OXFORD

International Journal of Neuropsychopharmacology, 2023, **26**, 80–90

https://doi.org/10.1093/ijnp/pyac076
Advance access publication 19 November 2022
Regular Research Article

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Muscarinic and NMDA Receptors in the Substantia Nigra Play a Role in Reward-Related Learning

Ewa Galaj, Eddy D. Barrera, Olivia L. Lynch, Rachel Diodati, Ashley Thomas, Piper Schneider, Hayley Lenhard, Apoorva Vashisht, Robert Ranaldi

Department of Psychological and Brain Sciences, Colgate University, Hamilton, New York, USA (Dr Galaj, Ms Lynch, Ms Diodati, Ms Thomas, Ms Schneider, and Ms Lenhard); The Graduate Center of the City University of New York, New York, New York, USA (Mr Barrera, Ms Vashisht, and Dr Ranaldi); Department of Psychology, Queens College of the City University of New York, Flushing, New York, USA (Dr Ranaldi).

Correspondence: Ewa Galaj, PhD, Department of Psychological and Brain Sciences, 13 Oak Drive, Hamilton, NY 13346, USA (egalaj@colgate.edu).

Abstract

Background: Reward-related learning, where animals form associations between rewards and stimuli (i.e., conditioned stimuli [CS]) that predict or accompany those rewards, is an essential adaptive function for survival.

Methods: In this study, we investigated the mechanisms underlying the acquisition and performance of conditioned approach learning with a focus on the role of muscarinic acetylcholine (mACh) and NMDA glutamate receptors in the substantia nigra (SN), a brain region implicated in reward and motor processes.

Results: Using RNAscope in situ hybridization assays, we found that dopamine neurons of the SN express muscarinic (mACh5), NMDA2a, NMDA2b, and NMDA2d receptor mRNA but not mACh4. NMDA, but not mACh5, receptor mRNA was also found on SN GABA neurons. In a conditioned approach paradigm, rats were exposed to 3 or 7 conditioning sessions during which light/tone (CS) presentations were paired with delivery of food pellets, followed by a test session with CS-only presentations. Intra-SN microinjections of scopolamine (a mACh receptor antagonist) or AP-5 (a NMDA receptor antagonist) were made either prior to each conditioning session (to test their effects on acquisition) or prior to the CS-only test (to test their effects on expression of the learned response). Scopolamine and AP-5 produced dose-dependent significant reductions in the acquisition, but not performance, of conditioned approach.

Conclusions: These results suggest that SN mACh and NMDA receptors are key players in the acquisition, but not the expression, of reward-related learning. Importantly, these findings redefine the role of the SN, which has traditionally been known for its involvement in motor processes, and suggest that the SN possesses attributes consistent with a function as a hub of integration of primary reward and CS signals.

Keywords: Conditioned reinforcement, substantia nigra, NMDA, mACh receptor, reward-related learning

Significance

This is the first demonstration, to our knowledge, that mACh and NMDA receptors in the SN are necessary for the acquisition of conditioned approach responses in rats.

INTRODUCTION

Motivated behaviors such as food or drug seeking involve reward-related learning during which animals learn associations between rewards and stimuli (i.e., conditioned stimuli [CS]) that predict or accompany those rewards. Those CSs provide information about where, when, and how to obtain rewards and are able to control adaptive or maladaptive behaviors. Not only are animals motivated by the reinforcing and energizing effects of primary rewards (e.g., food, sex, or drugs of abuse), but they are similarly motivated by the conditioned reinforcing effects and conditioned incentive motivational or energizing effects of stimuli associated with primary rewards (e.g., money) (Galaj and Ranaldi, 2021). Thus, to fully understand motivation it is imperative to understand how environmental stimuli acquire the ability to function as CSs that control reward-driven behaviors, including conditioned responses.

Midbrain dopamine (DA) neurons have been implicated in reward-related learning and initiation of approach behavior (Zellner and Ranaldi, 2010; Ranaldi, 2014; Galaj and Ranaldi, 2021). A series of studies has shown that CSs can increase calcium transients (an indicator of neural activation) of DA cells in the ventral tegmental area (VTA) (Saunders et al., 2018) and increase

Received for publication: August 1, 2022. **Accepted:** November 15, 2022. **Editorial decision:** November 14, 2022.
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DA cell firing (Kiyatkin and Stein, 1996; Schultz, 1997) and DA release in the nucleus accumbens (NAc) (Gratton and Wise, 1994; Ito et al., 2002; Stuber et al., 2008). Studies demonstrate that DA antagonists reduce conditioned responses elicited by drug- or food-associated CSs and reduce responses maintained by drug- or food-associated conditioned reinforcers (Di Ciano et al., 2001; Yun et al., 2004; Galaj et al., 2014). Our group has shown that the acquisition of conditioned approach learning requires cholinergic and glutamatergic inputs to the VTA and that the strength of reward-related learning depends on the degree of VTA DA cell activation (Sharf and Ranaldi, 2006; Sharf et al., 2006; Zellner et al., 2009; Ranaldi et al., 2011; Kest et al., 2012; Galaj et al., 2017).

Adjacent to the VTA is the substantia nigra, a home to DA and GABA neurons. The nigrostriatal DA pathway has received attention for its involvement in drug and food reward (Wise, 1981; Beninger and Ranaldi, 1993; Beninger et al., 1993; Quinlan et al., 2004) and associative learning (Han et al., 1997; Lima et al., 2017). Transgenic DAT-cre or Th-cre mice can learn to press a lever for optical intracranial self-stimulation that involves activation of SNc DA neurons (Ilango et al., 2014; Galaj et al., 2020), and cocaine cues can activate the nigrostriatal DA neurons (Ito et al., 2002).

Although neurons of the SN are known to receive cholinergic (Hong and Hikosaka, 2014) and glutamatergic inputs (Windels et al., 2000) and express mACh (Kayadjanian et al., 1994; Steidl et al., 2011) and NMDA receptors (Suárez et al., 2010), the subtypes of these receptors and their cell type–specific distributions are not clear. Importantly, although mACh (Sharf and Ranaldi, 2006; Sharf et al., 2006; Galaj et al., 2017) and NMDA receptors (Zellner et al., 2009; Ranaldi et al., 2011; Hachimine et al., 2016) of the VTA play an important role in reward-related learning, the role of these receptors in the SN in this type of learning has not been investigated.

Thus, the aim of this study was to (1) identify and characterize the cell-type specific distributions of mACh and NMDA receptors in the SN, using the RNAscope in situ hybridization (ISH) assay, and (2) investigate the role of SN mACh and NMDA receptor stimulation in the acquisition and expression of conditioned approach learning using an intracranial neuropharmacological approach. Although there are 5 subtypes of muscarinic receptors (Hulme et al., 1990) and a wide variety of NMDA receptor subtypes (Glasgow et al., 2015), we focused on muscarinic ACh4, mACh5 receptors, and glutamate NMDA2a, NMDA2b, and NMDA2d receptors because previous studies reported relatively moderate to high expression of these receptors in the midbrain (Levey et al., 1991; Suárez et al., 2010).

METHODS

This study was carried out in accordance with the guidelines established by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 2011) and was approved by the Colgate University Institutional Animal Care and Use Committee.

Animals

The subjects consisted of male and female Sprague Dawley rats (n = 124) taken from our in-house colony breeders purchased from Envigo (Altamont, NY, USA). The free-feeding weight of females was 250–350 g and of age-matched males was 350–450 g at the time of surgery. Post-surgery rats were housed individually on a reversed 12-hour-light/-dark cycle (lights on at 7 PM) in temperature- and humidity-controlled rooms. All rats had free access to food (LabDiet chow) and water at all times until a week prior to the start of experimental sessions, at which time access to food was restricted to daily rations that maintained their weight at 85% of their free-feeding values. All experimental procedures were conducted during the animals' active period (dark cycle). Nineteen rats were excluded from the final data analysis due to cannula misplacements or cannula clogging.

Drugs

AP-5 (a NMDA receptor antagonist) and scopolamine hydrobromide (a mACh receptor antagonist) were purchased from Tocris Bio-Techne (Minneapolis, MN, USA) and dissolved in saline. We selected doses of these compounds based on our previous studies (Ranaldi et al., 2011; Hachimine et al., 2016; Galaj et al., 2017). Scopolamine was administered intracranially into the SNr at doses of 0, 2.5, and 5 µg/0.5 µL/side and AP-5 at doses of 0, 1, 2, and 4 µg/0.5 µL/side. RNAscope probes and fluorescent multiplex kits were purchased from Advanced Cell Diagnostics (Newark, CA, USA).

Surgery

Prior to surgery, each rat was injected with atropine (0.05 mL 0.4 mg/mL, i.p.) to prevent mucus build-up and anesthetized with sodium pentobarbital (65 mg/kg, i.p.). The scalp of the rat was shaved and cleaned with Betadine, and an ophthalmic ointment (Paralube Vet ointment) was applied to the eyes to prevent corneal drying. During and post-surgery the rats were placed on heating pads to prevent hypothermia. The rats's heads were fixed in the stereotaxic apparatus, and a small incision was made on the midline of the scalp to expose the skull. Two holes were drilled on the skull above the SNr, and stainless-steel guide cannulae (23 gauge/16 mm long) were implanted bilaterally into the SNr using the following coordinates: AP –5.52, ML ±3.06, and DV –8.15 at a 10° angle away from the midline. The cannulae were permanently fixed to the skull using Gorilla superglue and dental acrylic anchored to 4 stainless-steel screws screwed into the skull while the rat was still in the stereotaxic apparatus. Obturators, extending 1 mm beyond the cannulae, were inserted into the cannulae to prevent blockage and remained there except during microinjections. After surgery, the rats were administered buprenorphine (0.5 mg/kg; SC) and were placed on a heating pad. They were closely monitored during the post-recovery and experimentation periods.

Conditioning Chambers

Conditioned approach training and testing took place in 8 conditioning chambers, each measuring 11 × 9 × 12" (H × W × L) and placed in a ventilated, sound-attenuating cubicle with an operating fan to mask outside noise. The conditioning chambers were equipped with a food trough into which food pellets (45 mg each; Bio-Serv, Inc., Flemington, NJ, USA) could be dropped from a food dispenser and a light and tone generator mounted above and to the left of the food trough. Photo-emitter/detector devices mounted in the food trough detected head entries (nose pokes) in the food trough.

Exp.1: Identifying Expression of mACh and NMDA Glutamate Receptors in the SN

To identify and characterize cell-type specific expression and type of mACh and NMDA receptors in the SN, we used RNAscope ISH to image *NMDA2a*, *NMDA2b*, *NMDA2d*, *mACH4*, *mACH5*, *DAT*, and *GAD67* mRNA in the SN pars compacta (SNc) and pars reticulata

(SNr). Two male rat brains were extracted by rapid decapitation and immediately submerged in 2-methyl-butane to be stored in –80°C until ready for use. Coronal sections of rat midbrain were collected at 16-µm thickness on Superfrost Plus slides and then dehydrated in graduated ethanol (PBS, 50%, 70%, and 100% ethanol). Using RNAscope Reagent Kit (Advanced Cell Diagnostics, Newark, CA, USA), the midbrain sections were incubated first in Protease-Pretreat 4 solution (room temperature, 20 minutes), rinsed twice in dH_20, and then treated with *Rn-Grin2a* (Cat No. 414621), *Rn-Grin2b* (Cat No. 420641), *Rn-Grin2d* (Cat No. 502941), *Rn-Chrm5-C2* (Cat No. 479161-C2), *Rn-Chrm4* (Cat No. 456671-C2), *Rn-Slc6a3* (Cat No. 319621-C3), or *GAD1-C3* (Cat No. 316401-C3) probes (Advanced Cell Diagnostics) for 2 hours at 40°C. After double-rinsing in wash buffer, the sections were treated with AMP 1 (30 minutes, 40°C), AMP 2 (15 minutes, 40°C), AMP 3 (30 minutes, 40°C), and AMP 4 Alt A (15 minutes, 40°C) and double-rinsed in phosphate buffer (PB) between each amplification step. Next, a drop of 4',6-diamidino-2-phenylindole (DAPI) was added to each slide, followed by fluorescent mounting medium (Fluoro-Gel, Electron Microscopy Science) and coverslip.

Images were taken on confocal microscope from 3 sections at 40× magnification from the SNc and SNr. The number of DAPI positive cells expressing NMDA and mACh mRNA were counted at 40× magnification using Image J software. We then followed up with a behavioral paradigm involving intra-SNr microinjections to assess the role of mACh or NMDA receptors, which are presumably on the dendrites of DA neurons. The SNr is known for an abundance of DA (and GABA) dendrites (Zhou and Lee, 2011).

Conditioned Approach Paradigm

The conditioned approach study consisted of both acquisition and expression (i.e., performance) parts. The acquisition part included 1 magazine training session (20 minutes), 3 conditioning sessions (1 hour each) with microinjections, 1 no US/no CS session (30 minutes), and 1 CS only test (1 hour). The expression part consisted of 1 magazine training, 7 conditioning sessions, 1 no US/no CS, and 1 CS only test with microinjections. Two days prior to the start of experimentation, rats were introduced to 30 food pellets (Bio-Serv, Flemington, NJ) in their home cages.

Exp. 2: Assessing the Role of mACh and NMDA Receptors of SN in Acquisition of Conditioned Approach

On Day 1, a magazine training session occurred to make the animals accustomed to the food dispenser sound and delivery of food pellets into the food trough. Twenty food pellets were released on average 1 every 24 seconds with no light/tone stimuli. During conditioning sessions, rats (group Ns = 4–6 males and 4–5 females) were exposed to thirty 3-second light/tone (conditioned stimulus [CS]) presentations delivered on a random time schedule with an average inter-stimulus interval of 150 seconds. Each light/tone presentation was immediately followed by a food pellet (unconditioned stimulus [US]) delivered into the food trough. Conditioning sessions occurred every other day. During the acquisition experiment, immediately prior to each conditioning session, obturators were removed and microinjectors, extending 1 mm beyond the guide cannulae, were inserted into the cannulae. Bilateral microinjections of scopolamine, a mACh receptor antagonist [0 (n = 9), 2.5 (n = 8) or 5 (n = 11) µg/0.5 µL/side; Exp. 2A], or AP-5, a NMDA receptor antagonist [0 (n = 9), 1 (n = 9), 2 (n = 9) or 4 (n = 8) µg/0.5 µL/side; Exp. 2B], was delivered over 60 seconds using a 10-µL Hamilton syringe and the Basi Bee Hive pump. Microinjectors were kept in place for an additional 2 minutes to allow the drug to diffuse. Next, the obturators were inserted back into the guide cannulae, the rats were placed into the conditioning chambers, and the session was started. Two days after the last conditioning session, all rats received one 30-minute session with no treatment, during which no US or CS presentations occurred. This was followed by a CS-only test session during which rats received 30 CS presentations only (light/tone only; no food) under the same schedule as during conditioning sessions. Nose pokes were detected by the photoreceptors and counted during nonCS, preCS, and CS periods.

Exp. 3: Assessing the Role of mACh and NMDA Receptors of SN in Expression of Conditioned Approach

Different sets of rats were used in the expression experiments. This experimental procedure was similar to the acquisition procedure described above, except that there were 7 conditioning sessions during which no microinjections were made. In this procedure, we used 7 sessions rather than 3 because under these conditions, it requires 7 sessions to achieve statistically stable asymptotic responding. Thus, at this point it is safe to assume that the conditioned approach behavior is well acquired, which allows us now to test the role of SNr mACh and NMDA receptors in the performance (i.e., expression) of this learned behavior. Intra-SNr microinjections of scopolamine [0 (n = 9), 2.5 (n = 9) or 5 (n = 8) µg/0.5µL/side; Exp. 3A] or AP-5 [0 (n = 9), 2 (n = 9), 4 (n = 8) µg/0.5 µL/side; Exp. 3B] were made prior to the CS-only test session, and rats' conditioned approach responses were measured.

Histology

After the CS-only test session, all rats were deeply anesthetized with sodium pentobarbital and perfused with 200 mL of saline followed by 100 mL of 4% paraformaldehyde and decapitated. The brains were removed and stored in 4% paraformaldehyde overnight, followed by transfer to 30% sucrose solution before being sliced in coronal sections and inspected for cannulae implantation and injection sites. Only rats with bilateral cannula placements in the SNr were included in data analyses.

Data Analysis

In the conditioned approach experiments, each conditioning session and the CS-only test session were divided into 3 types of periods: nonCS, preCS (6 seconds prior to onset of CS), and CS (6 seconds beginning with the onset of the CS) periods. The total numbers of nose pokes during the preCS and CS periods were counted for each session and used to calculate difference scores (CS-preCS nose pokes) for each session. During the conditioning sessions, responses made during the 6-second CS period included nose pokes that might be in response to the food as well as in response to the CS. During the CS-only test, nose pokes made during the 6-second CS period were responses made to the CS (conditioned responses/conditioned approach) because food was not present at that time. This difference score indicates the magnitude of the conditioned approach learning. These data were analyzed using a 2-way ANOVA with dose of scopolamine or AP-5 as between-groups factors and session as a repeated-measures factor. To address potential scopolamine or AP-5–induced motoric deficits, we also calculated the total number of non-CS nose pokes during conditioning sessions [all pokes – (preCS + CS nose pokes)] and compared groups using separate 2-way ANOVAs (dose × session). The CS-preCS difference scores for the CS-only

test session were analyzed using a 1-way ANOVA with scopolamine or AP-5 dose as a between-groups factor, followed by Tukey tests. Initial data were analyzed with sex as a biological factor, but no sex differences emerged. Thus, we combined data from males and females in our final analyses.

RESULTS

NMDA2a, NMDA2b, and NMDA2d mRNA Coexpressed With mACh5 mRNA on DA Neurons of SN

Although neurons of the substantia nigra are known to express NMDA and mACh receptors, direct cell-type–specific evidence of their subtypes is still lacking. To address this gap in knowledge, we examined the distribution of NMDA and mACh receptor mRNA in the SNr and SNc using RNAscope ISH. Figure 1A–D show that DAT-positive (i.e., DA) neurons of the SNc express high densities of NMDA2a, NMDA2b, and NMDA2d mRNA. We also observed colocalization of all NMDA receptor subtypes with mACh5 mRNA on DAT-positive neurons, suggesting that SNc DA neurons coexpress mACh5 and NMDA receptors. Similarly, we found that GAD67-positive (i.e., GABA) neurons of the SNr express high densities of NMDA2a, NMDA2b, and NMDA2d mRNA, but, to our surprise, we found no evidence of mACh5 mRNA on SNr GABA neurons (Figure 2A–D). Indeed, we expected to observe mAChR5 mRNA in GABA neurons because these neurons have been shown to receive cholinergic inputs (Breit et al., 2006; Hong and Hikosaka, 2014) and express nicotinic ACh receptors (Poisik et al., 2008).

Because there are reports that cholinergic inputs to the SN act through mACh4 receptors (Moehle et al., 2017), we also examined cell-type specific distribution of mACh4 mRNA in the SN and aimed to determine whether these mACh receptors co-localize with NMDA receptors. We confirmed our findings that SNc DA neurons (Figure 3A–D) and SNr GABA neurons (Figure 4A–D) express mRNA for NMDA2a, NMDA2b, and NMDA2d receptors but found no detectable expression of mACh4 mRNA in the SNc (Figure 3A–D) or SNr (Figure 4A–D). As a positive control, we performed RNAcope ISH on striatal brain sections and detected mACh4 mRNA on NAc neurons that also express NMDA2b mRNA (Figure 5), confirming that our assay can detect these receptors and suggesting that mACh4 receptors are expressed in the NAc but not SN.

NMDA and Muscarinic Receptors of SN Play Critical Roles in Acquisition of Conditioned Approach

To determine whether stimulation of mACh or NMDA receptors in the SN plays important roles in reward-related learning, we assessed the effects of intra-SNr scopolamine or AP-5 on the acquisition of conditioned approach. We aimed for the SNr because the SNr has an abundance of DA and GABA neuronal dendrites with receptor sites (Zhou and Lee, 2011).

Figure 6A shows a schematic diagram of the experimental procedure. Food-restricted rats were subjected to magazine training, followed by 3 conditioning sessions with intra-SNr microinjections of scopolamine, 1 no CS/no US session, and 1 CS-only test. As shown in Figure 6B, intra-SNr microinjections of scopolamine caused dose-dependent reductions in difference scores during conditioning sessions and during the CS-only test. Because we had found no sex differences in CS-PreCS nose pokes, we combined the data from males and females. A 2-way ANOVA on the CS-preCS difference scores during conditioning revealed a significant dose × session interaction ($F_{4,50} = 2.61$; $P = .046$). Tests of simple effect of scopolamine for each conditioning session revealed a significant dose effect on Day 3 ($F_{2,25} = 4.44$; $P = .022$) but not Days

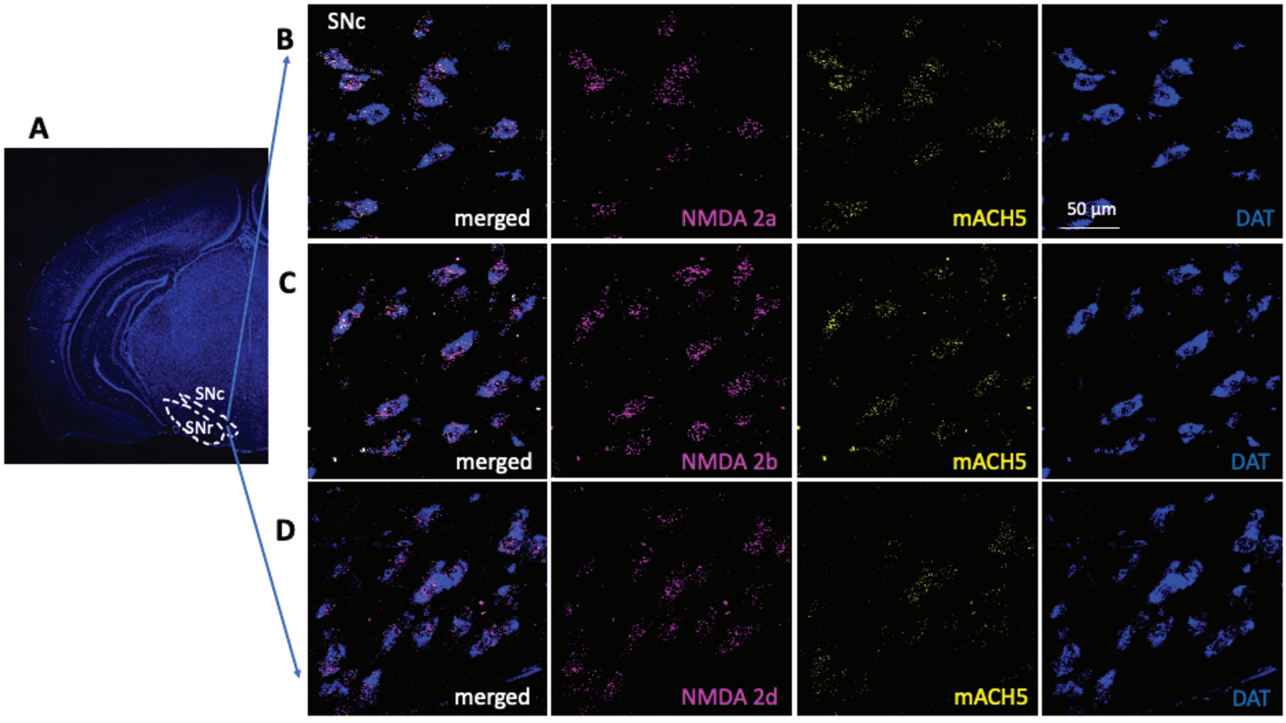

Figure 1. A Representative image of the rat midbrain under 4× magnification illustrating the substantia nigra pars compacta (SNc) and location where images were taken from. (B–D) Representative images under 40× magnification illustrating that Dopamine transporter, DAT(Slc6a3)-positive neurons in the SNc coexpress: (B) NMDA2a (*Grin2a*) and mACh5 (*Chrm5*) mRNA, (C) NMDA2b (*Grin2b*) and mACh5 (*Chrm5*), and (D) NMDA2d (*Grin2d*) mRNA.

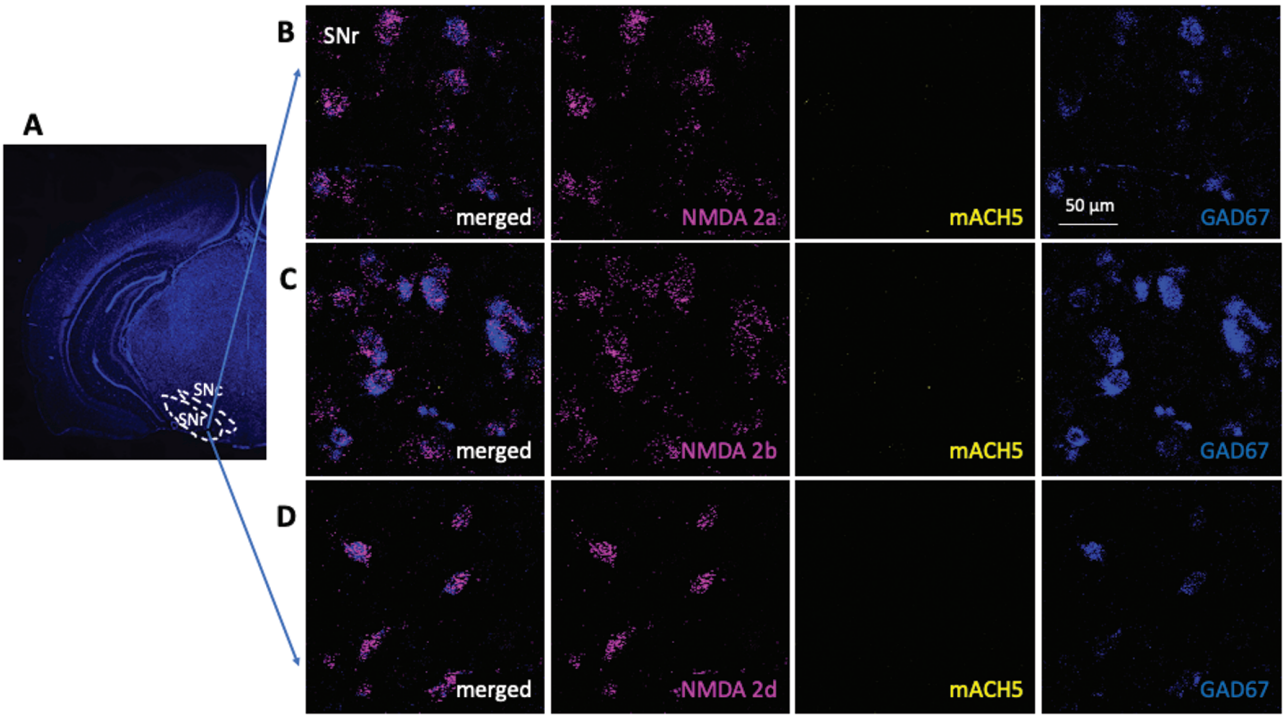

Figure 2. (A) Representative image of the rat midbrain under 4× magnification illustrating the substantia nigra pars reticulata (SNr) and location where images were taken from. (B–D) Representative images under 40× magnification illustrating that glutamic acid decarboxylase 67, GAD67 (GAD1)-positive neurons in the SNr express: (B) NMDA2a (*Grin2a*), (C) NMDA2b (*Grin2b*), (D) NMDA2d (*Grin2d*) mRNA but not mACh5 (*Chrm5*) mRNA.

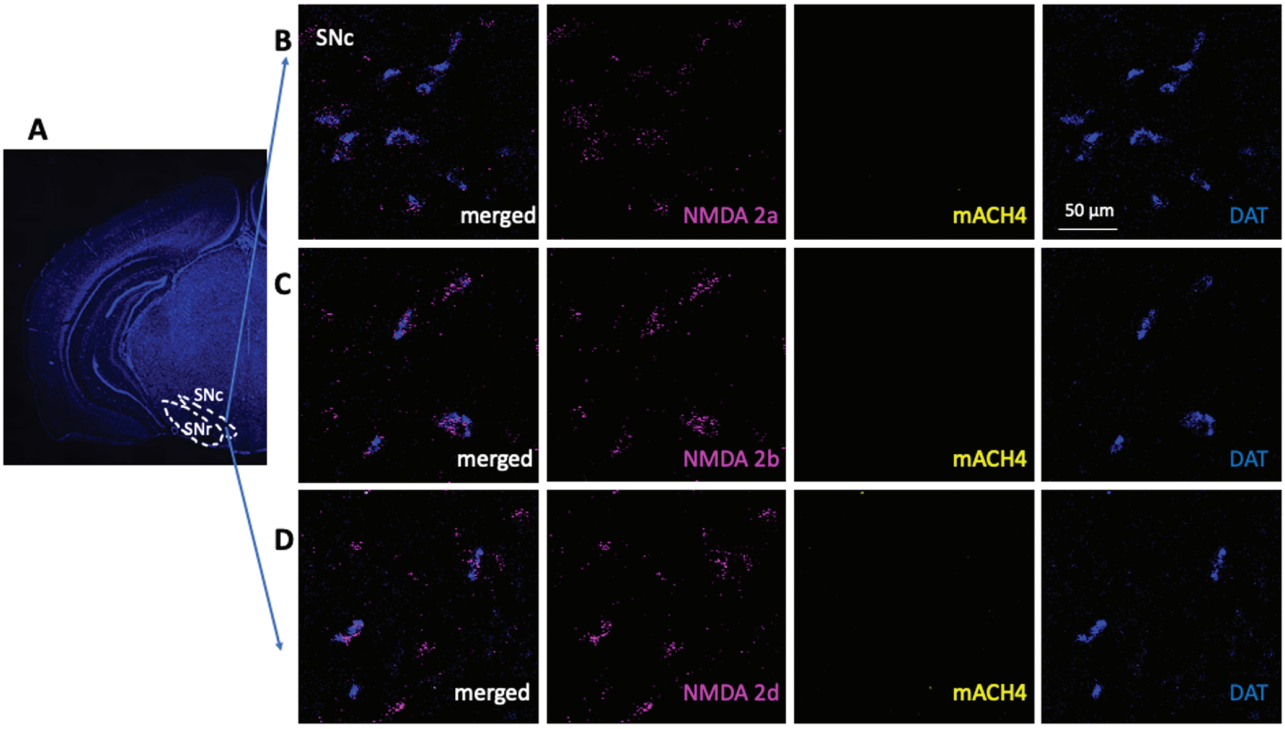

Figure 3. (A) Representative image of the rat midbrain under 4× magnification illustrating the substantia nigra pars compacta (SNc) and location where images were taken from. (B–D) Representative images under 40× magnification illustrating that DAT(Slc6a3)-positive neurons in the SNc express: (B) NMDA2a (*Grin2a*), (C) NMDA2b (*Grin2b*), (D) NMDA2d (*Grin2d*) mRNA but not mACh4 (*Chrm4*) mRNA.

1 ($F_{2,25}$ = 2.57; P = .96) or 2 ($F_{2,25}$ = 2.78; P = .08). A 1-way ANOVA on the CS-only test data revealed significant reductions in the difference score ($F_{2,25}$ = 10.71; P = .001) driven by the 2.5- and 5-µg doses of scopolamine (Tukey test: $P < .05$).

Because the SN is known for its role in motor processing, there is a concern that the observed reductions in the difference score reflect motoric impairment rather than impaired learning. To address this, we analyzed differences in total numbers of non-CS

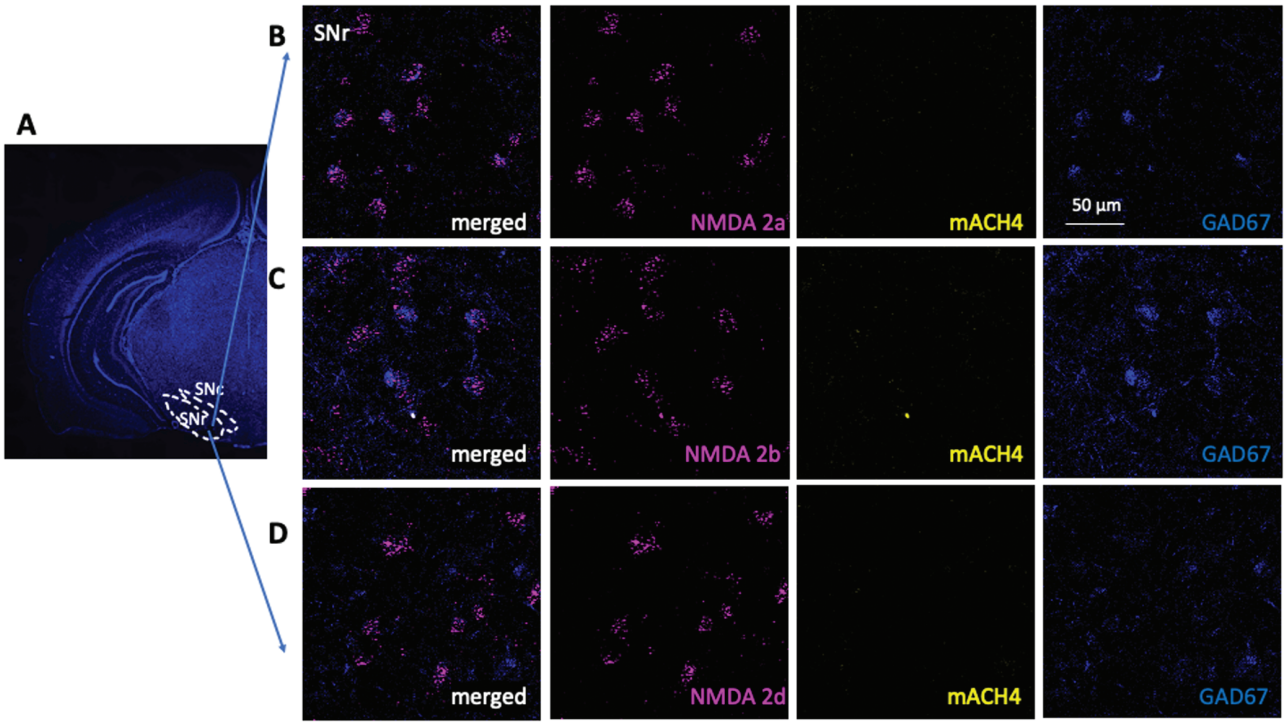

Figure 4. (A) Representative image of the rat midbrain under 4× magnification illustrating the substantia nigra pars reticulata (SNr) and location where images were taken from. (B–D) Representative images under 40× magnification illustrating that GAD67 (GAD1)-positive neurons in the SNr express: (B) NMDA2a (*Grin2a*), (C) NMDA2b (*Grin2b*), and (D) NMDA2d (*Grin2d*) mRNA but not mACh4 (*Chrm4*) mRNA.

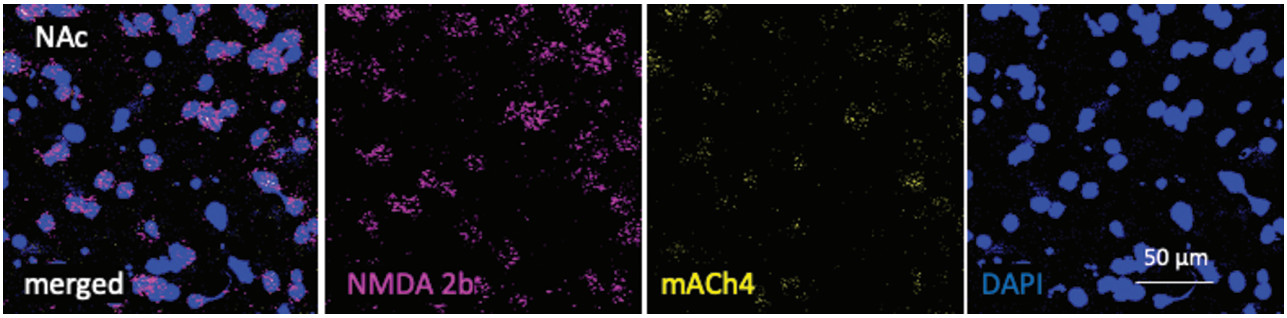

Figure 5. A. Representative images under 40× magnification illustrating that nucleus accumbens (NAc) neurons express mACh4 (Chrm4) and NMDA2b *(Grin2b)* mRNA.

nose pokes during each session. Non-CS pokes are all pokes excluding those during preCS and CS period. As shown in Figure 6C, intra-SNr scopolamine had no effect on nose poke responding during conditioning sessions, and rats showed no change in total non-CS nose pokes during the CS-only session. A 2-way ANOVA on the conditioning data revealed no main effects of session ($F_{2,50}$ = 0.78; P = .46), dose ($F_{2,25}$ = 1.38; P = .26), or session × dose interaction ($F_{2,50}$ = 1.64; P = .17). A 1-way ANOVA on the CS-only data revealed no significant differences in non-CS nose pokes between groups ($F_{2,25}$ = 0.72; P = .49).

We then evaluated whether intra-SNr microinjections of AP-5 during conditioning produce similar effects on the acquisition of conditioned approach. Figure 6D shows that with subsequent conditioning sessions, groups showed steady increases in the difference score; however, during the CS-only test, rats treated with AP-5 showed significant reductions in the CS-preCS difference scores. A 2-way ANOVA on the CS-preCS difference scores during conditioning revealed a significant session effect ($F_{2,62}$ = 34.58; P = .001) but no dose effect ($F_{3,31}$ = 1.39; P = .26) or dose × session interaction ($F_{6,62}$ = 0.48; P = .82). A 1-way ANOVA on the CS-only test data revealed significant dose-related reductions in the difference scores ($F_{3,31}$ = 3.22; P = .036) driven by the 2- and 4-µg doses of AP-5 (Tukey test: Ps < .05). No sex differences in CS-preCS scores were detected. We found no significant differences in total numbers of non-CS nose pokes made by AP-5-treated rats during conditioning but a very prominent ascending trend (Figure 6E). A 2-way ANOVA on the conditioning data revealed a significant session × dose interaction ($F_{6,62}$ = 2.11; P = .053). A 1-way ANOVA on the CS only data revealed no significant differences in total non-CS nose pokes between groups ($F_{3,31}$ = 2.08; P = .12).

At the end of behavioral experimentation, we performed histological analyses and mapped cannula placements according to the Paxinos and Watson atlas (Figure 6F).

Intra SNr-Scopolamine and AP-5 Have No Effect on Expression of Acquired Conditioned Approach

We then examined the role of SN NMDA and mACh receptors in the expression of already acquired conditioned approach behavior.

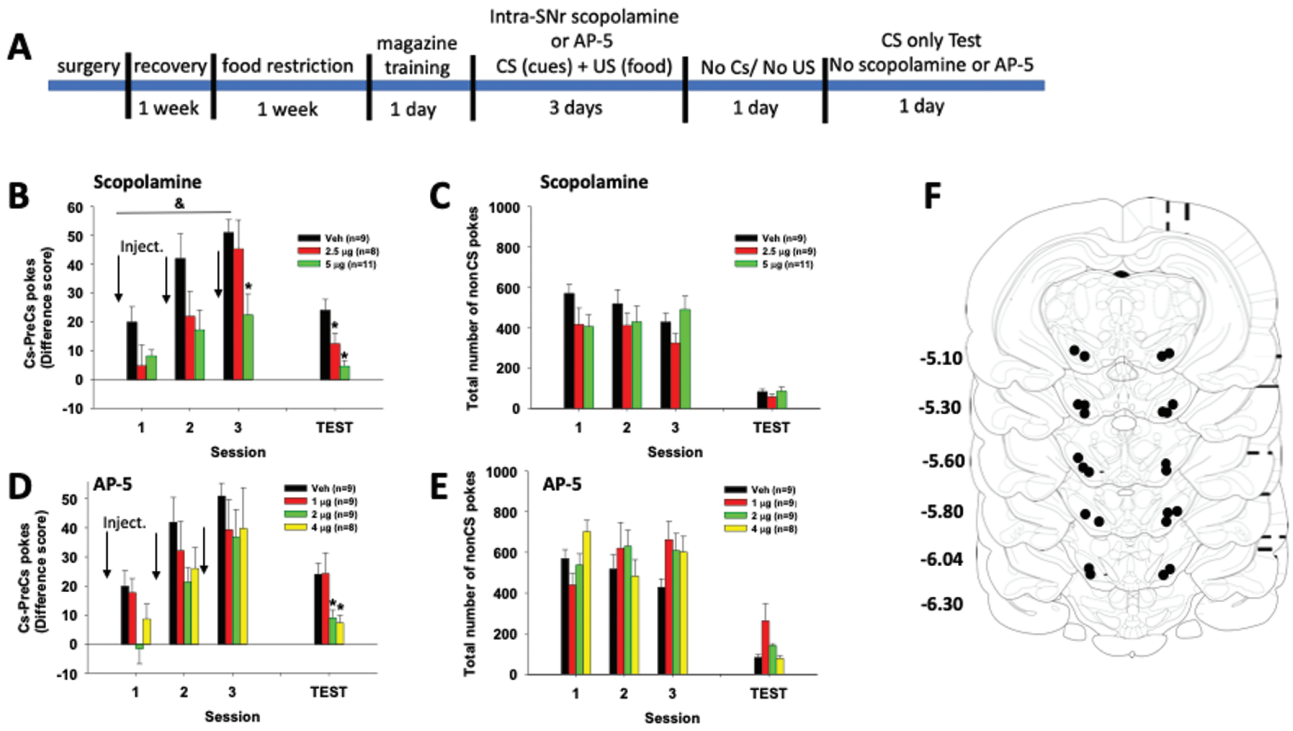

Figure 6. (A) Schematic timeline of behavioral experimentation testing the effects of intra-substantia nigra (SN) scopolamine or AP-5 on the acquisition of conditioned approach. (B) Intra-SNr scopolamine during conditioning caused significant reductions in CS-preCS difference scores during the CS-only test (C) without affecting overall non-CS nose poke responding. (D) Intra-SNr AP-5 during conditioning caused significant reductions in CS-preCS difference scores during the CS-only test (E) without affecting overall non-CS nose poke responding. (F) Post-experimental histology illustrating cannula placements in the rat SNr. *P < .05 compared with 0 µg/0.5µL/side dose; & session main effect at P < .05 indicates the timing of injection.

As shown in Figure 7A, food-restricted rats underwent magazine training followed by 7 conditioning sessions with no microinjections, 1 no CS/no US session, and a CS-only test with either microinjections of scopolamine or AP-5. Rats showed steady increases in CS-preCS difference scores during the 7 conditioning sessions and no difference in responding when treated with intra-SNr scopolamine during the CS-only test relative to vehicle (Figure 7B). A 2-way ANOVA on the conditioning data revealed a significant session effect ($F_{6,138}$ = 29.28; P = .001) but no dose effect ($F_{2,23}$ = 0.26; P = .76) or dose × session interaction ($F_{12,138}$ = 0.49; P = .92). A 1-way ANOVA on the CS-only data revealed no significant difference in the difference score between groups ($F_{2,23}$ = 0.66; P = .52). No sex differences in CS-preCS scores were observed. To examine the effect of intra-SNr scopolamine on overall responding, we analyzed differences in the total number of non-CS nose pokes made during the CS-only test and found the 5-µg scopolamine dose caused a significant increase in overall responding (Figure 7C). This observation was confirmed by a 1-way ANOVA ($F_{2,23}$ = 3.90; P = .035) and Tukey tests (P = .05).

As shown in Figure 7D, rats also showed steady increases in difference scores across the 7 conditioning sessions but no significant changes in responding under the AP-5 treatment during the CS-only test. A 2-way ANOVA on the conditioning data revealed a significant session effect ($F_{6,138}$ = 15.62; P = .001) but no AP-5 dose effect ($F_{2,23}$ = 0.07; P = .93) or dose × session interaction ($F_{12,138}$ = 1.00; P = .45). A 1-way ANOVA on the CS-only data revealed no significant difference in CS-PreCS difference scores between groups ($F_{2,23}$ = 0.11; P = .89). No sex differences in CS-preCS scores were observed. However, intra-SNr AP-5 enhanced overall non-CS nose poking, as shown in Figure 7E. A 1-way ANOVA on the CS-only data revealed a significant dose effect ($F_{2,23}$ = 5.57; P = .01), which was driven by the higher responding in the 4-µg dose group (Tukey test; P = .01). At the end of behavioral experimentation, we performed histological verification and summarized the results in Figure 7F and G.

Dopamine Neurons of the SN Receive Converging Acetylcholine and Glutamate Inputs

Both mACh and NMDA receptors located in the SNr, presumably on the dendrites of DA neurons, play a critical role in the acquisition of conditioned approach (Figure 8). Based on our data, we propose that DA neurons of the SNc receive converging acetylcholine and glutamate signals through mACh5, NMDA2a, NMDA2b, and NMDA2d receptors respectively.

DISCUSSION

In this study, we investigated the mechanisms underlying conditioned approach learning with a focus on the role of mACh and NMDA receptors in the SN, a brain region implicated in reward (Quinlan et al., 2004; Wise, 2009; Ilango et al., 2014; Galaj et al., 2020) and motor processes (Hodge and Butcher, 1980; Crocker, 1997; Chinta and Andersen, 2005). Using RNAscope ISH, we found that dopamine neurons of the SN coexpress mACh5, NMDA2a, NMDA2b, and NMDA2d glutamate receptor mRNA. GABA neurons of the SNr express NMDA2a, NMDA2b, and NMDA2d but not mACh5 receptor mRNA. Although we detected mACh4 receptor mRNA in the NAc, we found no detectable levels of mACH4 mRNA in the SNc or SNr.

In a conditioned approach paradigm, we found that microinjections of scopolamine (a mACh receptor antagonist) or AP-5 (a NMDA receptor antagonist), when administered prior

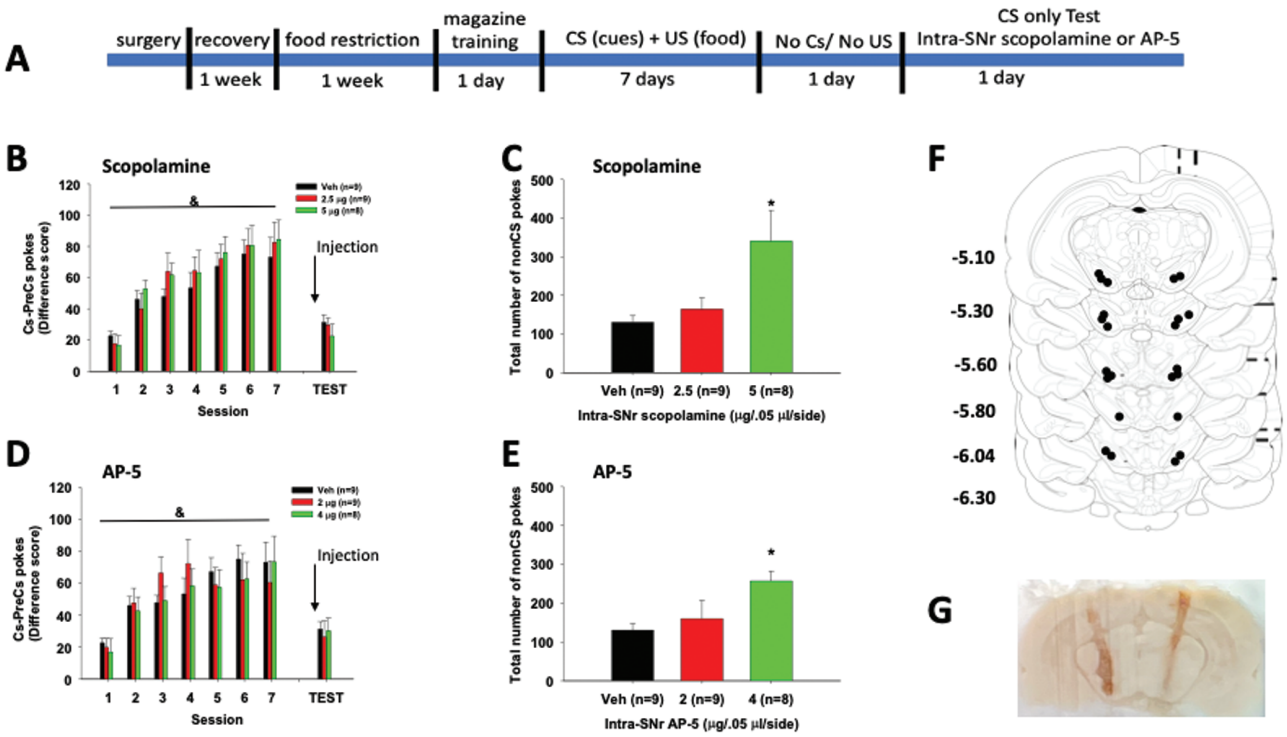

Figure 7. (A) Schematic timeline of behavioral experimentation testing the effects of intra-SN scopolamine or AP-5 on the expression of conditioned approach. (B) Intra-SNr scopolamine during CS-only test had no effect on CS-preCS difference scores (C) even though it increased non-CS nose poke responding. (D) Intra-SNr AP-5 during the CS-only test had no effect on CS-preCS difference scores (E) even though it significantly increased non-CS nose poke responding. (F) Post-experimental histology illustrating cannula placements in the rat SNr. (G) Representative image illustrating cannula placement. *P < .05 as compared to 0 µg/0.5µL/side dose; & session main effect at P < .05 indicates the timing of injection.

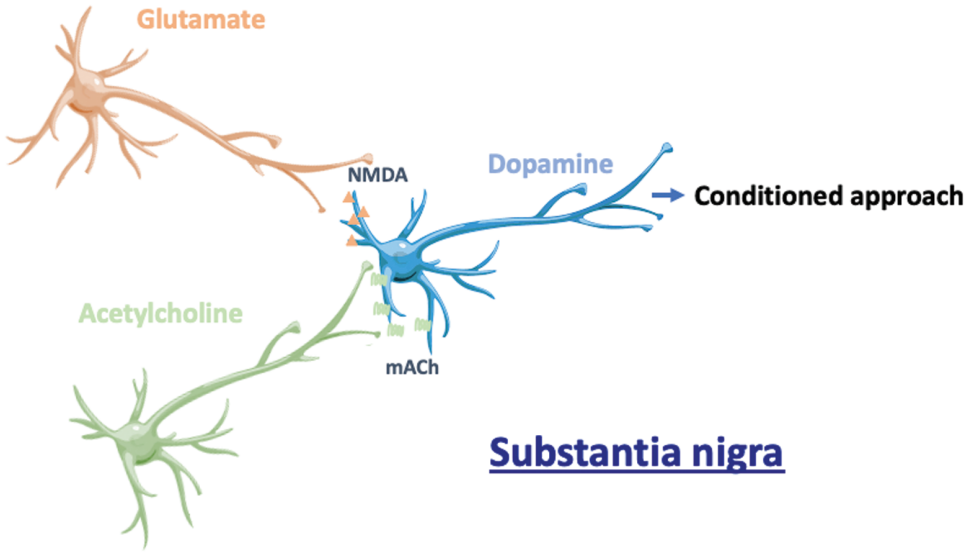

Figure 8. Our circuit model of reward-related learning. We propose that dopamine (DA) neurons of the substantia nigra pars compacta (SNc) coexpress muscarinic acetylcholine (mACh) and NMDA receptors. Convergent cholinergic and glutamatergic neurotransmission via mACh and NMDA glutamate receptors in the SN is needed for the acquisition of conditioned approach to occur.

to conditioning sessions, caused significant reductions in the CS-preCS difference scores, indicative of impaired reward-related learning. In contrast, intra-SNr scopolamine or AP-5 microinjections prior to the CS-only test (after animals already acquired the conditioned approach behavior) had no effect on the CS-preCS difference score. These data suggest that mACh and NMDA receptors in the SN play critical roles in the acquisition, but not performance (expression), of reward-related learning.

Given that the SN is involved in motor processing, there is concern that scopolamine- or AP-5-induced reductions in difference scores reflect motoric impairment rather than impairment in learning. To address this concern, we analyzed our data carefully and found that in contrast to their control counterparts,

rats treated with AP-5 or scopolamine overall made more non-CS nose pokes. These increases most likely represent nonselective responding due to impaired CS vs non-CS discrimination. Intra-SN scopolamine produced no significant change or enhanced overall responding. These data suggest that rats treated with intra-SN scopolamine or AP-5 were able to make as many nose pokes as their nontreated counterparts, strongly suggesting that the reduced CS-preCS scores in animals treated with intra-SN mACh or NMDA receptor antagonists during conditioning sessions resulted from impaired reward-related learning. When conditioned approach was well acquired, intra-SN scopolamine or AP-5 had no effect on the CS-preCS difference score despite enhancement in overall responding. Because nose poking as a measure of motoric deficits has its own limitations, future studies could further assess the effect of intra-SN AP-5 and scopolamine on locomotor activity.

Our data suggest that stimulation of mACh and NMDA receptors on SN DA neurons is important for reward-related learning. A major source of cholinergic input to the SN is the pedunculopontine tegmental nucleus (PPTg) that controls DA cells (Clarke et al., 1987). Muscarine (a muscarinic receptor agonist) depolarizes SNc DA cells (Lacey et al., 1990; Lacey, 1993), and optogenetic activation of PPTg cholinergic input into the SNc improves reversal learning in CHAT-cre mice (Ruan et al., 2022). In addition, SN DA neurons receive glutamatergic inputs from the subthalamic nucleus, cortex, PPTg and other brain regions (Watabe-Uchida et al., 2012; Beaudoin et al., 2018). SNc DA neurons respond to NMDA receptor stimulation (Suárez et al., 2010), and SNc DA activity is uniquely increased in response to drug cues (Ito et al., 2002).

Our findings are consistent with our previous reports regarding the role of VTA mACh and NMDA receptors in reward-related learning. In a series of papers, we have proposed and tested a neurobiological model of reward-related learning based on the propositions that VTA DA neurons can be activated by primary reward and associated CSs. Our model stipulates that CSs function as such because they acquire the capacity to activate midbrain DA neurons because of concurrent stimulation of mACh and NMDA receptors in the VTA (Zellner and Ranaldi, 2010; Galaj and Ranaldi, 2021). VTA DA neurons receive converging strong primary reward-related cholinergic signals and initially weak sensory stimuli (i.e., CS)-related glutamatergic signals that become strengthened by repeated joint stimulation of mACh and NMDA receptors. The strengthened CS signal can subsequently cause *conditioned* activation of VTA DA cells, leading to behavioral conditioned responses by sensory stimuli associated with rewards with these sensory stimuli now functioning as CSs. We have demonstrated that blockade of VTA mACh (Sharf and Ranaldi, 2006; Sharf et al., 2006; Galaj et al., 2017) or NMDA receptors (Zellner et al., 2009; Ranaldi et al., 2011) during conditioning impairs the acquisition of conditioned approach and conditioned reinforcement. The strength of this type of learning depends on the degree of VTA DA activation and requires joint stimulation of mACh and NMDA receptor stimulation during conditioning (Kest et al., 2012; Galaj and Ranaldi, 2018).

The findings of the present study suggest that reward-related learning also requires stimulation of mACh and NMDA receptors in the SN. These conclusions are based on the data from the CS-only test. SNc DA neurons coexpress mAChs, NMDA2a, NMDA2b, and NMDA2d receptors and blockade of SN mACh and NMDA receptor stimulation during conditioning, just like similar VTA manipulations, impairs conditioned approach learning. Given that SNr GABA neurons do not express mACh receptors, scopolamine-induced effects are likely mediated through mACh receptors on SNc DA neurons. Although at this point we cannot directly determine whether reward-related learning requires stimulation of SN NMDA receptors on DA or GABA neurons (as both types of neurons express NMDA receptors), we can deduce this conclusion based on our behavioral data and previous findings. Electrophysiological studies demonstrated that N-methyl-D-aspartate, an NMDA receptor agonist, induces bursts in SNc DA neurons (Li et al., 1996) and SNr GABA neurons (Ibáñez-Sandoval et al., 2007). Intra-SNr NMDA antagonism impairs conditioned approach learning and optogenetic activation of SNc DA neurons (Ilango et al., 2014; Galaj et al., 2020), but inactivation of SNr GABA neurons leads to the development of optical intracranial self-stimulation (Galaj et al., 2020). Thus, it is quite possible that reward-related learning is mediated by NMDA receptor–induced activation of SNc DA neurons. Based on our findings, we propose that acetylcholine and glutamate neurotransmission via mACh and NMDA receptors in the SN is necessary for the acquisition of conditioned approach learning. We propose that these actions are necessary for initiating the neuroplasticity whereby reward-associated environmental stimuli acquire the capacity to activate SNc DA neurons, similar to how USs would, and eliciting conditioned approach responses. We plan to test this proposition and explore our model further by identifying the specific cholinergic and glutamatergic inputs to the SNc involved in reward-related learning.

In addition, in this study we were not able to determine the role of SNr NMDA and mACh receptor subtypes in reward-related learning as AP-5 lacks selectivity for specific receptor subtypes (Buller et al., 1994; Monaghan and Buller, 1994) and scopolamine is a muscarinic nonselective antagonist (Renner et al., 2005). This is something that could be explored in future studies because these receptor subtypes are known for their roles in neuroplasticity related to learning and memory (Yeomans et al., 2001; Leaderbrand et al., 2016; Baez et al., 2018).

CONCLUSIONS

Although the exact neural substrates underlying reward-related learning have not been fully uncovered, the present study provides evidence supporting the role of mACh and NMDA receptors in the SN in the acquisition, but not expression, of conditioned approach learning. Importantly, these findings redefine the role of the SN, which has traditionally been known for its involvement in motor processes and suggest that the SN possesses the attributes to function as a hub of integration of primary reward and conditioned stimulus signals.

Acknowledgments

This work was supported by National Institute of General Medical Science of the National Institutes of Health under award number 1SC3GM130430-01 to R.R.

Contributions

E.G. designed the study, wrote the manuscript, and conducted surgeries. R.R. helped design the study and edited the manuscript. E.D.B. and A.V. conducted RNAscope experiments. A.T., O.L., P.S., R.D., and H.L. conducted behavioral assays and analyzed the data.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Behavioral data, images, Med-Associates output files, programs, and protocols used in this study are available upon request.

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