

Research paper

Ventral tegmental area muscarinic receptors modulate depression and anxiety-related behaviors in rats



Keri M. Small^a, Eric Nunes^a, Shannon Hughley^a, Nii A. Addy^{a,b,c,*}

^a Department of Psychiatry, Yale School of Medicine, New Haven, CT 06511, United States

^b Department of Cellular and Molecular Physiology, New Haven, CT 06511, United States

^c Interdepartmental Neuroscience Program, Yale University, New Haven, CT 06511, United States

HIGHLIGHTS

- We examined VTA acetylcholine effects in stress, anxiety and anhedonia behaviors.
- Enhancing VTA cholinergic tone produced pro-depressive and anxiogenic-like effects.
- VTA muscarinic AChR activation induced pro-depressive and anxiogenic-like behavior.

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ABSTRACT

Cholinergic and dopaminergic mechanisms within the mesolimbic dopamine system are suggested to play a role in the manifestation of depression and anxiety-related disorders. However, despite the fact that cholinergic mechanisms in the ventral tegmental area (VTA) highly regulate dopamine activity, the role of VTA cholinergic mechanisms in depression-related behaviors is relatively unknown. Here we sought to determine whether enhancing cholinergic tone in the VTA would alter depression and anxiety-related behavior in the forced swim test (FST), elevated plus maze (EPM) and sucrose preference test (SPT). Adult Sprague Dawley male rats received VTA infusion of the acetylcholinesterase inhibitor, physostigmine (0, 1, 2 $\mu\text{g}/\text{side}$), immediately prior to the FST, EPM, or SPT. Physostigmine administration increased immobility time in the FST, decreased time spent on open arms in the EPM, and decreased sucrose preference. We also examined whether activation of VTA muscarinic receptors was sufficient to alter behavior in the FST and EPM. Similar to physostigmine, VTA infusion of the muscarinic receptor agonist, pilocarpine (0, 3, 30 $\mu\text{g}/\text{side}$), increased immobility time in the FST and decreased time spent on open arms in the EPM. These data suggest that enhanced VTA cholinergic tone promotes pro-depressive and anxiogenic-like effects and demonstrate that specific activation of VTA muscarinic receptors is also sufficient to induce pro-depressive and anxiogenic responses. Together, these findings reveal a novel role of VTA cholinergic, and specifically muscarinic receptor, mechanisms in mediating responses to stress and anxiety.

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1. Introduction

Depression is a stress-related mood disorder that affects approximately 20% of adults in the United States [19,20]. Individuals diagnosed with major depressive disorder (MDD) experience various symptoms including anhedonia, fatigue, significant changes in appetite or weight, and thoughts or attempts of suicide [3].

In addition, anxiety is highly comorbid with depression, experienced by over 50% of individuals diagnosed with depression [19,27]. Dysfunction of the mesolimbic dopamine (DA) system is associated with both major depressive disorder (MDD) and generalized anxiety disorder (GAD) in humans [15,25,33]. Dopaminergic mechanisms also mediate depression and anxiety-related behavior in preclinical rodent models [16,25]. In addition to the dopaminergic system, the cholinergic system is also known to powerfully modulate depression symptomatology. Specifically, manipulations that increase brain cholinergic tone induce or exacerbate depressive-like symptoms while administration of cholinergic receptor antagonists induces antidepressant-like effects [1,9,17,18,23].

* Corresponding author at: 34 Park Street, 3rd Floor Research, New Haven, CT 06520, United States.

E-mail address: nii.addy@yale.edu (N.A. Addy).

Thus, while the etiology of depression is still unknown, current evidence strongly suggests that dopaminergic and cholinergic mechanisms likely play an important role in the pathogenesis of depression. Indeed, preclinical examinations of cholinergic mechanisms in depression have highlighted roles for cholinergic activity in several dopaminergic brain regions—including the prefrontal cortex (PFC) [24,36], the nucleus accumbens (NAc) [26,29], and the hippocampus [23]. However, the role of ventral tegmental area (VTA) cholinergic activity in depression and anxiety related behavior has not been extensively examined to date, despite the fact that DA cell bodies are localized in the VTA.

Within the mesolimbic system, recent studies in rodents have revealed a causal role for phasic DA activity in the VTA to NAc pathway in mediating both pro-depressive and antidepressant-like behavioral responses to stress [6,15,35]. These findings strongly suggest that mechanisms that regulate phasic DA activity could also play a critical role in mediating responses to stress. Indeed, findings from our laboratory and others have shown that VTA cholinergic mechanisms regulate both phasic DA cell firing and downstream phasic DA release in the NAc [14,22,38]. Furthermore, our recent findings revealed a novel role for cholinergic activity in the VTA in mediating both pro-depressive and antidepressant-like behavioral responses in the forced swim test (FST) [2]. However, it is unclear whether VTA cholinergic mechanisms also regulate other behavioral phenotypes associated with depression and anxiety. In the current study, our objective was to determine whether increasing cholinergic tone in the VTA of Sprague–Dawley rats would alter stress, anxiety, and anhedonia-related behaviors as measured in the FST, elevated plus maze (EPM), and sucrose preference test (SPT). Given our previous results showing the ability of muscarinic acetylcholine receptor (mAChR) blockade to reverse the pro-depressive effects of enhanced VTA cholinergic activity in the FST [2], we also examined whether direct VTA mAChR activation was sufficient to alter behavioral responses in the FST and EPM. Together, our findings provide novel insight into the role of VTA cholinergic, and specifically mAChR-mediated, mechanisms in response to stress and anxiety.

2. Methods

2.1. Animals

Adult male Sprague Dawley rats (250–280 g) were obtained from Charles Rivers Laboratories (Wilmington, MA, USA). Rats were group housed (2–3 per cage) and allowed to acclimatize to the housing facility for 1 week prior to surgical procedures. Animals were housed in a temperature-controlled room (22–24 °C) under 12-h light/dark cycle (lights on at 7:00 AM and lights off at 7:00 PM). Food and water were available ad libitum in home cages.

2.2. Surgery

Prior to surgery, rats were anesthetized with ketamine HCL (100 mg/kg, i.p., Sigma Aldrich, USA) and xylazine (10 mg/kg, i.p., Sigma Aldrich, USA). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for intra-cranial cannula implantation. Coordinates were obtained from the rat brain atlas [28] with anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) positions referenced from Bregma. A bilateral cannula apparatus with cannula spaced 1 mm apart (Plastics One, Roanoke, VA, USA) was placed 1 mm above the VTA (AP-5.2 mm, ML ±0.5 mm, DL –7.0 mm from dura) and secured to the skull using screws (Gexpro, High Point, NC) and dental cement (Dentsply, Milford, DE, USA). After surgery, rats were singly housed and allowed to recover for 5–7 days before behavioral testing. Animal proto-

cols were approved by Yale University Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.3. Drug Administration

In preparation for brain-region specific drug delivery, bilateral internal cannula containing the drug were inserted into the guide cannula and extended 1 mm beyond the guide cannula to target the VTA (–8.0 mm from dura). Drugs were delivered in a 0.5 µL total volume over 1 min via a micro-infusion pump (New Era Pump Systems, Farmingdale, NY) and syringe (25 gauge, Hamilton Syringe, Reno, NE, USA). After the 1 min drug infusion, the internal cannulae were left in place for an additional 1 min to allow for complete diffusion of drug into the brain tissue. Physostigmine (0, 1, or 2 µg, VWR, Bridgeport, NJ), an acetylcholinesterase inhibitor, and pilocarpine (0, 3, or 30 µg, Sigma Aldrich, St. Louis, MO), a selective mAChR agonist, were each dissolved in 0.9% saline and infused at behaviorally relevant doses that we and others have previously used in the VTA [2,21,42]. VTA infusion of vehicle or drug was performed immediately prior to the sucrose preference, elevated plus maze, forced swim or locomotor test session.

2.4. Forced swim test

The forced swim test (FST) was performed similar to protocols previously described by others [11,12]. During the pre-test, no pharmacological manipulation was given and behavior was not recorded. In the pre-test, a new cohort of rats naïve to any behavioral test rats were individually placed into a clear polypropylene, cylindrical water tank (diameter 30 cm; height 60 cm; water depth >40 cm; water temperature between 23 and 26 °C) for 15 min, to establish immobility for the subsequent test. The FST test session occurred during the second swim session, which took place 24 h after the pre-test. Rats were placed into the water tank immediately after intra-VTA drug infusion and immobility was scored from min 1 through min 6 (time: 1:00–6:00, 5 min total) of the 10 min test session. Each rat was randomly assigned to a specific drug administration group. FST was recorded by video camera and immobility was defined as an interruption of swimming behavior, when rats showed a lack of hind paw paddling. Thus, scoring of immobility time started when rats assumed a passive floating position, using only minimal movements required to keep their heads above water. For FST analysis, each test session was quantified by stopwatch by an experimenter blind to the treatment condition. At the end of the test session, rats were dried with a clean towel and monitored for 30 min in their home cage.

2.5. Sucrose preference test

The sucrose preference test was performed after a habituation period, where a new cohort of rats naïve to any behavioral test animals were given access to 1% sucrose solution (Sigma, St Louis, MO, USA) for 48 h in their home cage in place of the standard water bottle. Normal ad lib access to chow was maintained throughout habituation period. On test day, rats first underwent 4 h of water deprivation and were then given 1 h access to two identical bottles, one filled with 1% sucrose solution and the other with water. Sucrose and water consumption was determined by measuring the change in weight of bottles. The sucrose preference was defined as the ratio of the weight of sucrose vs. water consumed during the 1 h test. The relative consumption of 1% sucrose vs. water was calculated by weighing the bottles before and after 1 h test duration. Access to water bottles and chow was resumed immediately after the test.

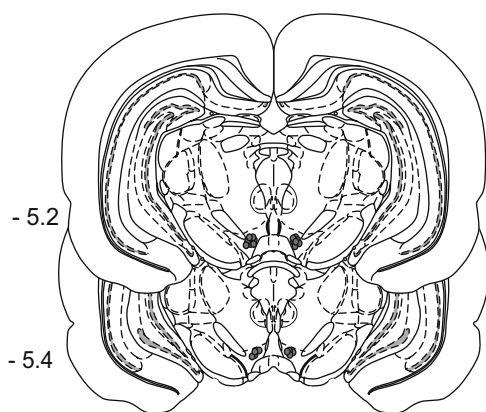


Fig. 1. Histological verification of cannula placements. Filled circles indicate representative placements for the physostigmine and pilocarpine experiments.

2.6. Elevated plus maze

The elevated plus maze test consists of placing rats that were naïve to any behavioral test in a plus-shaped maze with two open and two enclosed arms for a 5 min session. Each arm had an open roof and was elevated 40–70 cm from the floor. The time spent in the open arms and number of crosses into open arms was subsequently quantified using a stopwatch. Time spent in the open arm is defined as >50% of the rat's body has crossed the threshold of the defined open arm zone of the EPM. Animals were returned to their home cage at the end of the 5 min session.

2.7. Locomotor activity

Automated activity monitors (Digiscan animal activity monitor; Omnitech Electronics, Columbus OH) were used to assess locomotor activity. The monitors were equipped with two parallel rows of 16 sensors spaced 2.5 cm apart and were controlled by a computer using Micromax software (Omnitech Electronics). VTA drug infusions were performed immediately prior to the test—consistent with the FST, SPT, and EPM experimental design. Photobeam breaks were recorded, quantified and analyzed in 5 min bins.

2.8. Statistics

Time spent immobile in FST, sucrose preference in SPT, and time spent in open arms in the EPM were analyzed using a one-way analysis of variance (ANOVA), performed with GraphPad Prism 6 (Graph Pad Software, San Diego, CA). If the ANOVA revealed a significant main effect, a Tukey post hoc analysis was performed to compare between specific drug-administration groups. Locomotor activity was analyzed using a two-way repeated measures analysis of variance (ANOVA), with time as the repeated measure.

2.9. Histological verification

At the end of all behavioral experiments, rats were given a lethal dose of pentobarbital (150 mg/kg i.p.). Chicago blue dye was infused into the VTA (0.5 μ l volume), rats were sacrificed, and brains were removed and sliced into 1 mm sections using a brain matrix for histological verification of dye localization. Any subjects with misplaced cannula were excluded from the subsequent statistical analyses of the behavioral data (Fig. 1).

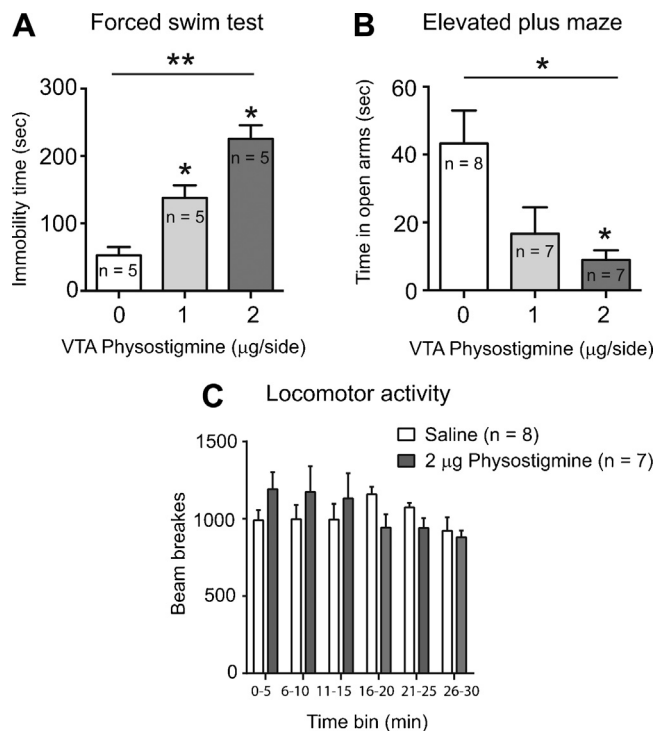


Fig. 2. Effect of intra-VTA physostigmine (0, 1, 2 μ g/side) on FST, EPM, and locomotor activity. Physostigmine increased immobility time in the FST (A), decreased time spent on open arms of EPM (B), and had no effect on locomotor activity (C). Data are expressed as mean \pm S.E.M.; (* p < 0.05, ** p < 0.01).

3. Results

In order to determine whether enhancing cholinergic tone in the VTA would alter stress or anxiety-related behaviors, we administered physostigmine (0, 1, 2 μ g/side) into the VTA of adult male Sprague Dawley rats and observed their behavioral responses in the FST and EPM (Fig. 2). Consistent with our previous findings, VTA physostigmine increased immobility in the FST, as revealed by a significant main effect of drug treatment ($F_{(2,12)} = 25.04$; $p < 0.01$, Fig. 2A). *Post hoc* analysis revealed that both doses of physostigmine significantly increased immobility time compared to vehicle ($p < 0.05$). In a separate cohort, VTA physostigmine infusion also altered behavioral responding in the EPM, as revealed by a significant main effect of drug treatment ($F_{(2,19)} = 5.744$; $p < 0.05$, Fig. 2B). *Post hoc* analysis revealed that the 2 μ g/side dose significantly reduced time spent in the open arms of the EPM compared to saline treated rats ($p < 0.05$). We also observed that the number of entries in the open arm positively correlated with time spent on open arm for each treatment group (data not shown). Moreover, no significant differences in locomotor activity ($F_{5,65} = 1.210$; $p = 0.3145$, Fig. 2C) were observed after physostigmine administration; suggesting that the effects of physostigmine were not due to non-specific locomotor effects. In addition, we have previously shown that the pro-depressive effect of physostigmine is site-specific, as administering physostigmine to a site 2 mm dorsal to VTA led to no significant differences in FST immobility time compared to vehicle treated animals [2]. Next, to determine whether an increase in VTA cholinergic tone mediates anhedonia-related behavior, physostigmine (0, 1, 2 μ g/side) was administered into the VTA and the sucrose preference was determined using the SPT. One way ANOVA revealed a significant main effect of drug treatment ($F_{(2,43)} = 3.228$; $p < 0.05$, Fig. 3) in the SPT. *Post hoc* analysis also revealed that the 2 μ g/side dose of physostigmine

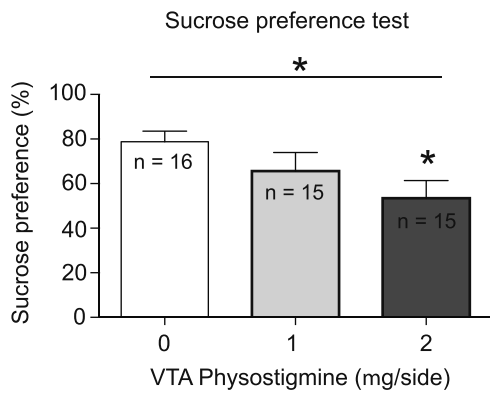


Fig. 3. Effect of intra-VTA physostigmine (0, 1, 2 μ g/side) on SPT. Physostigmine decreased sucrose preference. Data expressed as mean \pm S.E.M.; (* p < 0.05).

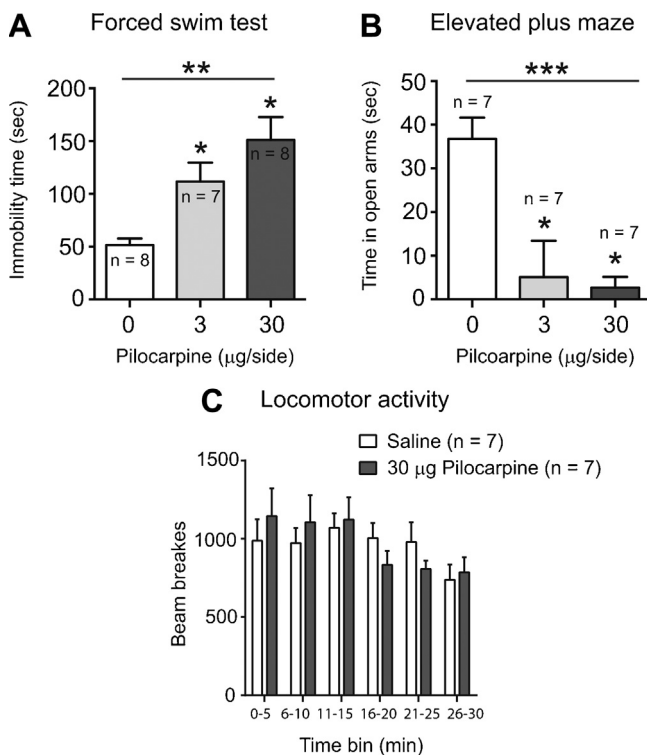


Fig. 4. Effect of intra-VTA pilocarpine (0, 3, 30 μ g/side) on FST, EPM, and locomotor activity. Pilocarpine increased immobility time in the FST (A), decreased time spent on the open arms of EPM (B), and had no effect on locomotor activity (C). Data expressed as mean \pm S.E.M.; (* p < 0.05, ** p < 0.01, *** p < 0.001).

significantly decreased sucrose preference compared to saline treated rats (p < 0.05).

We have previously shown that blockade of VTA mAChRs reverses the pro-depressive effects of VTA physostigmine in the FST [2]. Here, we wanted to determine if selective activation of mAChRs in the VTA was sufficient to alter behavioral responses in the FST and EPM – the two behavioral measures that were most sensitive to VTA physostigmine. Thus, pilocarpine (0, 3, and 30 μ g/side), a selective muscarinic agonist, was administered into the VTA and behavioral responses in the FST and EPM were observed (Fig. 4). One-way ANOVA showed a significant main effect of drug in the FST ($F_{(2,20)} = 9.654$; p < 0.01, Fig. 4A). *Post hoc* comparison revealed that pilocarpine, at both the 3 μ g/side and 30 μ g/side doses, increased immobility time compared to the saline group (p < 0.05, Fig. 4A). Pilocarpine administered into the VTA also altered behavioral responding in the EPM, as shown by a significant main effect of

drug ($F_{(2,18)} = 31.41$, p < 0.001, Fig. 4B). *Post hoc* analysis revealed that both doses of pilocarpine significantly reduced time spent on open arms of the EPM compared to saline (p < 0.05, Fig. 4B). We also observed that the number of entries in the open arm for each treatment group positively correlated with time spent on open arm of corresponding drug treatment (data is not shown). Further, the effect of pilocarpine on FST and EPM was not due to non-specific locomotor effects as VTA drug infusion of pilocarpine (30 μ g/side) led to no significant differences in locomotor activity ($F_{(5,60)} = 2.06$; $p = 0.0828$, Fig. 4C). Taken together, these results demonstrate that selective mAChR activation in the VTA is sufficient to promote pro-depressive and anxiogenic-like responses in the FST and EPM, similar to the effects of VTA physostigmine.

4. Discussion

To our knowledge, the present study is the first to examine the role of VTA cholinergic, and specifically mAChR, mechanisms in stress and anxiety-related preclinical behavioral models. Here we found that enhancing VTA cholinergic tone, with physostigmine, promoted pro-depressive-like, anxiogenic, and anhedonic-like behavioral responses in the FST, EPM, and SPT, respectively. Moreover, selective activation of VTA mAChRs was sufficient to increase immobility time in the FST and decrease open arm time in the EPM, which is similarly suggestive of pro-depressive and anxiogenic responses. Together, these results reveal a novel role for VTA mAChR activity in mediating responses to stress and anxiety.

Cholinergic mechanisms in the mesolimbic dopamine system are known to modulate depression and anxiety-related behaviors [5,24,29,37]. In addition, cholinergic mechanisms regulate phasic dopamine activity in the VTA to NAc pathway [13,32,38] that is known to causally mediate susceptibility to stress and anxiety [6,35]. However, depression and anxiety-related cholinergic investigations in the VTA to NAc pathway have focused primarily on the NAc [5,29,37] and have not included the VTA. While the effect of acetylcholinesterase inhibition in the NAc has not been directly examined in stress and anxiety-related behaviors, evidence from several studies suggests that cholinergic activity in the NAc promotes a pro-depressive phenotype, similar to what we observed with enhanced VTA cholinergic activity. Specifically, pharmacological blockade of M2 mAChR autoreceptors in the NAc increases ACh levels in the NAc and induces a pro-depressive-like response in the FST [5]. In addition, direct NAc infusion of corticotropin releasing factor (CRF), which robustly increases NAc ACh levels, also increases immobility time in the FST, decreases open arm time in the EPM, decrease center time in the open field test, and decreases sucrose preference [7]. Thus, enhanced ACh in the NAc is strongly associated with pro-depressive and anxiogenic responses across several behavioral paradigms. In contrast, decreased NAc ACh is associated with fluoxetine-induced antidepressant effects in the FST [5]. This association between decreased NAc ACh activity and antidepressant-like behavioral effects is consistent with our previous VTA investigations, where we found that pharmacological blockade of VTA AChRs induces an antidepressant-like response in the FST [2]. Thus, the current evidence suggests the enhancing cholinergic activity in either the VTA or NAc is sufficient to promote pro-depressive-like responses while decreasing cholinergic activity, in contrast, produces antidepressant-like effects.

As indicated earlier, cholinergic mechanisms in both the VTA and NAc modulate dopaminergic activity [13,32,38] that causally mediates susceptibility to stress and anxiety [6,35]. While we have demonstrated that increasing VTA cholinergic tone induces depressive-like and anxiogenic-like behavior, previous work has shown that VTA AChR activation is sufficient to promote DA burst firing patterns [41] that are known to facilitate DA release in the

NAc [34]. Similarly in the NAc, the ability of CRF to increase NAc ACh levels and to promote pro-depressive behavioral effects is accompanied by a significant increase in NAc DA levels [7]. Specifically, Chen et al. reported that direct NAc infusion of corticotropin releasing factor (CRF) robustly increased NAc ACh levels and increased immobility time in the FST, decreased open arm time in the EPM, decreased center time in the open field test, decreased sucrose preference, and showed significant increase in NAc DA levels [7]. Our findings compliment these previous studies and implicate the potential role of the VTA mAChRs in modulating DA activity to induce pro-depressive behaviors.

In contrast to enhanced cholinergic activity, VTA AChR blockade which is sufficient to decrease phasic DA release in the NAc [32,38] also induces antidepressant-like responses in the FST [2]. Together, these data show that VTA and NAc cholinergic mechanisms that enhance NAc DA are also sufficient to induce pro-depressive-like and anxiogenic-like behavioral phenotypes. A more recent study revealed NAc cholinergic effects that are in apparent contrast to previous NAc investigations. Specifically, Warner-Schmidt and colleagues found that optogenetic inhibition of NAc cholinergic neurons produced pro-depressive-like effects in the FST, SPT and tail suspension test [38]. However, the apparent ability of both increased NAc ACh levels and decreased NAc cholinergic activity to facilitate pro-depressive effects may be due to the complex mechanisms by which NAc ACh regulates dopaminergic activity. Tonic ACh activity in the NAc is known to inhibit DA release due to short term, synaptic depression and subsequent AChR inhibition removes this depression and allows for subsequent, enhanced DA release [8,13,41]. Thus, one potential mechanistic explanation for the pro-depressive effects in the Warner-Schmidt study is that optogenetic inhibition of NAc cholinergic neurons removed the short term DA-depression to facilitate an increase in DA levels, similar to the effects of enhancing ACh tone in the VTA or NAc, thus promoting a pro-depressive behavioral phenotype. However, this hypothesis has not been directly tested to date. Thus, additional work is needed to further elucidate the mechanisms through which cholinergic activity in the VTA to NAc pathway system mediates responses to stress and anxiety.

Concerning subtype specific VTA AChR mechanisms, we previously found that VTA mAChR blockade reversed the pro-depressive effects of VTA physostigmine in the FST [2]. Several studies had previously demonstrated a role of VTA mAChRs in multiple behaviors, including feeding behavior [30,31], conditioned reinforcement [39] and cue-induced drug-seeking [32,42]. However, our previous FST investigation was the first to suggest a role for VTA mAChR mechanisms in stress-related behavior. Here, our findings demonstrate that specific activation of VTA mAChRs is sufficient to promote pro-depressive and anxiogenic-like responses in the FST and EPM. These new mAChR findings are consistent with what one would predict based on previous examinations of VTA mechanisms in stress and anxiety. Specifically, recent work has shown that VTA hyperactivity is associated with general anxiety disorder (GAD) in humans [4]—consistent with our mechanistic hypothesis for the ability of mAChR activation to promote anxiogenic responses through enhanced VTA DA activity. Our VTA findings are also consistent with what has been observed with the downstream mAChR target, extracellular regulated signaling kinase (ERK). Specifically, Iniguez and colleagues found that overexpression of VTA ERK, which is activated downstream of VTA mAChRs, produces an anxiogenic-like responses in the EPM and a pro-depressive-like effect in the FST [16]—similar to our observed effect of VTA mAChR activation. These findings, together with our results, suggest that future VTA mAChR investigations are likely to reveal novel mechanisms that robustly mediate responses to stress and anxiety.

In closing, our findings provide novel understanding of the ability of VTA mAChR mechanisms to robustly modulate behav-

ioral responses to stress and anxiety. These results have important clinical implications, especially in light of the evidence demonstrating rapid antidepressant effects following systemic administration of the mAChR antagonist, scopolamine [10,24,40]. Based on our findings, we suggest that additional VTA mAChR examination may provide important, new understanding of the neurobiological mechanisms that underlie stress and anxiety-related disorders.

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