}$, which is extremely prone to seizures. $para^{bss}$ mutant flies are generally accepted as a model for intractable epilepsy due to the complexity of their seizures, including a tonic-clonic-like phase, and their significant resistance to AED treatments (38). *A. senegalensis* was used because of its strong reputation as a TM potentially effective for the treatment of seizures and because several studied performed in rodents have tested its anticonvulsant properties (19–24). In these studies, extracts of *A. senegalensis* were prepared with different methods (in general with polar solvents) and from different parts of the plant (leaf, root or stem). In all cases, an acute treatment with the extract, via intraperitoneal injection or via oral administration, produced a significant prophylactic effect against seizures induced by pentylenetetrazole, picrotoxin, pilocarpin or maximal electroshock (19–24). However, drug testing in mammals is costly and slow, reducing the number of compounds that can be subject to thorough analysis. Using *Drosophila* as a model system offers an opportunity to overcome such limitations and isolate active compounds in a high throughput and cost-effective manner.

$para^{bss}$ flies showed a strong seizure-like phenotype with significant differences between males and females. Approximately 85% of females were paralyzed after the mechanical stimulation, while only 50% of the males were. Despite this level of seizuring been below the one described in previous reports (36–38), the aminoacidic residue leucine in position 1699 was mutated to Phenylalanine in our $para^{bss}$ stock. This suggests that a repressor mutation in the X chromosome might be compensating for the L1699P mutation or, less likely, that our stock has lost an enhancer mutation that was generating the original fully penetrant phenotype.

Alternatively, this could be a consequence of the lack of controlled circadian synchronization in our experimental animals. In humans, it is known that the onset of seizures and the interictal epileptiform discharges (IED) have a tendency to occur in specific times of the day. For example, tonic-clonic seizures occur more frequently during sleep. This rhythmicity is controlled by both the circadian clock and sleep-wake state (43). In our experimental conditions, flies were inadvertently tested at different endogenous circadian time. This factor might have influenced the seizure threshold across animals, generating heterogeneity in the penetrance of the seizure phenotype. In this scenario the known behavioral differences in the rest-activity cycles between females (more active) and males might have resulted in the sexual dimorphism observed (44). It would be interesting to take advantage of the knowledge of circadian rhythms in *Drosophila* to further investigate the molecular and cellular mechanisms underlying the circadian and vigilance regulation of seizures.

Although presenting lower levels of seizuring than in the literature and sexual differences in phenotype penetrance, both sexes showed a measurable level of paralysis and recovered gradually, so they could be used to test the anti-seizure effect

TABLE 2 | Effect of *Annona senegalensis* treatments on the seizure-like phenotype of eas^{2F} flies.

Treatment eas^{2F} flies	Concentration (mg.ml-1)	% of recovery at $t = 0$					
		Females			Males		
		(Mean ± SEM)	*p*	*n* (# flies)	(Mean ± SEM)	*p*	*n* (# flies)
Control	0	6.73 ± 3.09		13 (115)	0.63 ± 0.63		16 (137)
A. senegalensis	2.67	0	ns	8 (73)	1.25 ± 1.25	ns	8 (69)
Leaf extract	26.6	0	ns	6 (54)	0	ns	8 (75)
A. senegalensis	2.67	0	ns	8 (73)	1.39 ± 1.39	ns	8 (73)
Stem Bark extract	26.6	1.39 ± 1.39	ns	10 (92)	4.26 ± 2.11	ns	8 (74)
		Mean recovery time					
Control	0	50.86 ± 2.73		105	79.17 ± 3.27		133
A. senegalensis	2.66	59.59 ± 3.58	ns	73	83.33 ± 4.83	ns	72
Leaf extract	26.6	70.00 ± 3.72	<0.001	54	81.20 ± 4.85	ns	92
A. senegalensis	2.66	58.29 ± 3.39	ns	70	77.67 ± 4.68	ns	73
Stem Bark extract	26.6	66.49 ± 3.71	0.001	74	70.87 ± 4.55	ns	69

Percentage of recovery at t =0. Kruskal-Wallis tests comparing each treatment were non-significant for females [$\chi^2_{(5)} = 6.45$, $p = 0.17$] and males [$\chi^2_{(5)} = 7.66$, $p = 0.1$]. The result of Dunn's post hoc test comparing each treatment against the control for each sex is included in the p column of the table. The n (# flies) column shows the number of replicates (n) and flies tested for each treatment.

Mean recovery time. Kruskal-Wallis test comparing each treatment showed a significant effect for females [$\chi^2_{(5)} = 23.98$, $p < 0.0001$] but not for males [$\chi^2_{(5)} = 4.34$, $p = 0.36$]. The result of Dunn's post hoc test comparing each treatment against the control for each sex is included in the p column of the table. The number of flies seizuring at t = 0 is shown in column n.

of *A. senegalensis*. Our experimental design was therefore suited to analyse the overall effect of drug treatments, from seizure induction to complete recovery. More precisely, we evaluated (1) the prophylactic effect against seizure induction and (2) the effect on recovery dynamics. We compared the effect of *A. senegalensis* extracts, to the commonly used AED phenytoin. Phenytoin was chosen as our positive control since its anti-convulsion properties have been documented in flies before. When administered chronically and at low doses (0.03–0.3 mg.ml^{-1}) it induced a decrease in MRT in a mixed population of males and females $para^{bss}$ (36).

In our experimental conditions (24 h drug exposure in the food, 0.91, 4.76 and 9.09 mg.ml^{-1}), phenytoin produced the expected improvement on seizuring phenotype. This was characterized by a clear prophylactic effect of all three doses, with fewer male or female flies showing seizures compare to controls at the beginning of the experiment ($t = 0$). The MRT was not improved at any concentration but this might be due to a slight toxic effect of phenytoin at 4.76 and 9.09 mg.ml^{-1}. At this concentration the MRT slightly increased and a few flies remained paralyzed for the entire duration of the experiment (1,200 s), a phenotype not observed in $para^{bss}$ mutants.

The treatment with *A. senegalensis* stem bark extract effectively improved the seizure-like phenotype. At the lower doses (below 2.67 mg.ml^{-1}) approximately 60% of the flies did not seizure at $t = 0$, showing a similar effect to the one quantified with phenytoin. We could not quantify a decrease in the MRT indicating that once the seizure has started the drug barely ameliorates the symptoms. In comparison, treatments with *A. senegalensis* leaf extracts were less effective than stem bark extracts, suggesting that the active compound of the plant is most concentrated in the stem bark.

These findings are significant because they lay the bases for future studies aiming to discover the active chemical compound present in *A. senegalensis* stem bark extract. In that direction, it would be possible to use this experimental approach to test the anti-seizure effect of samples coming from an extensive sub-fractioning of stem bark extracts. This would allow further purification and identification of the active compound.

One such simple sub-fractioning experiment was performed from *A. senegalensis* roots. It identified a putative candidate belonging to the diterpenoid family, Kar-16a-19oic acid, that showed promising sedative and anti-seizure properties in rodents (21). The candidate compound was enriched in an ethyl-acetate fraction and showed an acute toxicity in mice of $LD_{50} = 2154$ mg/kg compared to $LD_{50} = 150$ mg/kg for phenytoin (pubchem). Toxicity of an aqueous extract, like the one used in our experiments, in mice is lower, $LD_{50} > 5{,}000$ mg/kg (45), which would make it ideal for its clinical recommendation as a TM (9). This and other candidates could be further tested using the present animal model.

The difference in prophylactic effect observed on $para^{bss}$ and eas^{2F} mutant flies gives an indication regarding the possible mechanism of action of *A. senegalensis* extract. It has already been shown that the percentage of eas^{2F} mutants seizuring improves when treated with phenytoin (36) but that the mutants are resistant to treatment with several AEDs (36): carbamazepine a sodium channel blocker; ethosuximide that blocks T-type calcium channels, and may include effects on other classes of ion channel; vigabatrin that increases GABA in the brain blocking the activity of the gamma-aminobutyric acid aminotransferase and gabapentin which has recently been shown to selectively inhibit calcium channels containing the a2d-1 subunit (46). Because *A. senegalensis*

improves the phenotype of $para^{bss}$ but not eas^{2F}, it is likely that the active compound acts via a mechanism different than phenytoin, not acting on voltage gated sodium channels (40). On the other hand, an anticonvulsant effect of *A. senegalensis* leaf extract was shown on pentylenetetrazol (PTZ) induces convulsions in mice (19). PTZ antagonizes $GABA_A$ receptor Cl– channel complex (19) attenuating GABA-dependent inhibition and inducing seizures. It is possible that *A. senegalensis* effect is partially due to enhancement of GABAergic pathways. If this were the case, a high concentration of the plant extract could produce a sedative effect and induce more paralysis as observed when testing eas^{2F}. Ultimately, experiments to test the mechanism of action of *A. senegalensis* extract should be performed.

The simplicity, reproducibility, potential high throughput and low cost of the testing method reported here constitutes an ideal setup to isolate the active anti-seizure compound present in *A. senegalensis* and potentially other TMs. Molecules controlling the development and function of the nervous system (28, 29) in *Drosophila* and humans present high structural and functional conservation. *Drosophila* could provide an early neurophysiological characterization of the mechanisms of action of new candidate compounds with potential translational anti-seizure effects (25–27, 47, 48). Specific neurons whose neurotransmitter identity and connectivity are known could be patch-clamped in $para^{bss}$ and the effect of the drug tested (49). The contribution of inherited or *de novo* mutations in the etiology of epilepsy, including focal epilepsy (6, 50), is growing. Sodium channels, that play a crucial function for cellular excitability, have been associated with a wide range of epilepsies (5). Amongst them is *SNC1A*, the human ortholog of $para^{bss}$, with over 600 different mutations found in patients (8). Links to other ionic channels, receptors synaptic proteins and brain development pathways have also been proposed. The genetic malleability of *Drosophila*, where specific gene mutations can easily be generated, strengthens its validity for screening and early characterization of neural mechanism underlying hyperexcitability.

Screening TMs in several distinct *Drosophila* seizuring mutants will most likely help isolating new compounds for intractable epilepsy. This is exemplified by the lack of prophylactic effect induced by *A. senegalensis* extract on eas^{2F} mutants, which are insensitive to many EADs but might benefit from new drugs (36).

However, fruit flies and humans also show fundamental differences that need to be taken into consideration when interpreting screening results. From a circuit point to view, the fly brain is much simpler with no cortical and subcortical regions where most focal source of seizure are located in humans. From the aetiological perspective, the causes of epilepsy in humans are varied ranging from inherited (genetic) to acquire through brain injury (e.g., prenatal or perinatal causes, severe head or brain injury, stroke, infections) (11). Treatment with AEDs aims to re-balance neuronal activity but fly models of epilepsy might fail to capture the full spectrum of neural and brain dysfunctions. Limited behavioral phenotypes in flies suggest that only seizure resembling the "grand mal" with strong spams and muscular manifestations could be studied. More complex or subtle cognitive and emotional aspects of epilepsies are not accessible in flies. Finally, absence of a proper brain blood barrier, an open hemolymph circulation and the distinctive renal system of *Drosophila* compromises translation of pharmacokinetics and toxicity data to humans.

Ultimately, pre-clinical research in different animal models complemented with clinical trials in humans will be essential in establishing effectivity and safety profiles for TM compounds for the treatment of epilepsy.

In conclusion, our work is important because it confirms how powerful *Drosophila* is as a model system for screening of putative new AEDs. Regardless of the fact that there was little information about how to prepare the plant extract, we did not know the identity of the active compound, its effective dose, pharmacokinetic, or toxicity in flies, we were able to find a strong anti-seizure effect of *A. senegalensis* stem bark extracts on $para^{bss}$ flies. This method could be used for high throughput screening of many TM with suspected anti-convulsion properties, hence offering a robust platform for the identification and isolation of novel AEDs that could be the basis of new treatments for intractable epilepsy.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the use of Drosophila is not included the UK Home Office regulation.

AUTHOR CONTRIBUTIONS

SD and JB conceived and designed the experiments. SD, JB, JRC, and NH performed the experiments. EM and JB analyzed the data and EM did the statistics. PE identified the plants and prepared the extracts. EM and JB wrote the manuscript. 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/fneur.2020.606919/full#supplementary-material

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The Effects of Intravermis Cerebellar Microinjections of Dopaminergic Agents in Motor Learning and Aversive Memory Acquisition in Mice

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The cerebellum receives dopaminergic innervation and expresses the five types of described dopaminergic receptors. The cerebellar function involves both motor movement and cognition, but the role of cerebellar dopaminergic system on these processes remain unclear. The present study explores the behavioral responses to intracerebellar microinjection of dopaminergic agents in motor and emotional memory. For this, naïve Swiss mice had their cerebellar vermis implanted with a guide canula, received a intravermis microinjection of Dopamine, D1-like antagonist SCH-23390 or D2-like antagonist Eticlopride, and underwent a behavioral analysis of motor learning (by a Rotarod and balance beam learning protocol) or aversive memory acquisition (by the inhibitory avoidance task). The mixed-effects analysis was used to evaluate groups performance, followed by Tukey's *post hoc* when appropriated. In this study, Dopamine, SCH-23390 and Eticlopride at the doses used did not affected motor control and motor learning. In addition, the administration of Dopamine and SCH-233390 had no effects on emotional memory acquisition, but the animals that received the highest dose of Eticlopride had an improvement in aversive memory acquisition, shown by a suppression of its innate preference for the dark compartment of the inhibitory avoidance apparatus following an exposure to a foot shock. We propose that cerebellar dopaminergic D2 receptors seem to participate on the modulation of aversive memory processes, without influencing motor performance at the doses used in this study.

Keywords: cerebellum, dopaminergic agents, avoidance learning, motor activity, motor learning

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INTRODUCTION

Dopamine is a biogenic amine derived from hydroxylation and decarboxylation of tyrosine (Rodwell, 2003), and its most prominent neuronal portion is located in the ventral midbrain, sending projections to multiple areas of the central nervous system (CNS) (Chinta and Andersen, 2005). There are five characterized types of dopaminergic receptors (D1, D2, D3, D4, and D5), which are classified into two subgroups, according to its structural and functional similarities:

D1-like (D1 and D5), and D2-like (D2, D3, and D4) (Strange, 1993; Jaber et al., 1996). Dysregulations of dopaminergic metabolism can lead to several diseases, such as Parkinson's disease, epilepsy (Star, 1996), and schizophrenia (Dahoun et al., 2017). Hence, comprehending dopamine signaling pathways is the first step to discovery new therapies for these diseases (Klein et al., 2019).

The dopaminergic system is known to play a role in learning and memory processes in the prefrontal cortex (PFC) (Puig et al., 2014), anterior cingulate cortex, basolateral amygdala (Zheng et al., 2008), and hippocampus (Hamilton et al., 2010). However, despite the dopaminergic innervation (Panagopoulos et al., 1991) and expression of the five types of dopaminergic receptors in the cerebellum (Barili et al., 2000) its role on modulation of motor and emotional mnemonic processes is unclear.

The cerebellum has been traditionally associated with movement control but also it has recently related to cognition modulation (Koziol et al., 2014; Adamaszek et al., 2017), including learning and memory processes (Strata, 2015; Caligiore et al., 2019). The cerebellar vermis seems to represent an interface between sensorial stimuli, emotional processing, and motor responses, as a result of its connection with several important structures for the processing of such functions (Sacchetti et al., 2009). In a consensus article by Koziol et al. (2014), the authors suggest that the cerebellum acts establishing internal models for the coordination of movement and thought, modulating behavioral outcomes. Leggio and Molinari (2015) hypothesized that these internal patterns can be used to predict future components such as the body and the environment, explaining the cerebellar participation in learning processes.

Little is known about the role of cerebellar dopaminergic agents on the modulation of motor and non-motor function. Some studies showed the involvement of D1-like receptors on memory processes: Mysliveyek et al. (2007) observed an worsened spatial learning after i.p. administration of the D1 receptor antagonist SCH-23390 in mice with olivocerebellar degeneration, Locke et al. (2018) showed that the inhibition of D1 receptors of the lateral cerebellar nucleus (LCN) results in decreased spatial and working memory, and Heskje et al. (2020) suggests that the stimulation of D1 receptors of LCN may modulate frontal cortex circuitry and processing. Regarding the D2-like receptors, studies have focused on its motor role, showing an immediate decrease on spontaneous movement after intracerebellar administration of a D2-like agonist (Boulay et al., 2000; Kolasiewicz and Maj, 2001; Barik and Beaurepaire, 2005; Kolasiewicz and Ossowska, 2008; Shimizu et al., 2014).

Hence, we investigated how the intravermis infusion of Dopamine, D1-like receptor antagonist SCH-23390, and D2-like receptor antagonist Eticlopride influences in mice behavior during motor learning and inhibitory avoidance protocols. Based on the cerebellar dopaminergic innervation and on the important role of the cerebellum on processing and modulating motor and non-motor functions, we expect that such manipulation can modify both behaviors, and provide initial insights on the role of the cerebellar dopaminergic system in learning and memory.

MATERIALS AND METHODS

Animals

The experimental subjects were 201 male Swiss mice (Federal University of São Carlos – UFSCar, SP, Brazil), from 35 to 50 days old, weighing 25–35 g at testing. The mice were housed in groups of five animals per cage (31 cm × 20 cm × 13 cm) and maintained under a 12 h light cycle (lights on at 7:00 a.m.) in a controlled environment at a temperature of 23 ± 1°C and humidity of 50 ± 5%. All mice were experimentally naïve at the beginning of the study. The experimental sessions were conducted during the light period of the cycle (8:00 am – 4:00 pm) to minimize the influence of the circadian rhythm on behavioral responses.

This study was approved by the Animal Ethics Commission of the Federal University of São Carlos (protocol 4486110220), which follows the standards of the Brazilian Neuroscience and Behavior Society (SBNeC), based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs

Dopamine, SCH-23390 (D1 antagonist) and Eticlopride (D2 antagonist) (Sigma Chemical Co., St. Louis, MO, United States) were prepared in sterile 0.9% saline solution (SAL). The Dopamine solution was prepared at doses of 0.29, 0.86, and 1.5 nmol/0.1 μl; SCH-23390 at doses 0.31, 0.92, and 1.54 nmol/0.1 μl; and Eticlopride at doses 0.26, 1.32, and 2.65 nmol/0.1 μl. The solutions were stored in coded tubes until the microinjection, and the experimenter was blinded to the codes during behavioral and statistical analysis.

We selected doses that presented effects in previous studies with different types of behavioral protocols. For instance, Nasehi et al. (2016) performed intra BLA injections of SCH-23390 at doses 0.01, 0.1 and 0.5 μg/mouse, and observed that the higher dose of 0.5 μg (1.54 nmol) impaired memory acquisition at the passive avoidance test, but did not have any effect on locomotor activity. Boye et al. (2001) observed that intra-accumbens infusion of eticlopride at doses 0.25 and 0.5 μg resulted in inhibition of spontaneous activity, and the highest dose of 1.0 μg produced evidence of antagonism with nicotine, reducing locomotor activity.

Surgery and Microinjection

Mice received a general anesthesia with ketamine hydrochloride (100 mg/kg, IP) and xylazine (10 mg/kg, IP), and local anesthesia on the scalp (3% lidocaine with norepinephrine; 1:50.000), and then placed in a stereotaxic instrument. Mice cerebellar vermis (lobules 4-5) were implanted with a single 7-mm guide cannula (25-gauge; Insight Equipamentos Científicos, Brazil), according to the following coordinates from the mouse brain atlas of Franklin and Paxinos (2001): 6.5 mm posterior to the Bregma; 0 mm lateral to the midline; and 2.0 mm ventral to the skull surface. The guide cannula was fixed to the skull using dental acrylic (Blue dent, Brazil) and jeweler's screws. A dummy cannula (33-gauge) was inserted into the guide cannula to reduce the incidence of occlusion. Postoperative analgesia was provided by adding acetaminophen (200 mg/ml) to the drinking water at

a ratio of 0.2 ml acetaminophen to 250 ml water for a final concentration of 0.16 mg/ml.

On the third day post surgery (Curzon et al., 2009), saline or drug solutions were infused into the cerebellar vermis using a microinjection unit (33-gauge cannula; Insight Equipamentos Científicos Ltda, Brazil), which was attached to a 5 μl Hamilton micro syringe via polyethylene tubing, and an infusion pump that was programmed to deliver a volume of 0.1 μl over 60 s. Each animal received a microinjection of a single dose of the drug, according to the experimental group that they were randomly allocated.

Apparatus

Rotarod

The Rotarod apparatus consists of a dark acrylic box (450 mm × 540 mm × 350 mm) with an 8 cm diameter non-slip cylinder, transversely installed approximately 20 cm from the floor of the equipment. The box is divided into five bays (8 cm length each), allowing the analysis of five animals simultaneously. The cylinder rotation was driven by a motor in a pre-set acceleration, that might be set at any speed from 0 to 50 rpm. In this study, it ranged from speed 8 to 20 rpm within 5 min in an incremental test. The falling latency was automatically measured by a sensor located on the floor of the device.

Balance Beam

The Balance Beam is a wood beam (100 x 2.8 cm) with a flat narrow surface (0.6 cm), resting 50 cm above the countertop on two acrylic poles. A dark box containing nesting material from home cages was placed at the finish point of the beam. A nylon hammock was installed bellow the beam to cushion any possible fall. The time spent for crossing the beam central 80 cm was automatically measured by two motion detectors (fabricated by Visopia) placed at the start and finishing points of the beam.

Inhibitory Avoidance Apparatus

The apparatus consists in an acrylic box (48 × 24.5 × 25 cm) divided into two equally sized compartments: one light (under illumination of 450 lux), and one dark (covered with black acrylic), separated by a guillotine door (9 × 10 cm). The floor is made of stainless-steel rods (2.5 mm in diameter), spaced 1 cm apart, that delivers electric shocks of 1.5 mA for 5 s. The guillotine door and the shock delivery are triggered by a connected computer software (Insight Equipamentos Cientificos Ltda., Brazil).

Experimental Procedures

This study presents two distinct experimental procedures. The motor control and motor learning were assessed by the Rotarod and Balance Beam apparatus, and the aversive memory acquisition was assessed by the inhibitory avoidance apparatus. Each animal underwent only one experimental procedure, as described above.

Motor Control and Motor Learning

The combined use of Rotarod and Balance Beam allows the investigation of gross motor function and fine motor coordination (Curzon et al., 2009). This protocol was based on Song et al. (2006) and He et al. (2014) methodologies, with some modifications. It was divided into five steps, named habituation, microinjection, stage 1, stage 2, and stage 3. At habituation the animals were placed one time in the Rotarod apparatus up to 2 min or until they fall, and in the balance beam until crossing, which allowed the first contact of the animals with the apparatus. Twenty-four hours later, the microinjection procedure was performed using saline or one of the drugs doses, according to the experimental group. The stages 1, 2, and 3 were performed 5 min, 4 h, and 24 h after microinjection, respectively (Song et al., 2006). These timepoints permits the measurement of different motor learning processes, such as acquisition, and consolidation. For each stage, the animals were placed in the Rotarod in a crescent speed (8 to 20 rpm) up to 5 min or until falling, and in the balance beam until crossing. A five minutes interval was given between every exposure to the Rotarod and the balance beam. Overall, each animal performed three trials in Rotarod and three trial in balance beam per stage, totaling 9 trials per apparatus in the entire protocol (**Figure 1**). A mean of their scores in each stage was used in statistical analysis.

Aversive Memory Acquisition

The protocol started 5 min after the animals received a microinjection of saline or drugs, and was divided into habituation, aversive stimulus and memory acquisition test. In all stages, the mice were placed in the light compartment of the inhibitory avoidance apparatus, and the crossing latency to dark compartment was measured. For habituation, the crossing from the light side to dark compartment was allowed without any aversive stimulus, twice with a 30 min interval. In the next stage, the mice received a foot shock of 1.5 mA for 5 s as soon as they have crossed to dark compartment of the apparatus. The aversive memory acquisition test was performed 2 min after the foot shock delivery (**Figure 1**).

Histology

Following 60 to 90 min the end of the experiments, the animals received an anesthetic overdose, and were perfused transcardially with fixative (4% paraformaldehyde in 0.1 M phosphate buffer). The brains were removed and kept overnight in fixative. Coronal slices of 50 μm were cut with a vibratome and the injection sites were verified histologically according to the atlas of Franklin and Paxinos (2001). Animals with injection sites outside the cerebellar vermis were excluded from the study. Histological analysis confirmed that a total of 201 mice exhibited accurate positioning of the cannula placements in the vermal region of cerebellar lobules 4–5, mostly between bregma −6.25 and −6.55 (**Figure 2**).

Statistical Analysis

The statistical analysis was performed using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California, United States. Levene's test confirmed homogeneity of variance. The ROUT method (Q = 1%) was used to identify extreme outliers. The mixed-effects analysis was used to evaluate groups performance. The within-subjects effect was measured

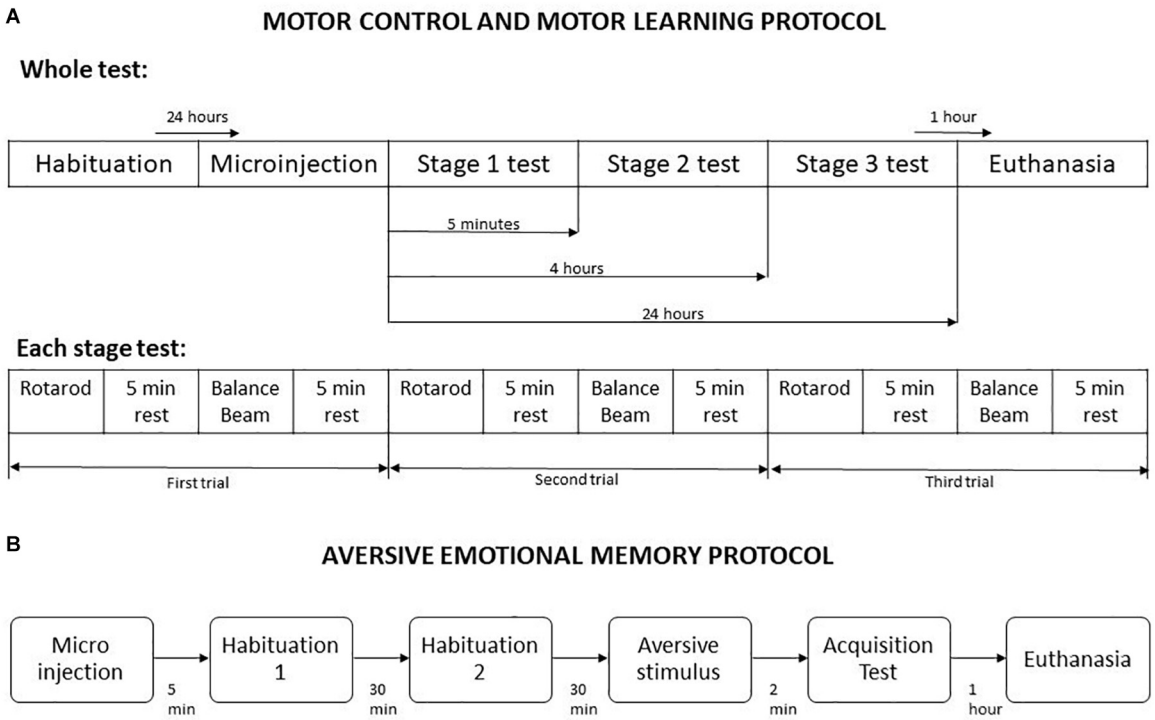

FIGURE 1 | Schematic representation of behavioral testing procedures. **(A)** Motor control and motor learning protocol. **(B)** Aversive emotional memory acquisition protocol. Adapted from Song et al. (2006).

by each exposure over time, and the between-subject factor was represented by the different groups of drug injection. Differences indicated by significant *P* values were further verified by *post hoc* Tukey's multiple range test. In all cases, $p < 0.05$ was considered significant.

RESULTS

Effects of Intravermis Cerebellar Microinjections of Dopamine on Motor Control and Motor Learning in Mice

No outlier was identified in the Rotarod analysis, and nine outlier measurements were identified and removed from balance beam experimental group. The statistical analysis revealed that all groups presented an increase of the latency to fall of the Rotarod ($F_{1.842,49.74} = 1.39$; $p < 0.0001$), and a decrease of time spent for crossing the balance beam ($F_{1.928,43.37} = 4.18$; $p = 0.02$) through the three stages. However, there were no differences between the groups that received Dopamine in different doses and the control group, for the rotarod ($F_{3,27} = 1.74$; $p = 0.27$), and balance beam ($F_{3,27} = 1.74$; $p = 0.18$) exposures over the stages, indicating that microinjection into the cerebellar vermis of Dopamine at doses 0.29, 0.86, and 1.5 nmol/0.1 ul did not show significant behavioral effects on gross motor function and fine coordination performance and learning in mice in this study (**Table 1** and **Figure 3**).

Effects of Intravermis Cerebellar Microinjections of Dopamine on Emotional Memory Acquisition in Mice

One outlier was identified and removed of this experimental group. The within groups comparison revealed that all groups showed appropriate aversive memory acquisition by an increase in crossing latency to the dark side of the inhibitory avoidance apparatus after the aversive stimulus ($F_{1,37} = 54.12$; $p < 0.0001$). However, there was no difference for the crossing latency among the groups that received Dopamine in different doses and the control group ($F_{3,38} = 2.35$; $p = 0.09$), which indicates that the microinjection in the cerebellar vermis of Dopamine in the doses 0.29, 0.86, and 1.5 nmol/0.1 ul had no significant behavioral effects on the emotional memory acquisition in mice in this study (**Table 2** and **Figure 3**).

Effects of Intravermis Cerebellar Microinjections of D1-Like Antagonist on Motor Control and Motor Learning in Mice

No outlier was identified in the rotarod and balance beam experimental groups. The statistical analysis indicated that all groups presented an increase of the latency to fall of the Rotarod ($F_{1.990,45.77} = 20.39$; $p < 0.0001$) and a decrease of time spent for crossing the balance beam ($F_{1.494,34.36} = 4.12$; $p = 0.03$)

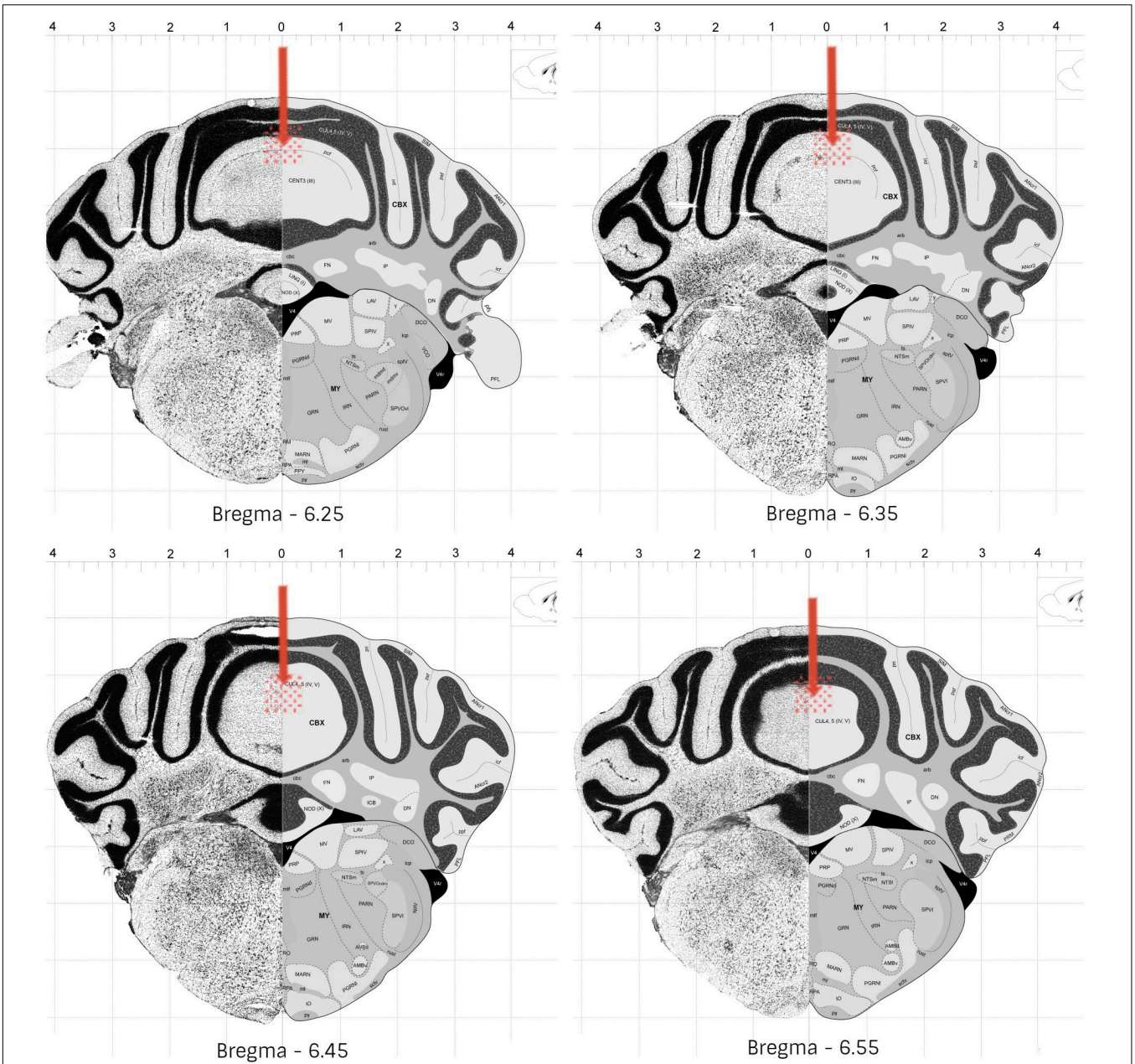

FIGURE 2 | Schematic representation of the most common cannula placement (represented by arrow) and dispersion areas (represented by filled circles), in mice cerebellar vermis (lobules 4-5). Adapted from © 2008 Allen Institute for Brain Science. The Allen Mouse Brain Atlas. Available from: http://mouse.brain-map.org/static/atlas.

through the three stages. However, there were no significant differences between the groups that received the D1-like receptor antagonist SCH-23390 in different doses and the control group in mice exposed to rotarod ($F_{3,23}$ = 0.44, p = 0.72) and the balance beam ($F_{3,23}$ = 2.16; p = 0.12). These results show that the intra vermis cerebellar microinjection of SCH-23390 did not promote significant changes in the motor performance and motor learning in mice at the doses used in this study (**Table 1** and **Figure 4**).

Effects of Intravermis Cerebellar Microinjections of D1-Like Antagonist SCH-23390 on Emotional Memory Acquisition in Mice

Two outlier measurements were identified and removed from this experimental group. The within groups comparison revealed that all groups presented an increase in crossing latency to the dark side of the inhibitory avoidance apparatus after the

TABLE 1 | Effects of intracerebellar microinjections of Dopamine (DOP), SCH-23390 (SCH), and Eticlopride (ETI) at different doses in the falling latency of the rotarod, and time spent for crossing the balance beam of mice exposed to motor control and motor learning behavioral protocol.

		Stage 1		Stage 2		Stage 3	
Groups	**N**	**Rotarod (s)**	**Balance beam (s)**	**Rotarod (s)**	**Balance beam (s)**	**Rotarod (s)**	**Balance beam (s)**
SAL	7	36.77 ± 13.73	41.38 ± 19.62	107.36 ± 42.60	36.14 ± 6.21	149.35 ± 34.73	26.51 ± 12.0
DOP 0.29	8	54.22 ± 38.26	35.35 ± 12.78	95.92 ± 22.34	20.87 ± 7.41	182.17 ± 35.91	20.60 ± 6.45
DOP 0.86	8	41.77 ± 7.42	22.1 ± 8.15	118.62 ± 23.69	9.73 ± 1.58	179.46 ± 28.97	15.91 ± 4.65
DOP 1.5	8	84.05 ± 17.50	17.11 ± 2.96	178.21 ± 21.95	9.43 ± 1.3	209, 0 ± 27.78	11.85 ± 2.52
SAL	6	142.58 ± 38.39	29.47 ± 7.68	198.59 ± 40.82	18.54 ± 4.83	246.9 ± 25.57	26.06 ± 8.22
SCH 0.31	7	103.28 ± 32.38	18.91 ± 2.65	182.32 ± 40.75	14.6 ± 2.06	211.30 ± 35.08	17.22 ± 4.74
SCH 0.92	7	96.41 ± 39.74	20.95 ± 4.56	223.99 ± 10.01	12.08 ± 3.73	195.61 ± 34.68	10.54 ± 1.12
SCH 1.54	7	130.7 ± 30.47	22.83 ± 4, 81	218.15 ± 34.29	13.0 ± 3.27	236.39 ± 34.81	27.59 ± 3.64
SAL	8	130.98 ± 24.81	14.91 ± 2.41	190.37 ± 35.70	12.92 ± 2.70	241.85 ± 35.64	12.83 ± 2.20
ETI 0.26	8	117.28 ± 35.4	17.68 ± 2.94	208.29 ± 36.65	12.99 ± 1.83	235.33 ± 33.46	10.22 ± 1.93
ETI 1.32	8	108.59 ± 24.71	18.90 ± 2.93	182.54 ± 32.89	16.95 ± 3.86	197.57 ± 45.24	18.46 ± 2.14
ETI 2.65	9	66.73 ± 22.39	29.91 ± 7.4	173.85 ± 31.21	24.31 ± 8.43	227.18 ± 31.16	21.31 ± 8.59

Mean ± SEM.

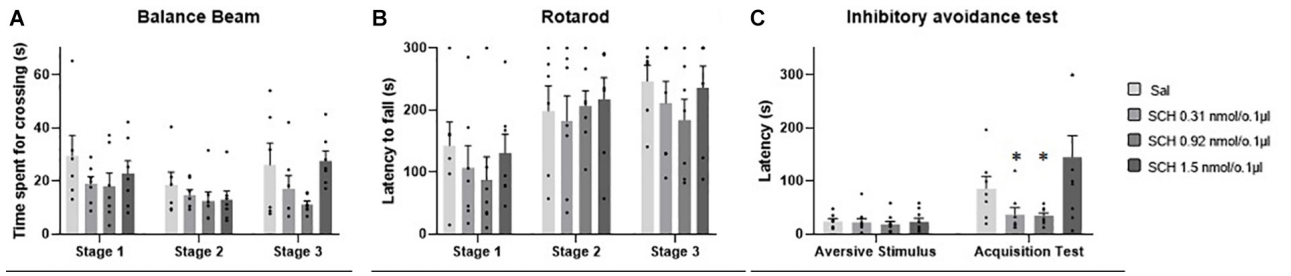

FIGURE 3 | Scatter plot and bar graph of the effects of intravermis cerebellar microinjections of Dopamine at different doses on motor learning and emotional memory acquisition in mice. **(A)** Balance beam. **(B)** Rotarod. **(C)** Inhibitory avoidance test.

TABLE 2 | Effects of intracerebellar microinjections of Dopamine (DOP), SCH-23390 (SCH), and Eticlopride (ETI) at different doses in the crossing latency do the dark side of the inhibitory avoidance apparatus of mice exposed to emotional memory acquisition behavioral protocol.

	N	**Habituation 1 (s)**	**Habituation 2 (s)**	**Aversive stimulus (s)**	**Acquisition test (s)**
SAL	8	20.44 ± 3.91	27.41 ± 4.14	21.81 ± 5.01	115.54 ± 22.40
DOP 0.29	14	46.56 ± 12.80	23.01 ± 4.92	29.02 ± 5.87	77.54 ± 19.29
DOP 0.86	9	51.54 ± 15.01	26.45 ± 5.14	23.18 ± 4.73	81.93 ± 12.45
DOP 1.5	11	33.51 ± 9.53	20.62 ± 4.28	14.73 ± 1.58	61.73 ± 14.69
SAL	7	29.05 ± 6.91	27.62 ± 6.83	24.13 ± 5.26	86.16 ± 22.59
SCH 0.31	9	34.84 ± 7.56	18.09 ± 6.45	22.23 ± 7.38	65.77 ± 31.47
SCH 0.92	10	27.29 ± 17.35	20.26 ± 4.92	18.43 ± 5.23	61.15 ± 26.94
SCH 1.54	9	3.95 ± 11.54	14.19 ± 3.63	23.59 ± 6.64	144.96 ± 40.51
SAL	9	53.66 ± 10.39	29.50 ± 3.79	24.04 ± 4.38	102.17 ± 13.61
ETI 0.26	9	54.17 ± 15.71	25.10 ± 3.95	16.90 ± 5.90	102.27 ± 30.85
ETI 1.32	8	25.64 ± 5.12	28.0 ± 4.88	35.61 ± 10.41	122.96 ± 21.79
ETI 2.65	7	36.11 ± 5.61	39.22 ± 7.35	46.76 ± 5.20	199.91 ± 28.96*

Mean ± SEM.

aversive stimulus ($F_{1,29}$ = 21.68; p < 0.0001). Moreover, a significant difference for the crossing latency was found between groups ($F_{3,31}$ = 4.07; p = 0.01). The multiple comparisons test revealed that there were no differences between the control group and the treated groups; however, the groups that received the D1-like antagonist SCH-23390 at the lower doses of 0.31 and 0.92 nmol/0.1 ul presented a lower crossing latency than the group that received the higher dose of 1.54 nmol/0.1 ul (p = 0.0004; p = 0.0002, respectively) (**Table 2** and **Figure 4**).

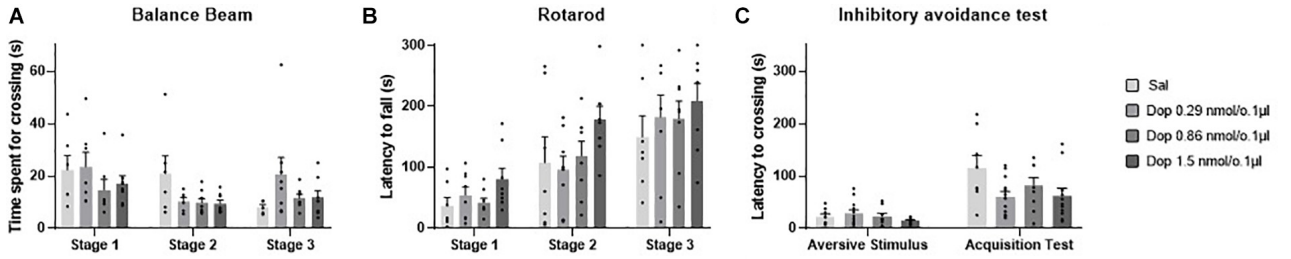

FIGURE 4 | Scatter plot and bar graph of the effects of intravermis cerebellar microinjections of D1-like antagonist SCH-23390 at different doses on motor learning and emotional memory acquisition in mice. **(A)** Balance beam. **(B)** Rotarod. **(C)** Inhibitory avoidance test. *Significant difference from SCH-23390 at 1.5 nmol/0.1 ul ($p < 0.001$).

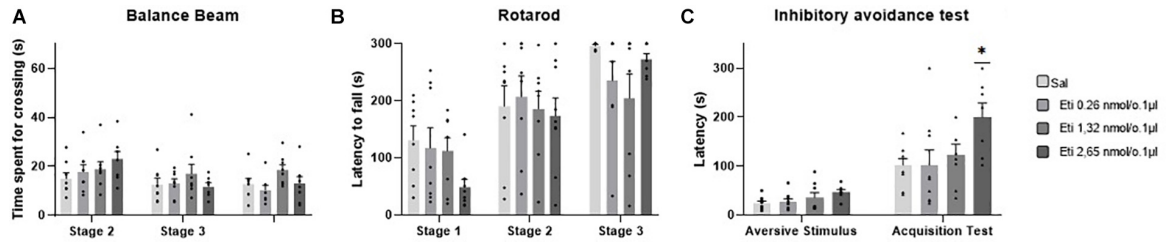

FIGURE 5 | Scatter plot and bar graph of the effects of intravermis cerebellar microinjections of D2-like antagonist Eticlopride at different doses on motor learning and emotional memory acquisition in mice. **(A)** Balance beam. **(B)** Rotarod. **(C)** Inhibitory avoidance test. *Significant difference from saline control group ($p = 0.002$), Eticlopride 0.26 nmol/0.1 µl group ($p = 0.002$), and Eticlopride 1.32 nmol/0.1 µl group ($p = 0.2$).

Effects of Intravermis Cerebellar Microinjections of D2-Like Antagonist Eticlopride on Motor Control and Motor Learning in Mice

Five outlier measurements were identified and removed from the rotarod, and four outliers were removed from balance beam experimental group. The statistical analysis revealed that all groups presented an increase of the latency to fall of the Rotarod ($F_{1.993,52.83} = 53.54$; $p < 0.0001$), and a decrease of time spent for crossing the balance beam ($F_{1.847,49.86} = 7.53$; $p = 0.001$) through the three stages. No significant differences were found between the groups that received the D-2 like antagonist Eticlopride and the control group, for the behavioral tests in Rotarod ($F_{3,29} = 0.49$; $p = 0.69$) and balance beam ($F_{3,29} = 1.15$; $p = 0.35$). These data suggest that microinjection into the cerebellar vermis of Eticlopride at doses 0.26, 1.32, and 2.65 nmol/0.1 ul did not lead to significant changes in the motor performance and motor learning in mice in this study (**Table 1** and **Figure 5**).

Effects of Intravermis Cerebellar Microinjections of D2-Like Antagonist Eticlopride on Emotional Memory Acquisition in Mice

No outlier was identified in this experimental group. Regarding the emotional memory acquisition, there was a significant difference observed within-subjects in all groups exposed to inhibitory avoidance protocol ($F_{1,29} = 64.23$; $p < 0.0001$). Moreover, the statistical analysis revealed that there were differences of crossing latency between-subjects ($F_{3,29} = 4.15$; $p = 0.01$), and Tukey's *post hoc* showed that the group that received the higher dose of the D2-like antagonist Eticlopride (2.65 nmol/0.1 µl) presented a significant increase in the latency of crossing to the dark side of the inhibitory avoidance apparatus when compared to control group ($p = 0.002$) and the groups that received Eticlopride at 0.26 nmol/0.1 µl ($p = 0.002$) and at 1.32 nmol/0.1 µl ($p = 0.02$). These data suggest that the intracerebellar microinjection of Eticlopride at the dose 2.65 nmol/0.1 µl improved aversive memory in mice exposed to inhibitory avoidance protocol in this study (**Table 2** and **Figure 5**).

DISCUSSION

In this study, we found that mice that received an intracerebellar microinjection of the D2-like antagonist Eticlopride at dose 2.65 nmol/0.1 ul had an improvement in aversive emotional memory, by a suppression of its innate preference for the dark compartment of the inhibitory avoidance apparatus following an exposure to a foot shock. However, the lower doses of Eticlopride, and the microinjection of Dopamine and D1-like antagonist SCH-23390 had no significant behavioral effect at the same test, that has been widely used to evaluate aversive emotional memory in rodents (Gold, 1986; Izquierdo and Medina, 1997; Arakawa, 2019).

Previous studies have shown that dopaminergic signaling is critical for emotional memory formation (Saito et al., 2020; Steinberg et al., 2020). In a review article, Likhtik and Johansen (2019) showed that dopaminergic neurons projects from different midbrain regions, including ventral tegmental area (VTA) and substantia nigra (SN), to lateral and central amygdala, modulating fear learning by responding to unexpected events and cues that predict them. At the same time, VTA sends dopaminergic projections to the cerebellar cortex (Ikai et al., 1996) and receives projections from Purkinje cells (Snider et al., 1976) and cerebellar nuclei (Watabe-Uchida et al., 2012; Carta et al., 2019), which might be an important path by which the cerebellum modulates information from other limbic-related structures (Ikai et al., 1994). Moreover, the cerebellum seems to be able to exert inhibitory control (Gil-Miravet et al., 2019) and modulate dopamine levels in the mPFC (Rogers et al., 2011), a key structure on the modulation of aversive memories (Canto-de-Souza and Mattioli, 2016), which in turn control the VTA dopaminergic systems that innervates amygdala and hippocampus (Izquierdo et al., 2016). In this way, we believe that the complex cerebellar-VTA-PFC connections might be a key element for dopaminergic modulation of aversive memory.

It is known that an increase in the cerebellar vermis activation occurs in face of an aversive stimulus (Ernst et al., 2019), but there is not enough information about how the cerebellar dopaminergic system acts on the modulation of memory processes. A recent study mapped the tyrosine hydroxylase – a dopamine precursor – at several cerebellar regions, including the posterior vermis, showing that catecholaminergic signaling, within a subset of Purkinje cerebellar cells, can modulate fear conditioning without affecting gross motor function on accelerating rotarod (Locke et al., 2020). Based on these findings, and in the results of the present study, we believe that the dopaminergic system – more specifically the D2-receptor - may play a role in the cerebellar modulation of the emotional memory.

Despite the Dopamine and D1-like antagonist SCH-23390 microinjections did not induce significant changes in the aversive memory acquisition in this behavioral test, its effects should not be neglected. The highest dose of Dopamine lead to an approximately 46% decrease in the crossing latency mean compared to control group, whereas the highest dose of SCH-23390 caused an 40% increase for the same variable compared to controls. On that basis, the present results do not entirely rule out the possibility of a D1-receptor role on the modulation of aversive memory acquisition.

Regarding the role of dopaminergic agents on motor learning, we found that the intracerebellar microinjections at different doses of Dopamine, D1-like receptor antagonist SCH-23390, and D2-like receptor antagonist Eticlopride had no significant effects on motor performance at the rotarod and balance beam behavioral tasks through the three stages learning protocol.

Some studies have demonstrated the dopaminergic role on motor performance and motor learning. For instance, the i.p. administration of D1-like antagonists have been related to deficits in motor coordination (Avila-Luna et al., 2016), and the absence of D2 receptors leads to severe impairments in motor coordination, locomotion, and motor learning (Bello et al., 2017; Lim et al., 2019). Furthermore, The D1 and D2 antagonists administered in the motor cortex impairs motor skill acquisition and synaptic plasticity (Molina-Luna et al., 2009; Rioult-Pedotti et al., 2015). However, the dopaminergic modulation of the cerebellar motor function is not clear.

As mentioned at the introduction section, some studies observed an immediate decrease on spontaneous movement after the administration of a D2-like agonist into the cerebellar lobules 9-10 (Kolasiewicz and Maj, 2001; Barik and Beaurepaire, 2005; Kolasiewicz and Ossowska, 2008; Shimizu et al., 2014), and no studies were found regarding the influence of dopaminergic agents in cerebellar motor learning. However, according to Fujita et al. (2020), the vermis presents several modules across its multiples lobules, which can be linked with brainstem nuclei in different ways, sub serving a variety of functions. We assume that the vermal region observed in the present study is not related to motor coordination and motor learning processes, but to the cognitive functions such as the passive avoidance, explaining the results of this study.

In conclusion, we believe that the cerebellum has a role as a modulator in adaptative behavior, such as the passive avoidance acquired after an aversive stimulus. Our view is that the cerebellum – by its connections with other brain structures and using its dopaminergic projections – might promote behavioral adjustments in similar ways of its mechanisms for adjusting voluntary movements. However, the specific cerebellar pathways involved in aversive emotional memory acquisition needs further investigation.

STUDY LIMITATIONS

This study has potential limitations. Despite some of the results did not reach statistical significance, a possible practical relevance of the drugs administration must be taken into consideration. A complete behavioral testing battery could provide elucidating data regarding the effects of dopaminergic system in such functions. Moreover, there is a lack of previous studies focusing on intracerebellar dopaminergic compounds administration, making the right drug dosage selection difficult. Further research should focus on the effects of different drug doses, providing a complete dose-dependent effect in learning and memory functions.

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 Animal Ethics Commission of the Federal University of São Carlos, which

follows the standards of the Brazilian Neuroscience and Behavior Society (SBNeC).

AUTHOR CONTRIBUTIONS

EG and AG designed the experiments. EG conducted the behavioral experiments, analyzed the data, and wrote the manuscript. AG reviewed and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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New Open-Source Tools: Using Bonsai for Behavioral Tracking and Closed-Loop Experiments

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The ability to dynamically control a behavioral task based on real-time animal behavior is an important feature for experimental neuroscientists. However, designing automated boxes for behavioral studies requires a coordinated combination of mechanical, electronic, and software design skills which can challenge even the best engineers, and for that reason used to be out of reach for the majority of experimental neurobiology and behavioral pharmacology researchers. Due to parallel advances in open-source hardware and software developed for neuroscience researchers, by neuroscience researchers, the landscape has now changed significantly. Here, we discuss powerful approaches to the study of behavior using examples and tutorials in the Bonsai visual programming language, towards designing simple neuroscience experiments that can help researchers immediately get started. This language makes it easy for researchers, even without programming experience, to combine the operation of several open-source devices in parallel and design their own integrated custom solutions, enabling unique and flexible approaches to the study of behavior, including video tracking of behavior and closed-loop electrophysiology.

Keywords: behavior, neuroscience, open source, visual programming, software

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INTRODUCTION

Quantifying animal behavior is crucial in many fields of biological research such as behavioral pharmacology, neuroscience, or ecology. By observing animal behavior in diverse settings, researchers try to extract information about internal states, aiming to understand the causal structure and dynamic properties of genetic and environmental factors (Gomez-Marin et al., 2014; Krakauer et al., 2017).

It is common to consider behavior as simply the set of all movements exhibited by an animal over time, and that the goal of the researcher is having the ability to predict the distribution of these movements under tightly controlled experimental conditions. Behavior, however, is precisely an act of resistance against a changing environment (Marken, 2009) and there is thus a "tug-of-war" between the experimenter who wants to control the environment of the animal and the animal who wants to have control over that same environment. For this reason, researchers often find themselves heavily constraining the opportunities for an animal to win over that control, to reduce the variability in animal movement. Head-fixation or even anesthesia are used both to make it easier to measure

internal physiological state and to simplify the analysis of the behavior. However, such extreme conditions can fundamentally change the nature of the relationship between the animal and its environment, with corresponding changes in the dynamics of neurophysiological activity and a non-negligible impact in the interpretation of experimental results.

In this article, we consider two alternatives to the classical head-fixation or anesthetized paradigms: virtual fixation and voluntary fixation. We discuss how these approaches can resolve fundamental issues in the design and analysis of behavioral experiments and introduce an emerging set of modifiable open-source tools aiming to make them increasingly more accessible to behavioral researchers. We include practical examples and tutorials using the Bonsai visual programming language to help researchers immediately start applying these methods to analyze and study brain circuits and behavior (see also **Supplementary Material** for basic tutorials).

This piece is part of the research topic "New Insights into Behavioral Pharmacology," organized as an extended forum for the workshop "From networks to behavior and back," a satellite event of the European Behavioral Pharmacology Society (EBPS) Biennial Meeting (August 2019, Braga, Portugal).

Virtual Fixation

One of the gold standards for tests of causality is reproducibility: any putative relationship between a causal variable and its effect should be reliably observed. To pick up on such statistical regularities, researchers try to establish comparable conditions under which the relationship can be recorded many times. In behavioral studies, it is common to consider the mapping between "perception" and "action." Perception is the information about the state of the environment that is accessible to the animal at any given time, and action is the set of movements performed by the animal to change its relationship with the environment (**Figure 1**).

To reduce the variability of this complex interaction, researchers often go to great lengths to constrain the variability in perception, in the hopes of reducing irrelevant variability in possible actions. Ironically, to do this effectively requires the action of the animal to be constrained in the first place, often by head-fixation, since even small changes to the position or orientation of the head relative to the environment will dramatically change the amount and type of stimulation reaching the sensors.

An alternative to head-fixation is the freely moving paradigm. In this situation, the animal is free to move in the environment, thus reinstating control over its perception. This situation is often considered by neurophysiology researchers to be a "harder" setting for behavioral neurophysiology, as precise control over the input stimulus is lost.

Surprisingly, however, the neurophysiology and mechanisms of specific brain systems, such as hippocampal navigation, only really started to emerge by allowing animals to be observed under freely moving conditions (O'Keefe and Dostrovsky, 1971; Kandel, 2014). The discovery and analysis of place cell activity rely on the ability to monitor neuronal firing during freely moving navigation. Rather than constraining the input/output mapping between stimulus and action, the researcher records the spontaneous behavior of the animal in detail together with the physiological data and then correlates neural activity across comparable conditions during the experiment, such as the animal location in space. We call this paradigm "virtual fixation."

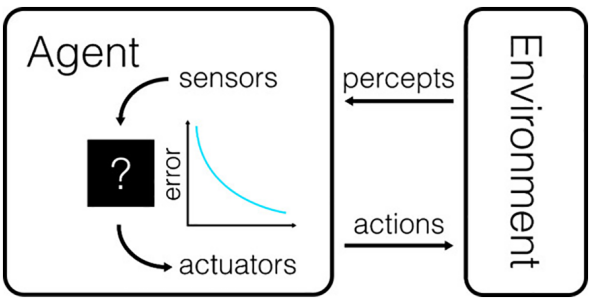

FIGURE 1 | The canonical model for the interaction between a behavioral subject and its environment. The agent receives information about the state of the environment through a variety of sensors and can control the state of the environment through the use of actuators. Actions are chosen to minimize the difference between the perceived state of the world and the intended state of the world. The amount by which this difference is reduced is also sometimes referred to as the utility of an action.

In virtual fixation, the goal is to identify reproducible conditions by precise continuous measurement of animal behavior over time. By identifying moments where the conditions of interest can be reliably compared, behavior becomes amenable to statistical analysis despite occurring spontaneously. Perceptual states can thus be fixed for analysis without artificially fixing the subject or the stimulus.

Of course, this approach is highly dependent on the accuracy of our behavioral measurements, and on the perceptual states we are interested in. Tracking the center of mass position of a single animal in the open field is enough to reconstruct reliable place cell activity. However, it is not enough if we are interested in measuring the distance between the nose and an object, the postural angles of the limbs during walking, or in knowing how many photons are hitting the retina at a given time point.

Fortunately, emerging data analysis techniques are expanding the scope of possible conditions which are amenable to virtual fixation. The use of machine learning technology has entered full-force into behavioral labs worldwide through the introduction of tools such as DeepLabCut (Mathis et al., 2018), which can lower the cost for tracking any user-labeled feature in video datasets. If the human eye can identify a feature of interest in a video, there is now a good chance we can automatically derive a tracker to reliably extract that feature for analysis. Markerless limb and body part video tracking used to be an approach limited to highly technical laboratories which is now much more accessible due to the open-source nature of these tools. Furthermore, first-order features can often be combined to yield other measures of interest. For example, tracking head position can be used to infer what portion of the visual field is accessible to the animal at each moment, thus allowing the researcher to precisely determine which visual information

FIGURE 2 | Bonsai workflow for recording video from a single camera.

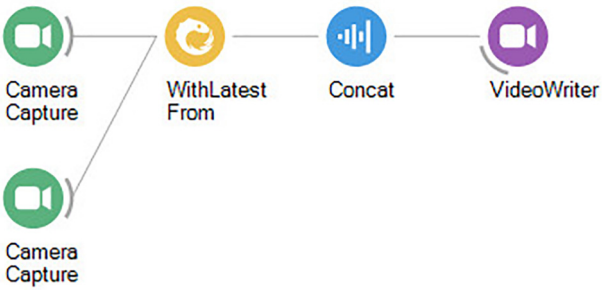

FIGURE 3 | Bonsai workflow for correlating video from two cameras simultaneously.

is accessible to the animal, despite freely moving conditions (Walter and Couzin, 2020).

Virtual Fixation in Practice

Until recently, the development and application of machine learning tools have required moderate programming experience (often in Python), or otherwise relying on standardized video analysis toolkits with a limited set of functionalities. However, the broad applicability of these techniques to diverse datasets has triggered widespread interest even in communities of researchers with no explicit technical training in computer science or computer vision.

Given the diversity of researchers' interests and the difficulties outlined above in defining what is behavior from a measurement perspective, such tools must ideally combine flexibility with ease of access for non-experts. For this tutorial, we will rely on the visual programming language Bonsai[1] (Lopes et al., 2015) to illustrate some basic principles of freely moving behavior measurement, analysis, and virtual fixation.

Although many approaches can be used to measure ongoing animal activity, we will start with video analysis as it remains the tool of choice for non-invasive, flexible, and unbiased investigation of behavior (in contrast to "lever presses" and "nose pokes," the video does not entail too many assumptions on what behavior is before making a measurement). It also does not require complicated hardware setups, as cameras can now be acquired very cheaply and can be placed virtually anywhere, provided that an adequate view of the animal can be obtained. Illumination and occlusion certainly pose a fair share of problems, but existing extensive collections of resources on photography and videography can help researchers to understand and resolve the majority of these issues.

Once the setup is in place, and a compatible camera is connected to the computer, video can be acquired in Bonsai with a simple workflow (**Figure 2**).

It can often be helpful to record videos from multiple perspectives to gain more information about the contingencies surrounding the animal in the freely moving condition. In this case, care should be given to ensure that it will be possible to correlate information from multiple perspectives frame-by-frame. This is possible in single-camera setups by using mirrors to direct light from different angles to the same sensor. In multi-camera setups, we need to correlate the image acquisition in time to ensure that information from one camera can be matched to information from the other cameras. Camera models supporting digital triggers can be used to precisely synchronize the exposure of each frame to an external pacemaker. Otherwise, we can correlate multi-camera acquisition in software by taking the latest exposure from the extra cameras, every time a new frame is collected (**Figure 3**).

Following video recording, data can be processed offline (see **Supplementary Material** for basic tutorials), but all examples shown here can also be used verbatim online to make real-time decisions on the conditions presented to the animal during closed-loop experiments (see "Voluntary Fixation" section).

Virtual fixation requires the reliable extraction of features from the video which we can use to establish comparable conditions for data analysis. By far the most commonly used feature is the spatial position of the animal (usually the center of mass), referenced to a fixed set-up (often a box or arena). The reason for such popularity can be justified by how much information can be derived from this simple metric relative to how easily it can be retrieved from the video (**Figure 4**).

Manipulating the resulting time-series of 2D positions can be used to gain insight into the recorded behavior. Binning the data spatially can generate occupancy maps, thus indicating where the animal spends most of its time; the numerical difference over time will give an approximation for speed or quantity of motion, thus indicating when the animal is active or quiet; and defining spatial entry or exit conditions allows identifying moments where the same path was taken (e.g., in a maze). If physiological data is available and synchronized with the video, it becomes possible to correlate animal position in the arena with physiological signal patterns (e.g., a spike from a specific cell); or the converse, what is the pattern of brain activity when the animal decides to enter a specific area within the arena.

By feeding back the result of tracking it is possible to dynamically crop a region of interest around the subject to obtain an ego-centric video where the animal is always in the center (**Figure 5**). From there we can analyze the video consistently for proximal cues surrounding the animal at any moment, at any point in the arena. For example, a common application of this strategy is to extract a sequence of frames used for training feature detectors by machine learning libraries such as DeepLabCut (Mathis et al., 2018). This way we can refine our

[1]All schematics included in the tutorial are fully functional workflows for the Bonsai programming language which can be executed in the development environment which is freely available online at https://bonsai-rx.org.

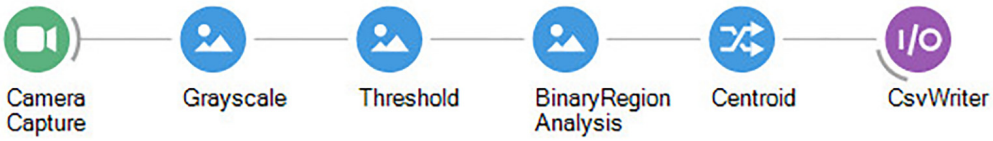

FIGURE 4 | Bonsai workflow for tracking the position (centroid) of a single animal in a well-lit arena, assuming a simple contrast threshold (black on white background).

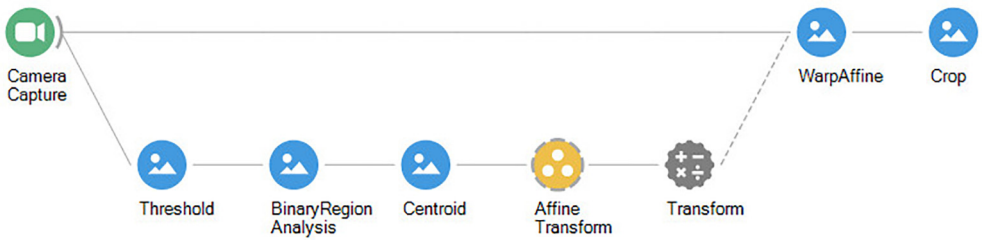

FIGURE 5 | Bonsai workflow for dynamic cropping of a region of interest around the center of the animal.

initial tracking to include specific body parts such as the nose, ears, or paws (**Figure 6**). Furthermore, cropping the video will reduce the dimensionality of the data, therefore significantly reducing training time and speeding-up inference performance for online analysis.

Expanding the basic center of mass tracking to specific body parts, using DeepLabCut or other simple image processing techniques, expands the possibilities for virtual fixation even further, allowing fixing specifically the video around the nose or head of the animal, and then calculating distances between those body parts and other points of interest (or other animals; Kane et al., 2020).

Ultimately, even failure modes in tracking can be interesting. If the tracking is well-calibrated or trained for a specific behavior pattern, then unexpected failures can work as outlier detectors and may well reveal moments of interest in the video (where the animal displays a novel behavior pattern or otherwise deviates from the usual path). In these situations, having an exploratory data analysis workflow with the ability to go from temporal information about a tracking failure back to the video can be extremely useful to assist with interpretation.

Voluntary Fixation

Behavior is often considered to be intrinsically variable, but the whole history of motor control tells us that the story is slightly more complicated than that (Lopes and Kampff, 2015). Behavior and motor control in most animals are incredibly precise, depending instead on the constraints of the body, the task, and most importantly, what the animal is trying to achieve. Interestingly, what tends to happen during training is that animals will shape their degrees of freedom around the exact constraints required to succeed at the task, leaving everything else variable. It is common for example in high temporal precision lever pressing tasks to see animals develop extreme stereotypical patterns which unfold through extremely reliable sequences, and yet will be highly idiosyncratic across individuals (Kawai et al., 2015).

We can observe this proposition by contrasting variability in movements (high), to variability in controlling desired state (not high). Animals will eat, drink and sleep when necessary in extremely reliable patterns, although the means to achieve those ends might be highly variable. If constraints are introduced that need to be overcome to achieve their goals, animals will reliably overcome them, even if the means to do so might surprise and frustrate the researcher. Means are variable, ends are less so, and indeed a large part of behavior is resisting external perturbations, no matter the cost, to achieve goals reliably.

It is thus not surprising to find researchers relying on naturally expressed drives and behaviors to challenge the animal to engage with the researcher's experimental apparatus. Water restriction or aversive stimuli remain common motivators of action in behavior studies such as the shuttling box (**Figure 7**). Another, arguably more humane, approach is to exploit the flexibility of the animal to adopt different goals, and indeed to be trained for different tasks using operant conditioning, where the animal is trained using secondary reinforcers to respond to specific stimuli in ways which are of interest to the researcher.

Both operant conditioning and intrinsic drives have been widely exploited in experimental psychology and experimental neuroscience as means of creating reproducible conditions. For our purposes, we group the collection of both approaches under the term "voluntary fixation." The main goal of voluntary fixation is getting animals to constrain their behavior without the need for pharmacological or physical restriction. Indeed, in some extreme cases, this might be the only way to even approach *in-vivo* fixation studies themselves. For example, rats are notoriously difficult to restrain in awake head-fixed preparations, as they will leverage their incredible arm strength and often damage their own skull in an attempt to escape. But

FIGURE 6 | Bonsai workflow for tracking a specific body part using the Bonsai-DLC package.

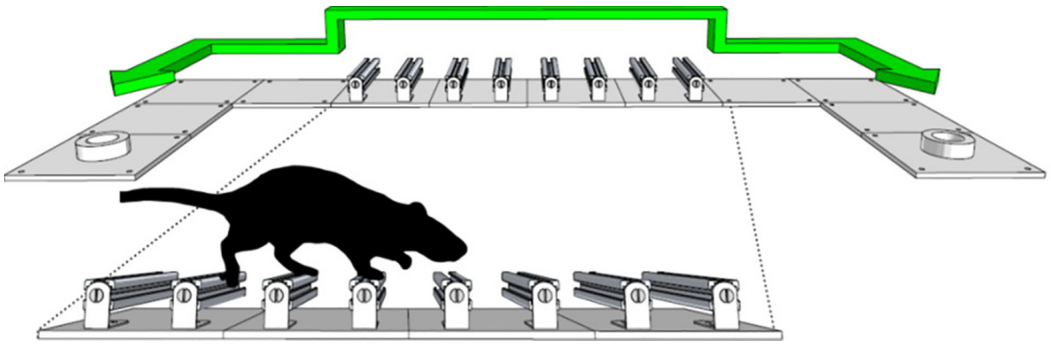

FIGURE 7 | Schematic of the shuttling box apparatus. Animals will forage for liquid reward in this environment, either sucrose or water if using water restriction, and will readily shuttle between the two ports (adapted from Lopes et al., 2016).

recent self-fixation studies have shown that rats will happily self-fix in awake imaging apparatuses in exchange for a reward, as long as it remains their own self-paced decision to do so (Scott et al., 2013).

As with virtual fixation, voluntary fixation places the burden on the researcher to design experimental apparatuses which will work with the animal, either accommodating their natural drives or using automation to deploy operant conditioning protocols where the animal can learn to constrain its behavior in exchange for a reward. In both cases, the technological investment can be too much to bear. Designing automated boxes for behavioral studies requires a coordinated combination of mechanical, electronic, and software design skills which can challenge even the best engineers, and for that reason used to be out of reach for the majority of experimental neurobiology and behavioral pharmacology researchers.

Voluntary Fixation in Practice

Following the advent of accessible hobby electronics platforms such as the Arduino[2], and the opening up of Ph.D. programs to multi-disciplinary candidates, neuroscience has now grown a healthy community of hackers and rapid-prototyping aficionados. Those early researchers who were sympathetic to the open-source and open-science movements ended up adopting those engineering practices while developing their work, resulting in open platforms and tools developed and shared broadly across the neuroscience community. These open devices can now be quickly assembled for monitoring animal actions such as licking and lever pressing or controlling the environment using motors, lights, and sounds (Freeman, 2015; White et al., 2019).

The challenge now remains on how to combine the operation of all these devices in a way that is easy to understand and customize for individual experiments. Ideally, we further want to combine this control with other tools for rich monitoring of behavior such as the techniques for virtual fixation based on the video discussed above. Most interfaces require bespoke programming skills to achieve the required control over the precise, moment to moment, the sequence of events in our experiment, and support closed-loop interactions, so we will again use Bonsai to design some simple, yet functional, illustrations of automated environments which can support voluntary fixation paradigms.

We will start by changing different aspects of the environment using an Arduino microcontroller, which provides different digital output ports which can be controlled directly in Bonsai with a simple workflow (**Figure 8**).

The state of each port can be modified simply by changing the state of Boolean inputs which can be either *True* or *False*. These will correspondingly change the voltage at the terminals of the

FIGURE 8 | Bonsai workflow for controlling a single Arduino digital output.

[2]Arduino is an open-source electronic prototyping platform described in more detail at https://www.arduino.cc/.

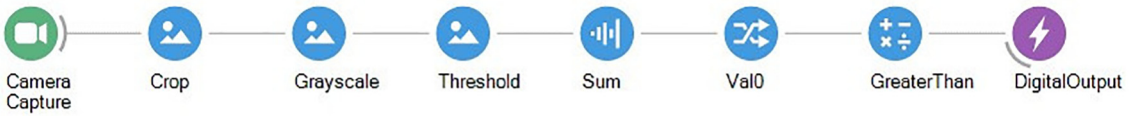

FIGURE 9 | Bonsai workflow for triggering a digital output based on a region of interest.

Arduino between +5 V and 0 V, which can be used to turn lights or lasers on or off, trigger reward, or open valves.

To support voluntary fixation, we need such changes to be triggered automatically so the animal can control the environment using its behavior. This means we need to be able to compute a change in logical level while the animal is moving freely in the environment. For example, to emulate the above foraging patches we can specify regions of interest in the arena which will activate a port whenever an animal enters the patch (**Figure 9**).

Changing the nature of the electronic devices wired to the Arduino allows a broad range of automated responses to be designed, from triggering lights and sounds to electrical or optogenetic stimulation. The online calculation can also easily be changed to operate on other features of animal behavior, such as the amount of motion (**Figure 10**), which will allow for output stimuli to become contingent on animal freezing or fast movements.

Such dynamic control procedures operated using low latency feedback allow the design of environments with interactive properties which can be directly operated by the animal in real-time. Crucially, the experimenter is now in control of the interaction and can modify the response characteristics of the closed-loop system to investigate animal behavior, for instance by delaying, suppressing, or amplifying the feedback response parametrically. Using Bonsai, virtual fixation techniques can themselves be used in the design of such closed-loop systems, for example by using the increasing computational capabilities of GPUs for real-time pose estimation (Kane et al., 2020).

Closed-loop systems are also not restricted simply to behavior and can be successfully used even for purely physiological investigations in living nervous systems. In the example shown below, Bonsai was used for a patch-clamp closed-loop experiment (**Figure 11**). *Ex-vivo* brain slices were prepared from transgenic mice expressing channelrhodopsin (ChR2) in cortical inhibitory interneurons [parvalbumin-positive interneurons (PV)]. Neighboring cortical pyramidal neurons (without ChR2) were patched to record spontaneous firing activity and Bonsai was used to count the number of action potentials fired by the pyramidal neuron in real-time. Upon every 10th action potential, Bonsai triggered a 488 nm fiber-coupled LED, leading to optogenetic stimulation of PV interneurons and inhibition of pyramidal neurons. This design can impose a new self-paced firing pattern where a period of ten action potentials is followed by a period of silence (5 s optogenetic induced silencing) using continuous real-time closed-loop feedback.

Finally, voluntary fixation paradigms can be extended not just to the study of the relationships between a single animal and its environment, but also to interactions between multiple animals. This is often perceived as much harder given the difficulty in identifying individual animals without complex computer vision algorithms. However, the use of simple features relying on invariant geometric properties can yield behaviorally meaningful and surprisingly robust metrics. For example, the following workflow will compute the distance between two animals in a single arena (**Figure 12**).

Since distance is a commutative quantity, we completely avoid the need to uniquely identify each animal, and thus easily achieve fast, real-time performance. By coupling this quantity to a digital output port in the Arduino in the same way as the above examples, we would now be able to trigger stimulation contingent on the distance to a conspecific.

CONCLUSION AND FUTURE PERSPECTIVES

In numerous tasks involving operant conditioning and intrinsic drives have been widely used in experimental psychology and neuroscience to reproducibly study animal behavior. Back in 1959, David Premack proposed that reinforcers should be seen not as stimuli but rather as opportunities to engage in a behavior (Premack, 1959). In his famous drinking vs. running experiment, he showed that thirsty rats prefer to drink rather than to run in the wheel, but when rats are not thirsty, they prefer to run rather than to drink. In other words, the activity should be regarded as the reinforcer, not the stimulus of water (Premack and Anglin, 1973). Of relevance to this discussion, Premack suggested that animals should be allowed to engage freely in activities. Accordingly, as behavioral neuroscientists, we should consider factors that may determine when, and how vigorously,

FIGURE 10 | Bonsai workflow for triggering a digital output based on movement.

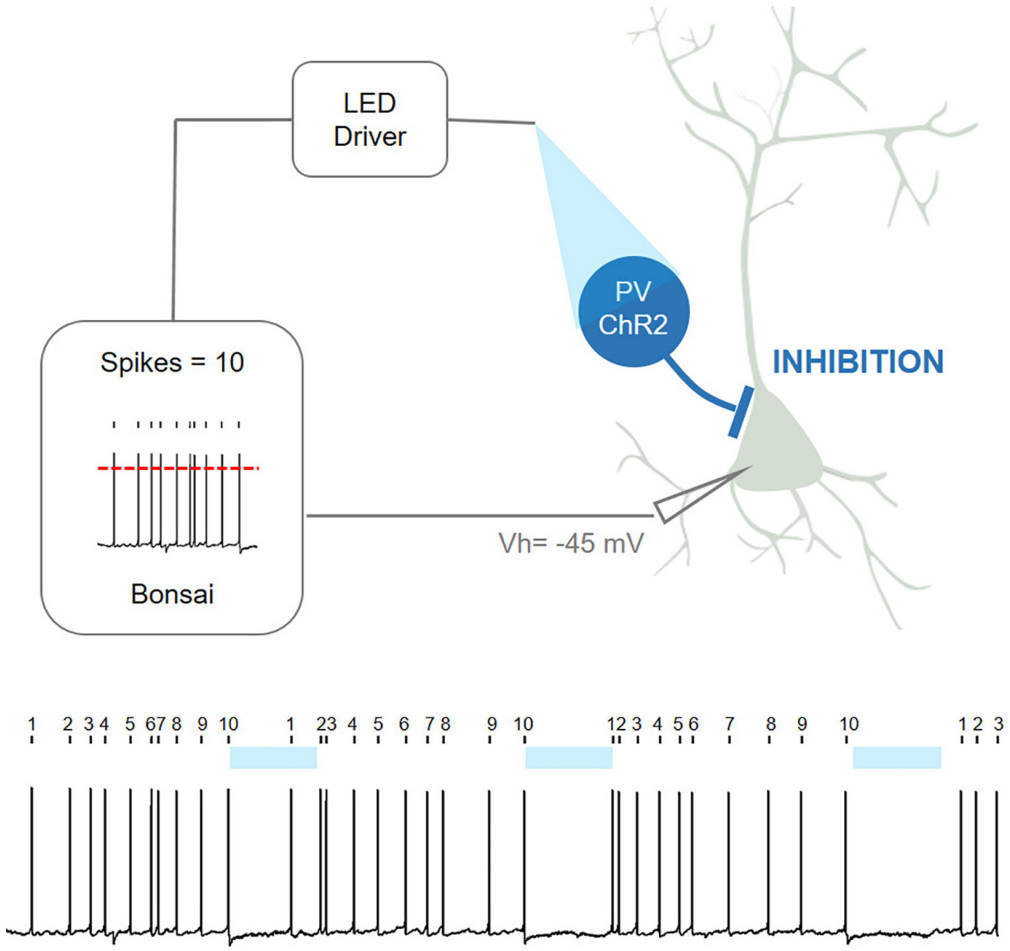

FIGURE 11 | Closed-loop patch-clamp experiment with Bonsai (contributed by Gonçalo Lopes, André Marques-Smith, Luis Jacinto, and Patricia Monteiro [unpublished]).

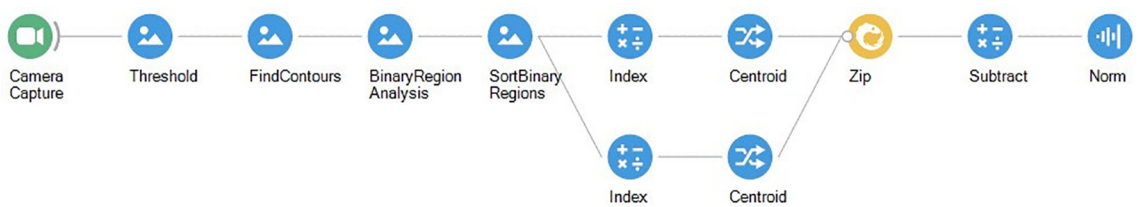

FIGURE 12 | Bonsai workflow for measuring the distance between the two largest objects.

responses will be freely performed and how exploitation of new tools and new behavioral paradigms might grant us experimental control over those responses.

New technologies and open-source tools for neuroscience are rapidly pushing the boundaries of what we can study, and how we study, the brains of awake behaving animals (Freeman, 2015; White et al., 2019). Opposite to proprietary tools that present limited collaborative development and restricted experimental designs, open-source tools allow customization and flexibility. Since its publication in 2015 (Lopes et al., 2015), Bonsai has been widely used by many labs worldwide not only for tracking animal behavior in different species of rodents, cephalopods, fish, and insects (Dreosti et al., 2015; Walker et al., 2015; Douglass et al., 2017) but also to control and acquire data from multiple streams. Being open-source software, Bonsai is free to use and not proprietary to any

one company, thus many users have adopted it to create their specific packages for EEG (Lopes, 2018), Miniscopes (Aharoni and Hoogland, 2019; Guo et al., 2020), Fiber photometry (Carvalho and Lopes, 2019), Open Ephys (Neto et al., 2016) and real-time video analysis with DeepLabCut (Kane et al., 2020). It also encourages good practices for experimental reproducibility by including a built-in package manager and support for portable deployment across rigs. This means that experimental workflow environments can be shared across labs while ensuring the behavior control software is reproducible. This has been leveraged with great success on large international collaborative projects such as the International Brain Laboratory (The International Brain Laboratory et al., 2020).

Leveraging on this exciting open-source hardware/software ecosystem, it is now possible to study animals' naturalistic behaviors while maintaining control over many other variables, and potentially also integrating it with large-scale housing environments (Castelhano-Carlos et al., 2017). In other words, instead of letting existing behavioral paradigms drive the research question, scientists can now design and implement custom behavioral neuroscience experiments with unprecedented control and intellectual freedom.

There are, however, challenges ahead. Because of their flexible nature and large degrees of customization freedom, open-source tools will always require some troubleshooting and experimental validation. Although user community help and development are more and more available, technical support for specific user problems might not be readily available. To overcome this challenge, we need to foster stronger communities and platforms for dissemination, sharing, and training in these open technologies. Only by understanding how such tools work can researchers fully leverage their advantage and realize a healthy open-source mindset for neuroscience. Towards this goal, it is also fundamental that funding agencies start to support and incentivize these development and dissemination efforts, which currently still rely mostly on the passion and determination of lone researchers to share the results of their work, often at great cost to their professional careers as researchers. To protect the future of open-source tools, the academic science community needs to recognize and value such contributions themselves.

Despite being in its infancy and despite all the above challenges, open-source tools have already demonstrated the benefits of shared neuroscience and currently play a significant role in the field of behavioral neuroscience. Their future is bright and adopting a collaborative mindset for the behavioral neuroscience field will prove itself crucial to driving our understanding of the brain. Ultimately, though, studying the neural basis of behaviors still depends on the ability to design the key experiment. It is up to researchers to ask the right questions.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GL and PM conceptualized and wrote the entire manuscript. All authors contributed to the article and approved the submitted version.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Assessing Impulsivity in Humans and Rodents: Taking the Translational Road

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Impulsivity is a multidimensional construct encompassing domains of behavioral inhibition as well as of decision making. It is often adaptive and associated with fast responses, being in that sense physiological. However, abnormal manifestations of impulsive behavior can be observed in contexts of drug abuse and attention-deficit/hyperactivity disorder (ADHD), among others. A number of tools have therefore been devised to assess the different facets of impulsivity in both normal and pathological contexts. In this narrative review, we systematize behavioral and self-reported measures of impulsivity and critically discuss their constructs and limitations, establishing a parallel between assessments in humans and rodents. The first rely on paradigms that are typically designed to assess a specific dimension of impulsivity, within either impulsive action (inability to suppress a prepotent action) or impulsive choice, which implies a decision that weighs the costs and benefits of the options. On the other hand, self-reported measures are performed through questionnaires, allowing assessment of impulsivity dimensions that would be difficult to mimic in an experimental setting (e.g., positive/negative urgency and lack of premeditation) and which are therefore difficult (if not impossible) to measure in rodents.

Keywords: impulsivity, behavior, self-report, translation, back-translation

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INTRODUCTION

Impulsivity has been defined in multiple and partially overlapping manners: (i) a tendency to act quickly, although often prematurely, and without appropriate foresight (Dalley and Robbins, 2017), (ii) predisposition to react in a rapid and unplanned manner to internal or external stimuli with reduced consideration for the negative impacts of such reaction (Fineberg et al., 2010), or (iii) a non-reflective stimulus, in opposition to a reward-driven action (Nigg, 2017). It is considered to be in the same spectrum as compulsivity, which can be defined as the repetition of choices or actions in an inflexible manner, despite changes of setting or negative consequences (Voon and Dalley, 2016). However, they are on opposing extremes of a continuum, being assessed with different tests (Hook et al., 2021) and characterized by dissociable psychological and neurological correlates (Voon and Dalley, 2016).

Impulsivity is commonly aggregated into two major categories: impulsive action and impulsive choice. Impulsive action, or rather its inhibition, is the transient suppression of a quick response to an internal or external cue, allowing slower cognitive processes to be able to operate to guide

the behavior (Winstanley et al., 2006). It has been suggested that impulsive actions can be divided into two types: action restraint or action cancelation, depending on the action being inhibited before or after its initiation, respectively (Schachar et al., 2007; Eagle et al., 2008). Impulsive choice, on the other hand, implies a decision-making component (Winstanley et al., 2006), mainly in two modalities: temporal discounting and reflection impulsivity. In the first, the preference for immediate smaller over delayed larger rewards reflects a higher impulsive choice. The second is the tendency to make fast decisions in the absence of sufficient evidence (Dalley and Robbins, 2017).

Impulsivity has a major adaptive role, but the balance between impulsivity and inhibition is labile, often depending on the situation. If an object falls off a table, the fast impulsive response of grabbing it is typically beneficial. However, if that object is at an extremely high temperature, such reflex can induce lesion. Such is also true for fast aggressive responses (in war vs. stable society contexts) or choice for a smaller immediate reward over a larger delayed one (in immediate need vs. comfortable living contexts). On the other hand, excessive impulsivity in a given context can lead to negative consequences such as physical injury, problems in maintaining relationships, or even imprisonment. At the pathological level, the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) classifies this trait as a diagnosis criterion, a feature, or a risk factor in multiple disorders, including attention-deficit/hyperactivity disorder (ADHD), gambling disorder, and disorders of alcohol or drug use, respectively (American Psychiatry Association, 2013). Indeed, a vast array of literature has associated impulsivity with disorders such as addiction (Mitchell, 2004; Jentsch et al., 2014; Herman and Duka, 2019), reactive aggression (Blair, 2016; Brennan and Baskin-Sommers, 2019), self-harm (McHugh et al., 2019), binge eating disorder (Giel et al., 2017), or ADHD (O'Neill et al., 2017). Comorbidities are also frequent, with impulsivity being a common factor between schizophrenia and aggression (Hoptman, 2015), ADHD and obesity (Cortese et al., 2016), or drug abuse, eating disorders, and self-harm in adolescents (Greydanus and Shek, 2009).

Thus, the assessment of impulsivity in a translational manner is of high importance. In this narrative review, we will systematize behavioral and self-reported measures of impulsivity in a critical manner. Considering that rodents are among the most widely used animal model, we will provide an analysis of tests commonly used for the assessment of impulsivity in humans and rodents and analyze their interspecies comparability. Regarding clinical validity, we will briefly mention results attained in pathologies of altered impulsivity, prioritizing literature with a higher degree of evidence (i.e., meta-analyses and systematic reviews).

BEHAVIORAL MEASURES OF IMPULSIVITY

Behavioral measures have the advantage of evaluating a given dimension of impulsivity in a direct and controlled manner. Also, tests developed for human usage can commonly be adapted for application in laboratory animals and *vice versa*. On the other hand, they are normally unable to assess impulsive behavior that occurs on more complex contexts, which is dependent on a specific emotional or physical state (e.g., impulsivity associated with states of high arousal), thus not capturing all of its dimensions (see self-reported measures for more information).

Impulsive Action

Tests for assessment of impulsive action typically involve a motor response, whose inhibition is rewarded. Considering their simplicity, they are easily adapted and applied to both rodents and humans (see **Table 1** and **Figure 1** for direct associations).

Go–noGo Task

The Go–noGo task assesses action restraint. In the *human* version, a sequence of images (typically letters) is shown to the experimental subjects, who are required to press a key whenever the signal is "Go" (e.g., the letter Z). In a subset of the trials, the "noGo" stimulus (instead of the "Go") is shown, and the subject is required to withhold the response (Winstanley et al., 2006; Winstanley, 2011). Failure to achieve this inhibition is counted as an impulsive response. Many variations of this task have been used, mainly for convenience purposes, or in order to fulfill concerns of each particular study, including using different stimuli (e.g., shapes, pictures, and sounds) (Kaladjian et al., 2011; Dambacher et al., 2015; Hege et al., 2015) or different numbers of Go and noGo stimuli (e.g., V as noGo and any other letter as Go) (Horn et al., 2003). It is also possible to manipulate the number of impulsive responses by altering the presentation proportions (i.e., typically noGo is presented in around 25% of the trials, but this value has been increased up to 50%) (Kaladjian et al., 2011) or by introducing pre-stimulus cues (Kaladjian et al., 2011; Fillmore and Weafer, 2013). Additional elements can be assessed in this test by including distracting images (Brown et al., 2015) or working memory components (Garavan et al., 1999). Because of its simplicity, Go–noGo can be applied to different ages and cognitive profiles. It also seems to be relevant for impulsivity-related disorders, as users of cocaine, MDMA, tobacco, and alcohol were shown to present higher impulsivity in this task, in a meta-analysis (Smith et al., 2014); in contrast, no effects were found in cannabis users, and internet-addicted subjects showed even better inhibitory control than controls (Smith et al., 2014). Also, it has been argued that this test assesses mostly attentional processes (Criaud and Boulinguez, 2013).

Its *rodent* version (also named Go–noGo) follows a similar principle, except that the stimuli are typically sounds or smells. The animal learns that responding to a Go stimulus is reinforced, while responses to the noGo stimulus are not, being considered impulsive. There are multiple variations of this protocol, including different rewards (e.g., food, sucrose, and drugs), cues (e.g., sounds, lights, and smells), apparatus (operant box vs. box with subdivisions), proportion of Go and noGo trials, or application of negative reinforcement (e.g., air puff or quinine). These are typically developed for either convenience purposes (e.g., type of apparatus available) or due to specific experimental concerns (e.g., assessment of the effects of drug or punishment administration on the behavior). Also, protocols in which the animals are head-fixed have been developed, allowing

TABLE 1 | Behavioral measures of impulsivity.

Impulsivity dimension	Impulsivity subdimension	Human version	Rodent version	Main construct
Impulsive action	Action restraint	Go–noGo	Go–noGo	Performing fast responses to Go signals. Withholding the response to a rare noGo signal
		5-csrtt adaptation	5-csrtt	Performing fast and specific responses to stimuli that can be shown at different locations. Avoiding to respond prematurely
		CPT	rCPT and 5C-CPT	Performing fast responses to rare target signals. Withholding the response to a common non-target signal
	Action cancelation	SST	SST	Performing fast responses to a signal. Inhibiting the initiated response upon presentation of a STOP signal
Impulsive choice	Temporal discounting	DD	DD	Choosing between a small immediate reward and a larger, delayed reward
	Reflection impulsivity	Beads task	N/A	Deciding on the amount of information that is sufficient to make a decision
Mixed		N/A	VDS	Performing fast responses to a stimulus. Avoiding to respond prematurely during a stable (impulsive action) or increasing delay (delay intolerance)

5-csrtt, five-choice serial reaction time task; CPT, continuous performance task; rCPT, rodent continuous performance task; 5C-CPT, five-choice continuous performance task; SST, stop signal task; DD, delay discounting; VDS, variable delay to signal.

usage of the Go–noGo task in experiments that involve imaging, electrophysiology, or similar protocols (Anker et al., 2009, rats; Moschak and Mitchell, 2012, rats; Jones et al., 2017, rats and mice; Kamigaki and Dan, 2017, mice; Schiff et al., 2018, mice; and Han et al., 2019, mice). In opposition to the human protocols, application in rodents requires extensive training, whose length will depend on the species (mouse or rat) and particularities of the Go–noGo version used. Indeed, these particularities may also need to be adapted according to species. For instance, Jones et al. (2017) showed that rats are able to learn a task in which a positive valence is associated with the Go cue and a negative valence is associated with the noGo cue, but not the opposite, while mice's learning is associated with the reverse. As in the human version, this task relies on additional functions beyond impulsivity, including attention, Pavlovian and instrumental conditioning, and working memory (Mitchell, 2004).

Five-Choice Serial Reaction Time Task

The five-choice serial reaction time task (5-csrtt) is another widely used task for assessment of action restraint in *rodents*. In this task, five response orifices are available, and lights are shown in each one individually. Nose poking in the illuminated hole is rewarded with a sugar pellet, while responses that occur before one of these five lights is on (i.e., during the inter-trial interval [ITI]) are considered impulsive (Carli et al., 1983, rats; Winstanley et al., 2006, rats; Bari et al., 2008, rats; Cope et al., 2016, rats and mice; Higgins and Silenieks, 2017, rats and mice). Also, continuous responses at the apertures after reward delivery are considered perseverative, which are more akin to compulsion, rather than impulsivity (Robbins, 2002). Several variations of this task are used, including alterations in stimulus or ITI duration (altering the propensity toward impulsive responses) or stimulus intensity (altering the attentional demand) (Higgins and Silenieks, 2017, rats). 5-csrtt usage is, however, quite homogenous. Performance on this task is very reliable after appropriate training, which is relatively simple (Higgins and Silenieks, 2017, rats), although prolonged, which may be an impediment for evaluating transient stages of development (e.g., adolescence). Also, other functions are necessarily involved in its performance (e.g., motor, attention, and motivation). Indeed, considering the small stimulus presentation times—down to 1 s or less (Zhong et al., 2018, rats; Bruinsma et al., 2019, rats)—and that the 5-csrtt is based on a human attentional task [continuous performance task (CPT); see below] (Winstanley et al., 2006, rats; Higgins and Silenieks, 2017, rats), attentional demand is very high, which can be seen as a potential confounding factor in the assessment of impulsivity, or as an outcome in itself—i.e., the task can be used for attention evaluation (Bari et al., 2008, rats). Of note, rats have been reported to perform more impulsive responses and to be more reliant on temporal than visual strategies (i.e., assess the time to response, instead of relying on aperture lights) compared to mice (Cope et al., 2016).

Regarding a *human* equivalent, despite being based on the CPT, the 5-csrtt is more akin to a recent back-translation to humans as it does not include a key part of the original task—non-target stimuli. Voon et al. (2014) created a direct adaptation of the 5-csrtt, in which four windows in a touch screen are shown. The trial initiates with the subject pressing a computer key, and upon fast presentation of a stimulus in one of the windows, the key must be released and the correct window selected. Premature release of the trial initiation key is considered an impulsive response (Voon et al., 2014). This task was shown, in the same work, to be relevant for subjects with substance dependence (alcohol, tobacco, methamphetamine, and cannabinoids), who presented higher impulsivity levels than controls (Voon et al., 2014). Of notice, an adaptation of this human version has also been developed for application during magnetic resonance imaging (MRI) (Neufang et al., 2016).

CPT

The *human* CPT (Rosvold et al., 1956) is very similar to a human Go–noGo with inverted frequencies of "Go" and "noGo" targets. Thus, stimuli are shown sequentially, and upon appearance of a rare target, the subject is required to respond. The main

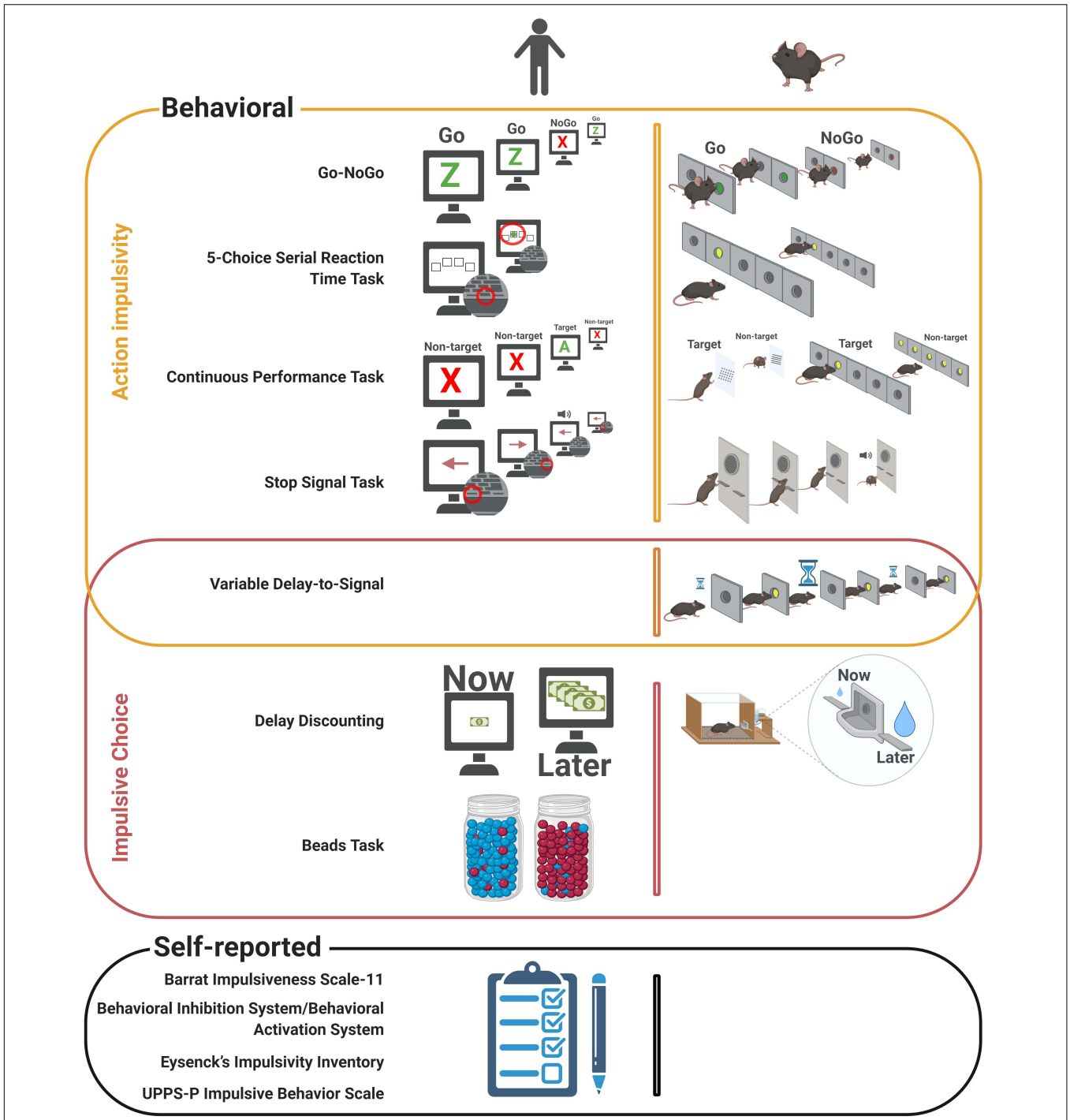

FIGURE 1 | Behavioral and self-reported measures of impulsivity. Behavioral (top) and self-reported (bottom) measures of impulsivity are shown in a schematic manner, highlighting the parallel between human (left) and rodent (right) methods. Image created with BioRender (www.biorender.com).

metrics are the error rate and the reaction time (Ballard, 1996; Roebuck et al., 2016). Although some aspects of impulsivity can be captured in this test, it was designed primarily for assessment of attention (Riccio et al., 2002; Roebuck et al., 2016), and that remains its most common usage (Riccio et al., 2002). It is also influenced by other factors that are particularly important in an attentional task and which can be associated with the surrounding environment (e.g., noise and temperature) or with subject state or trait characteristics (e.g., motivation, age, and personality) (Corkum and Siegel, 1993; Ballard, 1996). Multiple variations of this task have been used, including different stimuli (visual letters, auditory letters, or tones), which do not seem to

influence the outcome (Roebuck et al., 2016); different targets (e.g., an "X" that is shown after an "A," thus introducing a working memory component); frequencies of target presentation; or interstimulus intervals (altering the attentional component and the propensity for impulsive responses) (Riccio et al., 2002). In meta-analyses, the CPT showed good performance in the assessment of children with ADHD (Losier et al., 1996; Huang-Pollock et al., 2012), as well as in the distinction between treated and non-treated patients with this pathology (Losier et al., 1996).

The CPT has been adapted for *rodent* usage in a multitude of manners. The most commonly used for impulsivity assessment is the 5-csrtt (see section "5-Choice Serial Reaction Time Task"), although the rodent CPT (rCPT) and the 5C-CPT are more akin to the original task. As in the CPT, rodents performing the rCPT are required to respond to target stimuli and to withhold response to non-targets. The task is administered using a touch screen, where a pattern is shown. The animal should touch a target pattern and withhold response to a non-target pattern in order to receive a reward; each stimulus is presented 50% of the time (Kim et al., 2015, mice). Of note, as an adaptation of the CPT, the rCPT was developed as a task to assess attention (Kim et al., 2015, mice) rather than impulsivity, although it has also been used for that effect (Caballero-Puntiverio et al., 2019, mice). The five-choice CPT (5C-CPT; rodents) is very similar to the above-described 5-csrtt, with the target being a light that is shown in one of five apertures, but also including a non-target stimulus—the simultaneous presentation of all five lights—to which the animal must withhold response (Bhakta and Young, 2017, rats and mice; Higgins and Silenieks, 2017, rats). It has been developed aiming to assess attention, vigilance, and response inhibition (Bhakta and Young, 2017), although it is not commonly used, potentially due to being so recent (2017).

Stop Signal Task

In opposition to the above-mentioned tests which are designed to evaluate action restraint, the stop signal task (SST) is the only one able to assess action cancelation. In its typical format for *human* application, a sequence of visual stimuli is shown, to which one of two actions is requested (e.g., pressing a left button for a leftward arrow and a right button for a rightward arrow). In some trials (typically 25%) and at random delays, a second stimulus (e.g., an image of a cross or a tone) is presented after the first, signaling that the response must be inhibited (stop signal) (Smith et al., 2014; Verbruggen et al., 2019). The stop-signal reaction time is estimated from the probability of stopping upon presentation at different delays and reflects the time that is required to stop an initiated response (Smith et al., 2014). Multiple adaptations of the SST have been used, aiming to alter either the attentional load or the stop reaction time. These include alterations of the number of stimuli/responses in the "Go" condition, salience of the stop signal, or frequency of presentation of the stop signal. Aiming to homogenize conclusions, a consensus guide was recently published (Verbruggen et al., 2019). The construct of this task allows reduced interference of attention in the assessment of impulsivity, and several meta-analyses have found effects in pathologies typically associated with impulsivity, including ADHD (Alderson et al., 2007; Lipszyc and Schachar, 2010), pathological gambling (Smith et al., 2014; Chowdhury et al., 2017) (although Lipszyc and Schachar, 2010, did not find this effect), substance dependence (Lipszyc and Schachar, 2010), and schizophrenia (Lipszyc and Schachar, 2010). On the other hand, as SST is designed to elicit approximately 50% failures, it may be considered too difficult by some subjects and decrease motivation (Smith et al., 2014).

The *rodent* version of SST follows a similar construct. The animals are trained to, after a signal, press a first and then a second lever, sequentially, in order to receive a reward. On some trials (typically 20%), a tone (stop signal) is presented after pressing of the first lever, and the animal is required to withhold from pressing the second lever (Winstanley and Clark, 2016, rats). Because the task is very similar to the human one, impulsive-like behavior can easily be inferred; however, it requires extensive training.

Impulsive Choice

Tests for assessment of impulsive choice imply a decision between two potential actions, aiming to maximize the attained reward. Such implies complex processes which often hinder a direct translation of tasks between human and rodents (see **Table 1** and **Figure 1** for direct associations).

Delay Discounting

The delay discounting (DD) task is based on the assessment of reward value through a balance between its size and the delay to get it. Typically, across trials, one of these two variables is changed, eventually reaching a level at which the value of both rewards is similar (indifference point) (Vanderveldt et al., 2016). In *humans*, the task is often performed using a computerized platform. Variations may depend on the goal of the study, e.g., different types of reward (money, drugs, food, etc.), or aim to alter the levels of impulsive decision making by changing the sequence in which the values are presented (ascending, descending, balanced, or random—see Robles and Vargas, 2008, for effects) or the number/size of delays/rewards (da Matta et al., 2012). Also, the structure can be fixed for all participants or adapted to the performance, aiming to increase sensitivity (da Matta et al., 2012). Steeper discounting has been associated with obesity (Amlung et al., 2016) and abuse of alcohol, tobacco, cannabis, stimulants, opiates, or gambling (Amlung et al., 2017). While testing is simple and data analysis is well established, its simplicity in comparison with real-world decision making has been discussed (Vanderveldt et al., 2016). Indeed, the task typically contemplates one immediate reward and one associated with a delay, but not two different delays. Such would be associated with more complex processes of decision, in which a preference reversal is commonly observed, i.e., an initial preference for the larger, more delayed reward, which is reversed as the time of the smallest, less delayed one gets closer (e.g., the planning for starting a diet on Monday, aiming to achieve a healthier lifestyle, which is replaced by the desire for highly energetic food) (Vanderveldt et al., 2016).

In the *rodent* version, the animals are required to select one of two levers in order to receive the corresponding reward (typically sugared food) (Winstanley et al., 2006, rats). In alternative, it has been performed in a T-maze, instead of an operant box (i.e.,

the choice is made by selecting the left or the right arm, rather than a lever or a nose-poke hole) (Winstanley, 2011, rats; Feja et al., 2014, rats; Masuda et al., 2020, mice), but other variations include the way the delay evolves (preset or adjusting and within or between sessions), the type of reward (e.g., food or drugs), or its relative size (Winstanley, 2011, rats).

While the human and rodent tasks are quite similar in their construction, they often differ in key aspects, including (i) the type of reward—palatable food or drink, alcohol, or drugs in rodents—and hypothetical money (most common), real food/drink, real money, entertainment, activities, or social/sexual reward in humans (of note, non-monetary rewards used in humans have been shown to increase non-systematic responding, Smith et al., 2018); (ii) the delay—normally in the range of seconds for rodents and months or years in humans (although delays of seconds have been previously applied to humans, Pietras et al., 2003); (iii) the reward presentation—typically, animals experience the reward, while it is just communicated to humans; (iv) the waiting—rodents who choose the long delay have to endure it in a relatively small space with minimal entertainment, while humans are able to proceed with their normal activities during the delay (Vanderveldt et al., 2016).

Beads Task

The beads task, used in *humans*, assesses the "jumping to conclusions" bias, which is considered as a lack of reflection impulsivity, even though it is controversial whether this is a test of impulsive choice. In this task, two jars of beads are filled with equal but opposite ratios of different-colored beads (e.g., jar 1 has 85 red and 15 blue beads, and jar 2 has 85 blue and 15 red beads). The jars are hidden, and individual beads are shown in a predetermined order to the subject, who needs to decide from which jar the beads are being taken. Two main variables are assessed: the number of beads drawn before a decision is made, and the proportion of extreme responders (i.e., subjects who make a decision based on one or two beads) (Dudley et al., 2016). Common variations of the beads task include changes in the ratios or in the jar contents (Dudley et al., 2016), altering impulsive decision; or inclusion of distractor sequences (McLean et al., 2018), improving reliability and repeatability (McLean et al., 2018). In a meta-analysis, people with psychosis in comparison with healthy controls required less beads to make a decision and had a higher number of extreme responders. Also, people with delusions required less beads than people with psychosis but without delusions, also having more extreme responders (Dudley et al., 2016). In impulsivity-related pathologies, to the best of our knowledge, no systematic reviews or meta-analyses were performed, although data suggest alterations in this task in binge drinkers (Banca et al., 2016). There is no rodent equivalent of this task, nor is there a rodent task that claims to assess reflection impulsivity.

Mixed Tasks

One *rodent* task, the variable delay to signal (VDS), assesses both impulsive action and delay tolerance (impulsive choice) (Leite-Almeida et al., 2013, rats; Soares et al., 2018, rats). It was originally based on the 5-csrtt, but using only one response aperture. The animals are required to nose-poke in the aperture when its light is on but refrain from doing it in the delay that precedes presentation of such light (impulsive response). At a first stage of the task, this delay is maintained constant (3 s), and premature responses reflect action impulsivity (i.e., were associated with the 5-csrtt). The second stage includes three blocks of different delays: 3, 6/12, and again 3 s, and an increase in impulsive responses in consequence of the larger delays reflects delay intolerance (i.e., is associated with DD) (Leite-Almeida et al., 2013, rats; Soares et al., 2018, rats). To date, no variations of this task have been published, except for an adaptation of the delays after the first publications (Leite-Almeida et al., 2012, rats; Leite-Almeida et al., 2013, rats), which may be due to a utilization restricted to the original group (Leite-Almeida et al., 2012, rats; Leite-Almeida et al., 2013, rats; Melo et al., 2016, rats; Carvalho et al., 2017, rats; Cunha et al., 2017, rats; Soares et al., 2018, rats; Cunha et al., 2020a, rats; Cunha et al., 2020b, rats), with only one exception to date (Jiménez-Urbieta et al., 2020, rats). In comparison with other tasks, the VDS reduces the attentional bias, as well as potential effects of extensive training. Its reduced training time (7 days) also allows assessment of transient states (e.g., adolescence). Indeed, it was shown to be sensitive to age, sex, and strain differences (Soares et al., 2018, rats). On the other hand, it still relies on motor performance, and despite association with DD, its inclusion as a task for the assessment of choice impulsivity would be discussible, as it does not imply a choice *per se*, but rather a delay intolerance component. Although the VDS is based on the 5-csrtt (which is, in turn, based on the CPT), it does not have a direct human equivalent task, nor has it been used in mice.

SELF-REPORTED MEASURES OF IMPULSIVITY

Self-reported measures of impulsivity are attained using structured questionnaires regarding attitudes or feelings in different situations. Such allows the assessment of impulsivity within given contexts that cannot be reliably reproduced in a laboratory, and despite this dimension of subjectivity, they have often shown reliability and reproducibility (see below). On the other hand, subjective measures cannot be back-translated to rodents. We here summarize some of the most commonly used scales. For a more in-depth analysis of self-reported measures, please consult the recent review by Hook et al. (2021). **Table 2** summarizes the basic components of the here-described tests.

Barratt Impulsiveness Scale-11

The Barratt Impulsiveness Scale-11 (BIS-11) is one of the most widely used scales for the assessment of impulsivity. Its currently used version was designed by Patton et al. (1995) and assesses three main factors within 30 items, which can be further subdivided: attentional impulsiveness (attention and cognitive instability), motor impulsiveness (motor and perseverance), and non-planning impulsiveness (self-control and cognitive complexity), to which the subject responds through a scale that ranges from 1 (rarely/never) to 4 (almost always/always).

TABLE 2 | Self-reported measures of impulsivity.

Task	Response scale	No. of items	Impulsivity dimensions
BIS-11	1 (rarely/never) to 4 (almost always/always)	30	Attentional (attention and cognitive instability), motor (motor and perseverance) and non-planning impulsiveness (self-control and cognitive complexity)
BIS/BAS	1 (very true for me) to 4 (very false for me)	24	BIS and BAS (drive, reward, and fun seeking)
Eysenck's Impulsivity Inventory	Yes/No	54	Impulsiveness, venturesomeness, and empathy
UPPS-P	1 (agree strongly) to 4 (disagree strongly)	59	Negative urgency, positive urgency, sensation seeking, lack of premeditation, and lack of perseverance

BIS-11, Barratt Impulsiveness Scale-11; BIS, Behavioral Inhibition System; BAS, Behavioral Activation System; UPPS-P, Impulsive Behavior Scale.

Attentional impulsiveness items include statements such as "I don't pay attention" or "I am a steady thinker" (inverted). Motor impulsiveness is reflected in sentences such as "I act on impulse" or "I am future oriented" (inverted). Non-planning impulsiveness is assessed though statements such as "I say things without thinking" or "I like to think about complex problems" (inverted) (International Society for Research on Impulsivity, 2020). Meta-analyses have shown that BIS-11 motor impulsivity is altered in pathological gamblers (Chowdhury et al., 2017) and that all dimensions are altered in bipolar disorder (Saddichha and Schuetz, 2014). Also, a systematic review has shown an association with food addiction (Maxwell et al., 2020).

Behavioral Inhibition System/Behavioral Activation System Scale

The Behavioral Inhibition System/Behavioral Activation System (BIS/BAS) scale was developed by Carver and White (1994) and is based on the idea of two contrasting systems. One is associated with anxiety, is sensitive to negative outcomes, and is activated do avoid them (BIS), while the second is associated with appetitive motivation, is sensitive to positive outcomes, and is activated to approach them (Carver and White, 1994). This scale includes 24 items to which the subject responds in a scale that ranges from 1 (very true for me) to 4 (very false for me). BIS/BAS includes four subscales: (i) BIS, assesses the reaction to an anticipated punishment through sentences as "I worry about making mistakes" or "Criticism and scolding hurts me quite a bit"; (ii) BAS drive, directed at the pursuit of desired goals, including "I go out of my way to get things that I want"; (iii) BAS reward responsiveness, assesses the positive response to the anticipation of a reward, such as "When I get something I want, I feel excited and energized"; and (iv) BAS fun seeking, evaluates the desire for new rewards and the approach motivation toward potentially rewarding events, such as "I will often do things for no other reason than that they might be fun" or "I often act in the spur of the moment" (Carver and White, 1994). Despite its wide usage, to the best of our knowledge, no meta-analyses have been published to assess BIS/BAS effects on impulsivity-related disorders. One systematic review, however, was unable to find associations of BAS and food addiction, while the number of BIS studies was insufficient for conclusion withdrawal (Maxwell et al., 2020). Nonetheless, the literature suggests associations with alcohol (Studer et al., 2016) and nicotine (Baumann et al., 2014) use.

Eysenck's Impulsivity Inventory

Eysenck's Impulsivity Inventory, also known as the Impulsiveness, Venturesomeness, and Empathy (IVE) Questionnaire, was developed, in its current version (I7) by Eysenck et al. (1985). Although it is not as commonly used as the above-mentioned scales, we include it in this review due to its different construct. The questionnaire is composed of 54 items to which the subjects respond in a dichotomic manner (yes or no). These are organized into three subscales: (i) impulsiveness, including items as "Do you often buy things on impulse?" or "Before making up your mind, do you consider all the advantages and disadvantages?" (inverted); (ii) venturesomeness, including "Would you enjoy water skiing?" or "Do you find it hard to understand people who risk their necks climbing mountains?" (inverted); and (iii) empathy, including "Would you feel sorry for a lonely stranger?" or "Do you like watching people open presents?" (Eysenck et al., 1985). No meta-analyses or systematic reviews have assessed this scale's results in impulsivity-related disorders, although data suggest an association with obsessive-compulsive (Smári et al., 2008) and borderline personality (Mortensen et al., 2010) disorders, MDMA consumption (Morgan, 1998), and binge eating (Cuzzocrea et al., 2015).

UPPS-P Impulsive Behavior Scale

More recently, the UPPS-P Impulsive Behavior Scale was developed, having the particularity of assessing impulsive behavior that occurs under extreme positive emotions, i.e., positive urgency (Cyders et al., 2007). It is composed of 59 items (a shorter version of 20 items has also been developed, Cyders et al., 2014), to which answers range from 1 (agree strongly) to 4 (disagree strongly). Five dimensions are assessed: (i) negative urgency, including "When I feel rejected, I will often say things that I later regret" (inverted); (ii) positive urgency, as in "When I am in great mood, I tend to get into situations that could cause me problems" (inverted); (iii) sensation seeking, including "I quite enjoy taking risks" (inverted); (iv) lack of premeditation, such as "I like to stop and think things over before I do them"; and (v) lack of perseverance, including "Unfinished tasks really bother me" (International Society for Research on Impulsivity, 2020). Meta-analyses have been able to find associations between these subscales and impulsivity-related disorders, including alcohol (Berg et al., 2015) and substance (Berg et al., 2015; VanderVeen et al., 2016) abuse, nicotine dependence (Kale et al., 2018; Bos et al., 2019),

borderline personality traits, suicidality, aggression, and eating disorders (Berg et al., 2015).

DISCUSSION

Impulsivity is a multifaceted construct influenced by both biological (e.g., genetic, age, and sex) and environmental (familial, cultural, etc.) factors. For instance, impulsivity has been reported as sex dependent in both humans (Cross et al., 2011; Weafer and de Wit, 2014) and rodents (Weafer and de Wit, 2014; Soares et al., 2018), but the direction and strength of such effect depend on the assessed dimension. Additional variability may arise due to the mediation of other factors, as hormonal cycle in women (Diekhof, 2015) and female rats (Swalve et al., 2016), age (Soares et al., 2018, rats; Rosenbaum and Hartley, 2019, humans), genetics (Bezdjian et al., 2011, humans; Soares et al., 2018, rats; Jupp et al., 2020, rats), or environment (Bezdjian et al., 2011, humans; Kirkpatrick et al., 2013, rats). These influences, as well as their human–rodent parallels, are of high importance for the development of translational research. One additional relevant factor is attention, which is required for all the behavioral tasks presented above. It is widely acknowledged that attention to new environmental cues is critical for inhibiting the current flow of decisions and actions and for shifting toward a more appropriate flow (Bari and Robbins, 2013). Nevertheless, some of the available behavioral tasks for the assessment of impulsivity are frequently criticized by their excessive focus on attentional demand (e.g., 5-csrtt).

The here-described methods cover the spectrum of impulsivity dimensions and have been shown to reliably detect alterations in impulsivity in clinical contexts in which it is expected to be altered, including drugs (Jentsch et al., 2014) or alcohol (Herman and Duka, 2019) abuse, smoking (Mitchell, 2004), reactive aggression (Blair, 2016; Brennan and Baskin-Sommers, 2019) (often in the context of schizophrenia, Hoptman, 2015), self-harm (McHugh et al., 2019), binge eating disorder (Giel et al., 2017), or ADHD (O'Neill et al., 2017)—see text for details on meta-analyses and systematic reviews. Rodent task validation, on the other hand, is partially assumed by the similarities to their human counterparts, as most of these contexts can only be partially replicated in rodents. However, all the here-described rodent tasks have shown alterations of impulsivity associated with substances of abuse (e.g., 5-csrtt, Broos et al., 2017; 5C-CPT, Irimia et al., 2014; SST, Beckwith and Czachowski, 2016; DD, Harvey-Lewis et al., 2014; and VDS, Leite-Almeida et al., 2013).

Two main types of impulsivity assessments are here presented: behavioral and self-reported measures. Even though impulsivity is expected to vary with age, self-reported measures assess impulsivity as a trait—i.e., the scores are expected to remain relatively stable over time. They present several advantages, including the ability to assess different dimensions of impulsivity in the same questionnaire, which is applied in 5–15 min without the need for any equipment. They demonstrate satisfying psychometric characteristics, including good internal consistency and high test–retest reliability, and provide a context for the evaluated behaviors (e.g., "When I am in great mood, I tend to get into situations that could cause me problems" in UPPS-P). However, these behaviors are evaluated according to the subject's perception and are thus not necessarily objective and of limited application if self-perception is altered. Importantly, self-reported measures are not transposable to animals. On the other hand, behavioral assessments provide laboratory-controlled, objective measurements of a given impulsivity dimension. These measurements can be altered according to internal states (e.g., arousal and stress) and can thus be considered more akin to state impulsivity, being therefore more suitable for association with transient states (e.g., drug effects). They can often be paralleled between humans and rodents, allowing a translational evaluation of cellular, molecular, and network players. However, they fail to provide more complex contexts to the assessment. A concern related to these laboratorial assessments pertains to a limited external validity, particularly in animal models. On the other hand, the use of imagetic and simulated settings (e.g., the use of virtual reality, Pollak et al., 2009; Henry et al., 2012) is a recurrent strategy that diminishes this limitation on the behavioral assessment of impulsivity in human subjects. Of note is that the concordance between self-report and behavioral measures seems to be weak, suggesting that they are assessing different constructs (see for example, Cyders and Coskunpinar, 2012; Hasegawa et al., 2019).

One additional source of variability between studies is the multiple adaptations of established tasks for both humans and rodents. These are often performed in order to answer a specific experimental question (e.g., impulsivity when aiming to attain drugs instead of natural rewards) or to adapt the task to a specific population (e.g., using images instead of letters when studying a cohort of children or manipulating the number of Go and noGo trials in a population with altered attention). Although often necessary, these changes from the originally designed task create difficulties in comparability and may raise questions regarding validity, as validation of small changes is rarely performed. Even though several systematic reviews of the literature focusing on impulsivity exist—see for example (Smith et al., 2014) for Go–noGo and SST—they typically do not account for task variations, which may provide relevant insights regarding the most adequate manipulations to assess impulsivity under specific conditions/goals. Also, behavioral measures have been adapted from humans to rodents and *vice versa*. Such implies alteration in the structure of the task, including the applied stimuli (e.g., letters for humans and sounds for animals) or the rewards (e.g., hypothetical money for humans and physical sugar pellets for rodents). These adaptations are necessary, although interpretations of a parallel between human and rodent behaviors require careful consideration. Indeed, one can argue that the behavioral tasks here presented for rodents try to mimic human impulsivity in species who would not naturally present these behaviors. Reactive aggression, for example, is an ethological behavior in both humans and rodents, and being associated with impulsivity (Blair, 2016; Brennan and Baskin-Sommers, 2019), it could be used to establish this parallel.

There are also additional limitations that are different in rodents and humans. In rodents, whenever the reward is palatable food, caloric restriction is necessary, creating difficulties in the interpretation of impulsivity vs. satiety. Also, in rodents (and to a smaller degree in humans), there is a very high motor demand in some of the tasks, as 5-csrtt, where fast, premature responses are considered impulsive, hindering interpretation or requiring adaptation of the task for aged or injured animals. On the other hand, animal experiments are typically performed by a comparison of different groups (e.g., cortical lesion vs. controls), who should all be in the same satiety conditions and whose motor performance can be assessed and controlled for. In human subjects, however, a range of additional factors can influence impulsivity (see above), some of which, as cultural or familial environment, can be difficult to control.

CONCLUSION

Impulsivity can be considered as an umbrella term in which multiple processes are included. Importantly, not all forms of impulsivity can be objectively measured through laboratory-controlled tasks, whose complementarity with self-reported measures is evidenced by the poor correlation between them. Such self-reported measures, as well as some behavioral ones (e.g., assessment of lack of planning), imply complex reasoning that, even if potentially adaptable to rodent behavioral tasks, would be hard to interpret. Indeed, even in simpler tasks, such as the DD, there is a necessary adaptation of times and rewards, which brings out questions regarding the validity of such translation. These difficulties are added to the necessary consideration of additional factors whose disentanglement from the impulsive behavior is not clear, including attention, memory, and motivation, as well as the context in which the impulsive behavior occurs (e.g., urgency in positive or negative situations). There is, however, an evident effort to establish a parallel between tasks, creating multiple translations and back-translations and thus allowing the assessment of the core of the behavior in a translational manner.

AUTHOR CONTRIBUTIONS

ME performed the literature research. ME and HL-A designed the manuscript. ME, PM, and HL-A wrote the first draft. ME, PM, NS, and HL-A discussed, provided input, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Where Dopaminergic and Cholinergic Systems Interact: A Gateway for Tuning Neurodegenerative Disorders

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Historically, many investigations into neurodegenerative diseases have focused on alterations in specific neuronal populations such as, for example, the loss of midbrain dopaminergic neurons in Parkinson's disease (PD) and loss of cholinergic transmission in Alzheimer's disease (AD). However, it has become increasingly clear that mammalian brain activities, from executive and motor functioning to memory and emotional responses, are strictly regulated by the integrity of multiple interdependent neuronal circuits. Among subcortical structures, the dopaminergic nigrostriatal and mesolimbic pathways as well as cholinergic innervation from basal forebrain and brainstem, play pivotal roles in orchestrating cognitive and non-cognitive symptoms in PD and AD. Understanding the functional interactions of these circuits and the consequent neurological changes that occur during degeneration provides new opportunities to understand the fundamental inter-workings of the human brain as well as develop new potential treatments for patients with dysfunctional neuronal circuits. Here, excerpted from a session of the European Behavioral Pharmacology Society meeting (Braga, Portugal, August 2019), we provide an update on our recent work in behavioral and cellular neuroscience that primarily focuses on interactions between cholinergic and dopaminergic systems in PD models, as well as stress in AD. These brief discussions include descriptions of (1) striatal cholinergic interneurons (CINs) and PD, (2) dopaminergic and cholinergic modulation of impulse control, and (3) the use of an implantable cell-based system for drug delivery directly the into brain and (4) the mechanisms through which day life stress, a risk factor for AD, damage protein and RNA homeostasis leading to AD neuronal malfunction.

Keywords: acetylcholine, dopamine, Alzheimer's and Parkinson's disease, impulse control, encapsulated cell-based system

INTRODUCTION

Although research in neurodegenerative disorders have been focusing for many years on individual neuronal circuits and neurotransmitter systems [e.g., dopaminergic one in Parkinson's disease (PD) and cholinergic in Alzheimer's disease (AD)], it is increasingly accepted that different neurotransmitter systems are interrelated and affected under neurodegenerative conditions leading to deficits in related brain functions.

For instance, while PD research is commonly focused on the motor deficits resulting from the loss of nigrostriatal dopaminergic neurons (Paolone et al., 2015), a majority of PD patients suffer from non-motor symptoms such as cognitive and emotional disorders (Chaudhuri et al., 2006). These disturbances are, at least in part, related to a loss of basal forebrain cholinergic neurons but also increased cholinergic tone within the striatum, which temporally coincides with the loss of midbrain dopaminergic neurons (Bonsi et al., 2011; Yarnall et al., 2011). Many PD patients also have a tendency to fall and suffer from a freezing of gait, impairments in posture control and movement efficacy that are not treatable with L-DOPA. Relative to controls and non-falling patients, these individuals have greater reductions of cortical cholinergic activity (Bohnen et al., 2018). Preclinical studies support these findings with demonstrations that a concomitant loss of cholinergic and striatal dopamine afferents disrupts posture control and movement efficacy (Kucinski et al., 2013).

Similarly, memory impairment is the cardinal feature of AD, yet the clinical symptoms of this disorder also include a marked loss of motor function. Moreover, many AD and PD patients suffer from mood deficits, such as depression, a disease state where deficits in monoamines (e.g., dopamine), are found.

It is increasingly recognized that brain functions, from the executive and motor functioning to memory and emotional responses, are strictly regulated by the integrity of multiple interdependent neuronal circuits, the above clinical profiles in PD and AD, neurodegenerative disorders with different etiology, are likely the result of an intricate interplay of multi-system degenerations extending beyond the loss of nigrostriatal dopaminergic neurons in PD and the cholinergic denervation in AD (Paolone, 2020; Policastro et al., 2020).

To this aim, this mini-review will briefly discuss the main topics covered in a symposium from the 18th biennial meeting of the European Behavioral Pharmacology Society held in Braga, Portugal in August 2019 that focused on our current state of knowledge regarding functional interactions and cooperation of cholinergic and dopaminergic systems in motor and non-motor behaviors, as well as innovative developments in intracranial drug delivery in PD models and new insights into the role of chronic stress in AD models. This mini-review will start with describing electrophysiological and behavior data demonstrating the involvement of striatal cholinergic interneurons (CINs) in murine PD models, followed by a brief discussion on behavioral data indicating dopaminergic and cholinergic modulation of impulse control in rats. Subsequently, a novel encapsulated cell-based system for neurotrophic delivery directly into the brain will be introduced that demonstrated therapeutic effects in neurological and degenerative diseases. The mini-review will end with cellular data highlighting the effects of chronic stress on the dysregulation of proteostasis and RNA homeostasis in AD.

STRIATAL CHOLINERGIC INTERNEURONS AND PARKINSON'S DISEASE

Marianne Amalric provided an update on the role of striatal CINs in the expression of motor, cognitive and motivational impairments in neurological disorders (Bonsi et al., 2011). The degeneration of nigrostriatal dopaminergic (DA) neurons in PD leads to an imbalance between the dopaminergic neuronal and CINs activity in the striatum that is thought to be associated with the emergence of rigidity, tremor and bradykinesia (Obeso et al., 2000; Aosaki et al., 2010). Anticholinergic drugs were the first therapeutic treatment for PD suggesting that an increased cholinergic tone in the striatum could result from striatal DA denervation (Duvoisin, 1967). Despite comprising less than 2% of all striatal neurons, they are potent modulators of medium spiny neuronal (MSNs) excitability, due to their widespread connections to output neurons. Modulation of MSNs by CINs may therefore appears as a critical player to reduce the imbalance between striatal DA and ACh activity (Calabresi et al., 2006; Pisani et al., 2007).

Although PD is traditionally classified as a movement disorder, it is increasingly recognized that non-motor symptoms frequently appear in the early stages or even during the pre-motor phase of the disease (Chaudhuri et al., 2006; Aarsland, 2016). A variety of non-motor symptoms, ranging from neuropsychiatric to cognitive impairments and loss of inhibitory control, are commonly observed in Parkinsonian patients. Although reciprocal interaction of acetylcholine and dopamine may underlie motor symptoms observed in pathophysiological conditions (Aosaki et al., 2010; Lester et al., 2010; Gittis and Kreitzer, 2012; Rizzi and Tan, 2017), much less is known on DA/Ach interactions in non-motor functions in the early stages of the disease. By combining a series of optogenetics, electrophysiological and pharmacological studies, Amalric and colleagues investigated the impact of striatal DA denervation in rodent models of PD on striatal CINs reactivity and behavioral outcome. The activity of striatal CINs is mainly driven by dopaminergic modulatory inputs and excitatory glutamatergic cortical and thalamic inputs. Nicotinic and muscarinic receptors are expressed at different levels of the striatal microcircuit where they modulate striatal afferent and efferent neuronal systems. In particular, the high level of expression of muscarinic acetylcholine receptors (mAChRs) in the striatum raised the question of their role in the regulation of the striatal network. *In vitro* studies in animal models of PD reveal that DA denervation of the striatum increases CINs excitability (Fino et al., 2007) and ACh release (Duvoisin, 1967; Bonsi et al., 2011) and contributes to the reorganization of striatal microcircuitry (Tozzi et al., 2016). The impact of CINs modulation *in vivo* on motor and non-motor symptoms in rodent models of PD is less known, however. Therefore, Amalric and colleagues investigated

how optogenetic manipulation of CINs may affect the basal ganglia circuitry in different murine models of PD and how it translates to behavioral changes. To specifically express the opsins in striatal CINs, they performed stereotaxic injections of a Cre-inducible adeno-associated virus (AAV) vector carrying the gene encoding channelrhodopsin (ChR2) or halorhodopsin (eNpHR) into the striatum of transgenic mice expressing Cre-recombinase under the choline acetyltransferase (ChAt) promoter. *In vitro* recordings of CINs and MSNs revealed that photoactivation of ChR2 increased CINs firing activity in a light-locked manner while photoactivation of the inhibitory eNpHR opsin reduced firing activity. *In vivo* electrophysiological results in anesthetized mice, showed a normalization of the abnormal firing activity measured in the substantia nigra *pars reticulata*, the main output structure of the basal ganglia, in Parkinsonian conditions. Furthermore, it was found that photoinhibition of CINs activity primarily affected the transfer of cortico-striatal information by enhancing the activity of the direct striatonigral pathway, rather than reducing the activity of the indirect pathway (Maurice et al., 2015). Behavioral studies confirmed the critical contribution of striatal CINs in the various rodent models of late PD stage. In a pharmacological model (neuroleptic-induced catalepsy), photoinhibition of CINs reduced the akinetic symptoms, while their photoactivation did not modify the cataleptic behavior. In the lesional model of late PD (extensive DA lesions), CINs photoinhibition reversed all the asymmetric motor deficits, while the same optogenetics manipulation was ineffective in sham-control animals (Maurice et al., 2015; Ztaou et al., 2016). In a model of early PD stage, low dosage of the neurotoxin 6-OHDA induces an average of 30–40% loss of nigral DA neurons affect short-term memory in object and social recognition tests (Bonito-Oliva et al., 2014; Ztaou et al., 2016, 2018). Emotional deficits are also measured in the elevated cross maze in partially lesioned mice. CINs photoinhibition of transgenic mice expressing eNpHR in cholinergic neurons with similar partial 6-OHDA lesions alleviated the social recognition and cognitive deficits and reduced anxiety level, but did not affected the behavior of non-lesioned animals. These results suggest that even with a moderate striatal DA depletion, CINs reactivity may account for the cognitive and emotional symptoms measured in lesioned mice. Reducing their activity locally in the striatum may thus appear to be an alternative therapeutic target to reduce non-motor symptoms early in the disease in addition to alleviate motor impairments in the late-stage of PD (Ztaou et al., 2018; Ztaou and Amalric, 2019).

To decipher the mechanisms of ACh action on striatal postsynaptic M1 and M4 mAChRs, additional experiments were performed to pharmacologically block these receptors in normal and mutant mice lacking M4 receptors specifically in direct pathway MSN-D1 neurons (M4-D1 knockout mice). Blocking either M1 or M4 mAChRs in the dorsal striatum with telenzepine and tropicamide (M1 and M4 mAChR antagonists, respectively) reproduced the beneficial effect of optogenetics manipulation of CINs on motor symptoms. Interestingly, tropicamide had no effect in M4-D1 knockout mice. Postsynaptic M4 receptors expressed on direct MSNs output pathway may thus be preferentially involved in tropicamide action (Ztaou et al., 2016). The occurrence of motor and non-motor symptoms in PD may thus involve cholinergic activation of M1 and M4 muscarinic receptors of the striatum.

In summary, optogenetic inhibition of striatal CINs alleviates motor and non-motor deficits in rodent models of early and late PD stages. Optogenetic modulation of striatal CINs may thus provide new tools to treat both motor and cognitive symptoms of Parkinsonian patients.

DOPAMINERGIC AND CHOLINERGIC MODULATION OF IMPULSE CONTROL

Tommy Pattij described their efforts to elucidate the roles of DA and ACh function in impulse control, noting that impulse control disturbances are important features in psychiatric disorders such as attention-deficit/hyperactivity disorder and substance use disorder (Moeller et al., 2001). In particular, he focused on inhibitory response control as one of the behavioral and cognitive phenomena of impulse control (Bari and Robbins, 2013). In view of this mini-review it is important to note that impulse control disturbances can also develop as non-motor symptoms in PD, and particularly can arise as a result of dopamine replacement therapy (Seppi et al., 2019). These impulse control disorders can develop in up to one out of five PD patients on dopamine replacement therapy and can manifest themselves as, for example, compulsive buying, hypersexual behavior and pathological gambling (Weintraub and Claassen, 2017).

There is an extensive preclinical literature on dopamine modulation of impulse control, that started decades ago with the observation that challenges with the psychostimulant amphetamine impair inhibitory response control (Cole and Robbins, 1987). Since then, many other studies have further elaborated on this and earlier work from Pattij and colleagues demonstrated the critical involvement of DA and, more specifically, of dopamine D1-like and dopamine D2-like receptors in inhibitory response control (Van Gaalen et al., 2006). Subsequent functional neuroanatomical approaches, including intracranial microinfusions of dopamine ligands and sophisticated rodent micro-positron emission tomography (PET) studies with dopamine D2/D3 ligands, have pinpointed the ventral striatum as a main brain region where dopamine D1-like and dopamine D2-like receptors modulate impulse control (e.g., Dalley et al., 2007; Pattij et al., 2007; Pezze et al., 2009; Besson et al., 2010; Caprioli et al., 2013; Jupp et al., 2013; Pattij and Vanderschuren, 2020). Importantly, the pre-clinical data are paralleled by clinical observations. Recent PET work found that human trait impulsivity correlates with enhanced amphetamine-evoked DA release in the ventral striatum and lower dopamine D2/D3 receptor availability in the midbrain (Buckholtz et al., 2010) and, moreover, with lower dopamine transporter availability in the ventral striatum (Smith et al., 2019). Thus, collective preclinical and clinical data have uncovered a striatal dopamine D2-like receptor mechanism subserving impulse control.

With regard to cholinergic modulation of impulsivity, pharmacological challenges with nicotine impair inhibitory

response control (Hahn et al., 2002; Kolokotroni et al., 2011; Wiskerke et al., 2012), an effect that appears to depend on DA receptor activation (Van Gaalen et al., 2006). Thus, a functional interaction between the ACh and DA neurotransmitter system explains the effects of nicotine on impulse control. As such, it is well known that activation of somatodendritic nicotinic receptors on DA neurons in the ventral tegmental area evokes DA release in the ventral striatum explaining this functional interaction (e.g., Imperato et al., 1986; Barik and Wonnacott, 2009). Interestingly, although different from the acute pharmacological effects of nicotine on the brain, subchronic adolescent but not adult nicotine exposure resulted in long-lasting impairments in inhibitory response control as well as disturbances in attention in rats (Counotte et al., 2009, 2011). Strikingly, these adolescent nicotine effects on impulse control were accompanied by increased electrically-evoked DA release from the prefrontal cortex and not ventral striatum (Counotte et al., 2009). Further work pinpointed terminals of glutamatergic synapses in the medial prefrontal cortex as the brain locus where adolescent nicotine could have impacted impulse control and attention (Counotte et al., 2011). Recent optogenetic approaches have provided further insight into the roles of basal forebrain cholinergic neurons and prefrontal cortical CINs in inhibitory response control and attention. For this, the inhibitory opsin archaerhodopsin was expressed in ChAt-expressing interneurons in either the medial prefrontal cortex or basal forebrain cholinergic neurons in transgenic rats expressing Cre-recombinase under the ChAt promoter. As such this approach in rats is complementary to the murine PD-model experiments conducted by Amalric and colleagues described above (Maurice et al., 2015; Ztaou et al., 2016). Optical inhibition of basal forebrain cholinergic projections to the mPFC as well as CINs in the prefrontal cortex reduced attentional function, albeit at different time scales (Obermayer et al., 2019). In the same study, inhibitory response control was not affected by inhibiting the activity of either of these two types of ChAT containing neurons. Taken together, these results highlight the interplay between the dopaminergic and cholinergic neurotransmitter systems in modulating impulse control, either by activation of nicotinic ACh receptors on DA neurons or on terminals of glutamatergic synapses.

ENCAPSULATED CELL THERAPY: TARGETING DOPAMINERGIC AND CHOLINERGIC STRUCTURAL ALTERATIONS WITH NEUROTROPHIC FACTORS AS A NEW STRATEGY IN THE PATHOPHYSIOLOGY OF NEURODEGENERATIVE DISORDERS

Giovanna Paolone has exploited an encapsulated cell technology that, following to implantation into the brain, provides a targeted, continuous, *de novo* synthesized source of proteins that can be distributed directly to the desired brain region (Lindvall and Wahlberg, 2008; Emerich et al., 2019; Paolone et al., 2019). These studies were based on the use of human ARPE-19 cells that had been genetically modified to produce trophic molecules including glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF). Prior to implantation, the cells were "encapsulated" within semipermeable, immunoisolatory hollow fiber membranes to facilitate their implantation, allow their retrieval for confirmation of function, and minimize immunological rejection. Initial studies evaluated the potential of GDNF in pre-clinical models of epilepsy. GDNF is a particularly interesting candidate for epilepsy as it is physiologically found within the temporal lobe, is upregulated in response to seizure activity, and local delivery can reduce seizures in animal models (Kanter-Schlifke et al., 2007). Extensive *in vivo* studies were conducted in a pilocarpine rat model of epilepsy. Animals with established seizures received bilateral implants of GDNF-secreting devices into the hippocampus and were tested on a battery of neurological tests over several months. Results included:

(1) Controlled, stable, and long-term (at least 6 months) delivery of GDNF to the hippocampus in a well-tolerated manner.
(2) GDNF significantly reduced (>90%) pilocarpine-induced seizures while also normalizing changes in anxiety-like and cognition over several months. In addition to reducing behavioral seizures, it was also found that GDNF significantly reduced seizures as measured by EEG.
(3) The benefits of GDNF were both symptomatic and disease-modifying as the reductions in seizures persisted even when the devices were retrieved.
(4) The functional benefits were associated with protection of the hippocampus against the pathological changes brain anatomy that accompany epilepsy, including hippocampal atrophy, cell degeneration, loss of parvalbumin-positive interneurons, and abnormal neurogenesis. The neuronal protection was associated with GDNF receptor activation (Paolone et al., 2019).

The versatility of this system was confirmed when similar benefits were observed when delivering BDNF to the temporal lobe of pilocarpine-treated rats. In these studies, the frequency of spontaneous seizures was reduced by more than 80%, cognitive performance was improved, and the neurological benefits of BDNF were associated with reductions in degenerating cells and normalization of hippocampal volume and neurogenesis (Falcicchia et al., 2018).

Dr. Paolone further described studies using GDNF as a potential treatment for PD. While GDNF has a relatively long and promising pre-clinical history as a potent neuroprotective agent in models of PD (Choi-Lundberg et al., 1997; Kordower et al., 2000; Kirkeby and Barker, 2019; Whone et al., 2019) its clinical utility has been difficult to test. To be effective, GDNF needs to be delivered selectively in a long-term and stable manner while covering the nigrostriatal system. Implants of encapsulated GDNF cells one week prior to intrastriatal 6-OHDA injections in rats protected DA neurons in the substantia nigra, preserved

DA fibers in the striatum and protected against declines in motor performance. To quantify behavioral extent of the lesion as well as the benefits of GDNF implants, rats behavior was assessed prior to device implant, prior to 6-OHDA lesion and again two and four weeks post lesion using the cylinder, placing and stepping test. When cell-based delivery of GDNF occurred four weeks post 6-OHDA lesions (i.e., a neurorestorative model), improvement in the forelimb use was observed as early as four weeks post GDNF treatment and continued to grow for over one year (62 weeks). Similarly, impressive distribution of GDNF and positive effects on DA function were observed when larger, clinical-sized devices were implanted for three months into the putamen of minipigs. Implantation of two devices, separated by 5 mm, resulted in distribution of GDNF throughout the putamen and caudate that robustly upregulated the expression of tyrosine hydroxylase staining in the regions covered by GDNF diffusion (Wahlberg et al., 2020).

Although the mechanisms are not completely understood, proper function of cholinergic neurons located in the basal forebrain, relies on the supply of NGF retrogradely transported from the cortex and hippocampus (Salehi et al., 2004). Neurons in the medial septal nucleus, the nucleus of the diagonal band of Broca, the nucleus basalis of Meynert, and the substantia innominata, including their cortical and hippocampal projections are severely lost in AD contributing to memory and attention deficits. In rats, NGF cells survive long-term (1 year) and protect cholinergic cells in lesioned and aged animals (Winn et al., 1994). Similarly, in non-human primate, NGF protects septal neurons in lesioned and aged monkey (Emerich et al., 1994; Kordower et al., 1994, 1996). The safety and tolerability of this technology as well as the biological effects, have also been explored in patients with mild to moderate AD to deliver NGF directly to the basal forebrain to restore cholinergic function (Wahlberg et al., 2012; Ferreira et al., 2015; Karami et al., 2015).

In rats, performance of a Sustained Attention Task (SAT) induces a performance-associated increase in cortical cholinergic neurotransmission depending on the integrity of the cholinergic inputs to the prefrontal or posterior parietal cortex. Furthermore, attentional performance is enhanced by the stimulation of the mesolimbic circuitry, particularly the shell of the nucleus accumbens (NAc) through the activation of basal forebrain corticoperal projections (St Peters et al., 2011; Paolone et al., 2012, 2013).

Given that these results support the potential use of encapsulated trophic factor-secreting cells in human diseases such as PD, AD and epilepsy, future studies might focus on the simultaneous delivery of multiple factors to more fully treat the pathology mosaicism that occurs in multisystem disorders such as neurological diseases.

THE INTERPLAY OF CHOLINERGIC INNERVATION AND CHRONIC STRESS IN AD NEUROPATHOLOGY

Investigation in PD provided a model for the pursuit of the selective neuronal vulnerability in the AD brain which was originally focused on cholinergic neurons. In 1970's, the first evidence suggested a selective reduction of the activity of the acetylcholine synthetic enzyme choline acetyltransferase (ChAT) as well as the acetylcholinesterase (AChE) in the brain area of hippocampus, a region known to participate in memory functions, as well as in cortex and amygdala (Davies and Maloney, 1976) while other studies described a relationship between ChAT activity and mental ability in demented subjects (Perry et al., 1978). Today, it is widely accepted that cortical cholinergic denervation in the AD brain represents one of the earliest and most severe transmitter changes while drugs that boosting cholinergic system (e.g., by AChE inhibition) are widely used for mild/moderate AD patients. Overall, the cholinergic hypothesis has been implicated in the AD etiology and it is based on the degeneration of cholinergic neurons of basal forebrain which can cause memory deficits. Interestingly, the cholinergic system is also involved to the response to stress and the regulation to stress-related hypothalamic-pituitary-adrenal (HPA) axis (Saswati et al., 2015) while loss of cholinergic input to the hippocampus is suggested to induce AD hippocampal vulnerability aggravating memory deficits caused by stress (Craig et al., 2011). Thus, the work described by Dr. Ioannis Sotiropoulos in the EBPS 2019 meeting focused on the recent evidence about the interplay of chronic stress and AD on novel neurodegenerative mechanisms in hippocampus with particular attention on Tau protein which seems to be the converging protein between chronic stress and AD brain pathologies. For instance, exposure to chronic stress or high levels of major stress hormones including glucocorticoids (GC) increases the levels of aberrantly hyperphosphorylated Tau together with neuronal atrophy, synaptic malfunction, reduced neurogenesis, and memory deficits (Sotiropoulos et al., 2011; Lopes et al., 2016; Dioli et al., 2017; Pedrazzoli et al., 2019). Importantly, the hyperphosphorylation occurred at certain Tau epitopes that are strongly implicated in cytoskeletal dysfunction and synaptic loss (e.g., pSer262) (Callahan et al., 2002) and hippocampal atrophy (e.g., pThr231) (Hampel et al., 2005) in AD patients. Related to synaptic malfunction and loss, chronic stress causes the missorting of hyperphosphorylated Tau to synapses which subsequently become dysfunctional (Lopes et al., 2016; Pinheiro et al., 2016). The missorting of Tau to synapses is now acknowledged as an early event in AD, preceding the manifestation of detectable neurodegenerative processes related to excitotoxic synaptic signaling and malfunction (Ittner et al., 2010). Intriguingly, Tau deletion prevents the aforementioned stress-induced signaling as well as neurostructural and behavioral deficits (Lopes et al., 2016), suggesting that Tau is the "final executor" of stress/GC induced neurotoxicity, similar to the reported role for Tau as a mediator of Aβ-driven neurotoxicity in AD (Ittner et al., 2010).

In vitro and *in vivo* studies suggest that stress and GC reduce the degradation of Tau in hippocampus, thereby increasing its accumulation (Sotiropoulos et al., 2008) *via* dysregulation of molecular chaperones (responsible for Tau proteostasis) (Sotiropoulos et al., 2015). More recent efforts have focused on the impact of chronic stress and high GC on two essential degradative mechanisms of Tau, the endolysosomal pathway (Vaz-Silva et al., 2018) and autophagy (Silva et al.,

2018). The endolysosomal pathway has been implicated in neurodegenerative diseases such as AD and PD in which Tau accumulation is a pathological feature (Kett and Dauer, 2016; Small et al., 2017). Current work by Sotiropoulos and colleagues has identified Tau as a substrate of the endolysosomal degradation pathway (Vaz-Silva et al., 2018) while it demonstrated that *in vitro* or *in vivo* exposure to high GC levels blocks this pathway, accompanied by the accumulation of Tau. Further, they showed that the involvement of the small GTPase, Rab35, and the endosomal sorting complexes required for transport (ESCRT) machinery that delivers Tau to lysosomes *via* early endosomes and multivesicular bodies (MVBs). Importantly, high GC suppress *Rab35* transcription resulting in Tau accumulation due to its impaired degradation while overexpression of Rab35 reverses GC-induced Tau accumulation and related neuronal atrophy in the hippocampus (Vaz-Silva et al., 2018). Based on the suggested signaling interplay between cholinergic and GC receptors, future studies should monitor whether cholinergic signaling participates in this GC action on the endolysosomal degradation pathway.

Though its ability to degrade long-lived and misfolded proteins such as Tau, autophagy and its interruption is causally related to the accumulation of Tau protein aggregates in the AD brain. Recent studies presented by Dr. Sotiropoulos at EBPS meeting demonstrated for the first time, that both, chronic stress and high GC levels inhibit the autophagic process *via* activation of mTOR signaling providing another mechanism through which these conditions contribute to the accumulation and aggregation of Tau and downstream neurodegeneration (Silva et al., 2018). These findings are in line with previous reports that chronic stress stimulates mTOR activity (Polman et al., 2012), an event associated with increased total Tau levels in the brains of AD subjects (Pei and Hugon, 2008). Furthermore, inhibition of mTOR signaling is shown to ameliorate Tau pathology (Jiang et al., 2014) while our studies show that inhibition of mTOR blocked the GC-driven Tau accumulation and aggregation (Silva et al., 2018). Interestingly, autophagy is related to the degradation of stress granules (SG) that are conserved cytoplasmic aggregates of ribonucleoprotein complexes (RNPs) implicated in the regulation of RNA translation, storage, and decay (Wolozin and Ivanov, 2019). While the formation of SGs is considered a protective mechanism against cellular stress (e.g., oxidative stress), prolonged SG induction can become pathological and neurotoxic. For instance, in AD neurodegeneration, SG promote the accumulation of Tau aggregates in a vicious cycle wherein Tau stimulates SG formation, with the RNA binding protein TIA1 playing a lead role in Tau misfolding and aggregation (Wolozin and Ivanov, 2019). Dr. Sotiropoulos showed that chronic stress and high GC increase the protein levels of various RBP and SG markers in soluble and insoluble fractions in both cellular and animal models of Tau pathology. Specifically, chronic stress increased cytoplasmic (soluble and insoluble) levels of several RBPs and SG-associated markers (e.g., TIA-1, PABP, G3BP, FUS, DDX5) that contributed to the formation of insoluble Tau inclusions and Tau accumulation (Small et al., 2017). As noted above, TIA-1 plays a prominent role in Tau aggregation (Vanderweyde et al., 2016; Apicco et al., 2018). Under stressful conditions, TIA-1 is trafficked from the nucleus to the cytospasm where it interacts directly with Tau (and other RBPs) to stimulate its aggregation and accumulation (Pei and Hugon, 2008). Tau missorting and accumulation in the dendritic compartment, such as is found in AD pathology, is also triggered by chronic stress/GC exposure (Lopes et al., 2016; Pinheiro et al., 2016). Thus, the above findings highlight the important role of chronic stress and GC signaling in the hippocampal neurodegeneration in AD brain adding to the suggested complexity between different factors/parameters that contribute to precipitates of AD brain pathology.

CONCLUDING REMARKS

This mini-review briefly describes recent developments in behavioral and cellular neuroscience as part of a symposium outcome and indicates that neurodegenerative diseases such as PD and AD have complex, multi-system changes in neuronal circuits that underlie the disease's characteristic neurobehavioral changes. Our understanding of the molecular, neurochemical, intraneuronal, and circuitry pathology underlying these diseases has advanced considerably with developments in analytical techniques and convergences in disciplines including model development, molecular biology, engineering, and pharmacology. Highlighted in this mini-review is the importance of continued refinements in behavioral pharmacology where understanding the functional consequences of disease manifestation will lead to more rapid developments in medical advancements. In particular, the presented optogenetic data from transgenic mice and rats expressing Cre-recombinase under the ChAt promoter indicate (1) the interplay between the CINs and dopaminergic system in the striatum in motor and non-motor behavior in murine 6-OHDA-PD models, as well as (2) new insights into cholinergic modulation of attention in the prefrontal cortex by directly comparing basal forebrain cholinergic inputs and CINs in this cognitive function. Novel intracranial drug delivery methods have revealed neuroprotective effects of GDNF and NGF on dopamine and achetylcholine degeneration.

The novel and powerful tools of regulation of cholinergic and dopaminergic innervation would offer novel and solid evidence about their individual contribution in neuronal pathology and behavioral impairment in different brain areas and circuits of the neurodegenerative brain in different stages of the disease.

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MA, TP, IS, DE, and GP wrote the manuscript. MA, TP, IS, JS, NS, SZ, DE, and GP were involved in the conceptualization of the studies. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: LW is the CEO of Gloriana Therapeutics, Inc., a for-profit biotechnology company that is developing the encapsulated cell technology to treat CNS diseases.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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