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***The Endogenous Negative Allosteric Modulator, Lynx1, Inhibits Nicotinic Cholinergic
Signaling in the Ventral Tegmental Area to Regulate Nicotine Reinforcement***

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) underlie many aspects of cholinergic signaling, including as essential contributors to learning and memory function. However, the functional impact of endogenous nAChR modulators remains largely unclear. Thus, the current investigations sought to define the function of *lynx1* in reward-related reinforcement behaviors. First, male and female mice with constitutive *lynx1* knockout (*lynx1*^{-/-}) or their wildtype littermates (*lynx*^{+/+}) were examined with intravenous nicotine self-administration across a full dose response function. Knockout of *lynx1* induced an increase in nicotine intake at lower self-administered doses, with dose-specific effects in males and females. These actions appear to be selective for nicotine, as no phenotypic differences were found with operant food training. We next examined whether *lynx1* in the ventral tegmental area (VTA) may underlie the increase in nicotine intake, and we found that administration of a low dose of nicotine increased cellular activation in the VTA of *lynx1*^{-/-} mice. To determine which nAChRs could be involved in *lynx1*'s actions, co-expression of *lynx1* with nAChR subunits in the VTA was assessed. *Lynx1* was found to co-localize in VTA cells expressing $\alpha 4$, $\alpha 6$, $\alpha 7$, and $\beta 2$ and $\beta 3$ nAChR subunits, with male *lynx1*^{-/-} mice exhibiting an increase in $\alpha 4$ expression compared to wildtype mice. Finally, site-specific *lynx1* knockdown in the VTA recapitulated the behavioral phenotype found in the *lynx1* global knockout mice, in which an increase in nicotine reinforcement was evidenced. Taken together, these findings reveal that *lynx1* acts on nAChRs in the VTA to dampen the reinforcing properties of nicotine.

Significance Statement: Lynx1 plays a critical regulatory role in cholinergic signaling by modulating the function of neuronal nicotinic acetylcholine receptors. Our data show that loss of lynx1 enhances nicotine self-administration, particularly at lower doses, through actions in the ventral tegmental area. Together, these findings position lynx1 as a key gatekeeper of nicotine reward, thereby providing critical insight into the neural mechanisms that drive nicotine intake and identifying a potential target for therapeutic intervention.

Introduction

Cholinergic signaling via nicotinic acetylcholine receptors (nAChRs) underlies numerous physiological processes across the central and peripheral nervous systems, and dysregulation of such signaling represents a significant hallmark of multiple neurological diseases (Picciotto et al., 2012; Chen et al., 2022). The cholinergic system also contributes to the initiation and maintenance of substance dependence, with a key role in nicotine use (Fowler et al., 2011; Picciotto et al., 2012; Le Foll et al., 2022). Indeed, driven by nicotine's agonist actions on nAChRs, tobacco product use continues to be a leading cause of preventable chronic disease and death worldwide (Le Foll et al., 2022). Thus, understanding the diversity of endogenous molecular factors that regulate cholinergic signaling has the potential to elucidate mechanisms that are broadly applicable to various facets of cognitive function.

Neuronal nAChRs are comprised of various pentameric combinations of α and β subunits, which include the $\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$ subunits (Gotti and Clementi, 2004; Dani, 2015). The nAChR subtypes exhibit diverse pharmacokinetic properties, along with cell- and brain region-specific expression patterns (Gotti and Clementi, 2004; Picciotto and Kenny, 2021; Mogul et al., 2025). Nicotine or acetylcholine binding to a resting state nAChR induces a conformational change that allows cation influx during an open state, which is followed by a transition to a closed, desensitized state that is resistant to ligand-induced actions (Gotti and Clementi, 2004; Dani, 2015; Wittenberg et al., 2020). Of relevance, we have found that lynx proteins act as endogenous negative allosteric modulators of nAChRs (Miwa et al., 2006). The lynx1 protein is part of the ly6/uPAR superfamily of genes, which exhibit a high level of conservation across species (Miwa et al., 2019). *In vitro* studies have characterized lynx1 as a negative allosteric modulator due to its actions in reducing agonist efficacy and enhancing the transition into the nAChR desensitized conformational state, which has been mainly demonstrated with $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs (Ibanez-Tallon et al., 2002;

Miwa et al., 2006; George et al., 2017; Miwa et al., 2019). Through associations in the endoplasmic reticulum, lynx proteins can bias $\alpha 4\beta 2$ nAChRs toward a lower sensitivity configuration and decrease $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR plasma membrane trafficking (Nichols et al., 2014; Wu et al., 2015), resulting in an overall dampening of cholinergic signaling. Receptors containing the $\alpha 5$ -, $\alpha 7$ -, and $\alpha 6$ -nAChR subunits have also been shown to be affected by lynx1, and lynx1 is expressed in brain regions implicated in reward-related reinforcement processing (Thomsen et al., 2016; Parker et al., 2017; Sherfat et al., 2021). Given that lynx1 appears to be a critical negative regulator of nAChR function, understanding its role in nicotine-mediated behaviors is essential to provide key insights into cholinergic agonist-mediated signaling underlying motivated behaviors.

Here, we aimed to assess the role of lynx1 in regulating reinforcement for nicotine and food reward. We first hypothesized that increasing nicotinic signaling via elimination of the 'molecular break' imposed by lynx1 would enhance overall nicotine intake. Mice with null mutation in the *Lynx1* gene (*lynx1*^{-/-}) and wildtype littermates (*lynx1*^{+/+}) were examined with intravenous nicotine self-administration across a full dose response function. *Lynx1*^{-/-} mice exhibited dose-dependent increases in nicotine intake, with differential effects in males and females, compared to *lynx1*^{+/+} mice. Interestingly, these effects on reinforcement behavior were specific to nicotine, as no phenotypic differences were found with operant food training. We next found an increase in nicotine-mediated cellular activation in the VTA of *lynx1*^{-/-} mice, and thus, we then sought to determine whether site-specific VTA knockdown of lynx1 would recapitulate the phenotype found in the knockout condition with sex-specific differences, which was confirmed. Together, these findings identify lynx1 as a critical regulator of nicotinic receptor driven behaviors in the VTA and reveal a previously unrecognized, brain region specific mechanism underlying nicotine reinforcement.

Materials and Methods

Animals

Male and female adult mice on a C57BL/6J background with null mutations in the *Lynx1* gene and their respective wildtype littermates were bred in our animal facilities. Wildtype mice on a C57BL/6J background were either bred in our animal facilities or obtained from the Jackson Laboratory. All mice were at least 6 weeks of age at the beginning of the experiments and were maintained in an environmentally controlled vivarium on a 12:12 hr reversed light:dark cycle. Food and water were available *ad libitum* until behavioral assessments commenced. All behavioral assessments were conducted during the active, dark phase of the light cycle. During self-administration studies, mice were mildly food restricted to 85%-90% of their free-feeding body weight, with water provided *ad libitum*. All procedures were conducted in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Lynx1 Genotyping

At 21 days of age, pups were weaned, and tail clips were obtained. DNA was extracted and analyzed by PCR for determination of genotype. The following primers were used to genotype *lynx1* mice: *Lynx1* WT Forward (CTGGAGTGCCACGTGTGTGCC), *Lynx1* KO Forward (GCCAGCTTGCGTGAAGTTCC), and *Lynx1* WT/KO Reverse (CGTTTGAGTGGATCTGGCTTGGGG). The band for the *lynx1* wildtype allele was detected at 470 bp and for the *lynx1* knockout allele at 200 bp.

Drugs

(-)-Nicotine hydrogen tartrate (MP Biomedicals, catalog # 0215355491) was dissolved in 0.9% sterile saline and adjusted to pH 7.4. Nicotine doses are represented as the free base form.

Food and Intravenous Nicotine Self-Administration

Subjects were mildly food restricted to 85-90% of their free feeding body weight. Mice were trained to acquire operant food self-administration across an ascending fixed ratio (FR) schedule beginning at FR1 and up to FR5 to earn 20 mg food pellets (5TUM grain-based pellets, TestDiet), up to a fixed ratio 5, time out 20 second schedule of reinforcement (FR5TO20). Responses on the inactive lever were recorded but did not have any consequences. Data were automatically recorded by the Med Associates software. For intravenous self-administration, mice were surgically implanted with a catheter into the right jugular vein, as previously described (Fowler and Kenny, 2011; Chen et al., 2023). Catheters comprised of a 6 cm length of silastic tubing fitted onto a guide cannula bent at a curved right angle and encased in dental acrylic. Subjects were anesthetized with an isoflurane (1%-3%)/oxygen vapor mixture and prepared for catheter implantation under aseptic conditions. Catheter tubing was subcutaneously passed from the animal's back into the right jugular vein. A 1 cm length of the catheter tip was inserted into the vein and secured with surgical silk suture. Thereafter, animals were provided ≥ 48 hrs to recover from surgery. Intravenous nicotine self-administration was acquired during 1 hr daily sessions, 6-7 days per week, at the standard training nicotine dose (0.03 mg/kg/infusion) for 7 days, followed by the moderate nicotine dose (0.1 mg/kg/infusion) for 5 days. To obtain the dose response, mice were assessed across a range of doses from 0 to 0.25 mg/kg/infusion in a modified Latin square design with 5 days on each dose, and in between each dose, mice were required to re-establish baseline responding at the training dose with at least 2 sessions (Fowler and Kenny, 2011). The

mean number of infusions for the final 2 days of each dose was used for statistical analyses to derive the dose response function. Nicotine infusions were delivered through tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each session was performed with the presentation of 2 retractable levers extended 1 cm into the chamber (one active, one inactive). Five lever presses on the active lever delivered a 0.0367 mL intravenous nicotine infusion over 3 seconds, followed by a 20 second time out period with cue light activation (FR5TO20s schedule). Responses on the inactive lever were recorded but had no scheduled consequences. Behavioral responses were recorded automatically by the Med Associates software.

Stereotaxic AAV Injections

Subjects were anesthetized with an isoflurane (1%-3%)/oxygen vapor mixture and positioned at the flat-skull position in a Kopf stereotaxic frame. Viral microinfusions were administered bilaterally to the ventral tegmental area (VTA) with a Hamilton syringe. Infusion volume was 275 nL per side, and the AAV constructs included AAV8-U6-*scrambled control*-GFP and AAV8-U6-shRNA $Lynx1$ -GFP (titers $>1 \times 10^{13}$ GC/mL; Charles River/Vigene Biosciences). The flow rate for all injections was 250 nL/min, and injectors remained in place for an additional 10 min after each infusion to prevent viral spread with injector removal. Coordinates for the VTA were: flat skull, AP -3.3 mm, ML $\pm$ 0.42 mm, DV -4.1 mm. Mice were permitted to recover for ~3 weeks to allow for viral expression prior to behavioral assessments. At the conclusion of the behavioral assessments, mice were anesthetized with intraperitoneal ketamine-xylazine and perfused through the ascending aorta with 0.9% saline, followed by 4% paraformaldehyde in 0.1M phosphate buffered solution (PBS), pH 7.4. Brains were then collected, cryoprotected in 30% sucrose, and then sliced on a cryostat and mounted on slides for image analysis to verify viral spread. Fifteen mice were excluded from analysis due to the viral spread extending beyond the

VTA region boundaries. Viral expression was also analyzed to assess possible retrograde transport, but no GFP expression was detected in other afferent brain regions.

Immunohistochemistry

Mice were subcutaneously injected with 0.125 mg/kg nicotine, and 2 hrs thereafter, mice were anesthetized with intraperitoneal ketamine-xylazine and perfused through the ascending aorta with 0.9% saline then 4% paraformaldehyde in 0.1M phosphate buffered solution (PBS), pH 7.4. Brains were then removed and postfixed in paraformaldehyde for 2 hrs. Thereafter, brains were cryoprotected in a 30% sucrose solution for at least 72 hrs prior to sectioning in a cryostat at 35 μ m. Floating sections were stored in 0.1M PBS with 0.01% sodium azide. For c-fos immunolabeling, one out of every 4 sections was processed for staining. Sections were rinsed in 0.1M PBS, and then blocked in 10% normal donkey serum (Jackson Immuno, catalog # 014-000-121) and 0.5% Triton X-100 in 0.1M PBS at room temperature for 1 hr. Afterward, sections were incubated at 4°C overnight with rabbit anti-cfos IgG (1:5000 dilution; ABCAM, catalog # ab190289) in 10% normal donkey serum and 0.5% Triton X-100 in 0.1M PBS. Thereafter, sections were rinsed in PBS and incubated in biotin donkey anti-rabbit IgG (1:200 dilution; Jackson Immuno, catalog # 711-065-152) in 0.1% Triton X-100 in 0.1M PBS for 2 hrs at room temperature. After rinsing in PBS, sections were incubated with solution from the Elite ABC kit (Vector Laboratories, catalog # PK-6100) for 90 mins at room temperature. Immunoreactivity was revealed using 3-diaminobenzidine (DAB) with nickel (Vector Laboratories, catalog # SK-4100). Sections were then rinsed, mounted, and dehydrated. Lastly, slides were cover slipped with Permount (Fisher Chemical, catalog # SP15-500). To ensure rigor, sections from multiple subjects across all groups were concurrently processed in the same staining dish for each experiment (with separate experiments being conducted for the saline-injected and nicotine-injected studies). To derive a measure of c-fos positive cell density, the number of cells and brain region volumes

(VTA) were quantified using unbiased stereological methods with the optical fractionator probe and Stereo Investigator software (MicroBrightField) by experimenters blinded to the experimental conditions. Given the experimental requirements for accurate quantification with the automatic optical fractionator (Mouton et al., 2017; MBF, 2026), the sampling parameters were first adjusted to achieve a coefficient of error of ≤ 0.1 , as prior (Fowler et al., 2005; Fowler et al., 2011). Systematic random sampling and strict stereological counting rules were applied to minimize bias associated with cell size, shape, and sectioning angle. The resulting parameters were as follows: Bilateral VTA quantified along the rostro-caudal extent based on the mouse brain atlas (Paxinos and Franklin, 2013), Section cut at 35 μm , Section evaluation interval at 4, Resulting coronal sections per animal 5-7, 40x magnification during quantification, Non-matched starting point in the VTA, Consistent lighting conditions across sections within each experiment, Guard size 2 μm , Grid size 120 μm x 125 μm , and Counting frame 60 μm x 60 μm . Six mice were excluded from the study due to an insufficient number of VTA sections across the full rostro-caudal extent due to tissue damage during section cutting/mounting. Cell density ($\text{cells}/\mu\text{m}^3$) was subsequently calculated by dividing the estimated cell population number by the corresponding estimated VTA volume, and these values were multiplied by 100,000 for graphical presentation.

RNA Scope

Brain sections from wildtype mice were examined for colocalization of *Lynx1* with $\alpha 4$ (*Chrna4*) and $\beta 2$ (*Chrb2*) in the VTA. Subjects were anesthetized with ketamine-xylazine and perfused through the ascending aorta with saline (0.9% w/v) followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Thereafter, brains were removed and postfixed for 2 hrs in paraformaldehyde, followed by cryoprotection in 30% sucrose for at least 72 hrs. Brain sections were cut on a cryostat at 35 μm and then processed for RNA Scope Multiplex Fluorescent assay (Advanced Cell

Diagnostics) as previously described (Sherafat et al., 2020). Sections were placed in an incubator at 60°C for 30 min and then treated at 100°C for 6 min in the target retrieval solution. Sections were dehydrated in 100% ethanol and treated with protease (Advanced Cell Diagnostics, catalog #322380). RNA hybridization probes included *Lynx1* (Advanced Cell Diagnostics, catalog #449078), *Chrna4* (Advanced Cell Diagnostics, catalog #429871-C3), and *Chrnb2* (Advanced Cell Diagnostics, catalog #449231-C3) which were labeled with Opal 520 or Opal 570 (PerkinElmer). Slides were then counterstained and coverslipped with Vectashield with DAPI (Vector Laboratories) and imaged with a Leica microscope.

Single Cell-Transcriptomics

Transcriptomic data were obtained from an open-source database published by Macosko and colleagues at the Broad Institute with VTA tissue obtained from wildtype mice (Langlieb et al., 2023). For the current analysis, the applied inclusion criteria were predetermined as > 100 TH-positive cells for the VTA of each subject. VTA datasets were extracted for analysis, and cells were then identified based on expression of *Lynx1* mRNA. Expression of the nicotinic acetylcholine receptor (nAChR) subunit transcripts for *Chrna2* ($\alpha 2$), *Chrna3* ($\alpha 3$), *Chrna4* ($\alpha 4$), *Chrna5* ($\alpha 5$), *Chrna6* ($\alpha 6$), *Chrna7* ($\alpha 7$), *Chrnb2* ($\beta 2$), *Chrnb3* ($\beta 3$), or *Chrnb4* ($\beta 4$) was then determined. The percentages of cells with nAChR expression were calculated by dividing the number of cells in a category containing a given nAChR subunit transcript by the total number of cells expressing *Lynx1* transcripts. For instance, to determine the percentage of *lynx1* cells containing $\alpha 4$, the equation contained a numerator of the total number of cells that co-expressed *lynx1* and $\alpha 4$ and a denominator of the total number of *lynx1* expressing cells; the resulting number was multiplied by 100 to derive the percentage shown.

RT-qPCR

Tissue from the VTA of flash frozen brains stored at -80°C was dissected, and RNA was processed as previously described (Chen et al., 2018). RNA was extracted from homogenized tissue, and the quality of the RNA was determined by a NanoDrop 2000 spectrophotometer (ThermoScientific). For each sample, total RNA was reverse transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories). RT-qPCR was performed targeting mRNA of the *lynx1*, nAChR subunits, *c-fos*, and the housekeeping gene, β -actin. TaqMan Universal Master Mix II was used with real-time PCR gene expression assays for *Lynx1* (Thermo Fisher, Mm01204957_g1), *Fos* (Thermo Fisher, Mm00487425_m1), *Chrna4* (Thermo Fisher, Mm00516561_m1), *Chrna7* (Thermo Fisher, Mm01312230_m1), and *Actb* (Thermo Fisher, Mm02619580_g1). Samples were tested in duplicate and quantified with a CFX96 RT-qPCR system (Biorad). Normalized gene expression ($2^{\Delta Ct}$) was multiplied by 100.

Statistical Analyses

All data were analyzed using GraphPad Prism software (La Jolla, CA) with either a t-test, one-way ANOVA, repeated measure (RM) two-way ANOVA, or RM three-way ANOVA, as appropriate. Significant main or interaction effects were followed by Bonferroni or Tukey's post-hoc test, as appropriate, with correction for multiple comparisons. The criterion for significance was $\alpha=0.05$.

Results

Lynx1 inhibits nicotine reinforcement at lower doses

We first sought to determine whether the absence of the lynx1 protein mediates nAChR signaling to alter nicotine reinforcement behavior. Mice with null mutation in the *Lynx1* gene (*lynx1*^{-/-}) and their wildtype littermates (*lynx1*^{+/+}) were examined across the nicotine dose response function. Male *lynx1*^{-/-} mice exhibited enhanced reinforcement behavior at lower nicotine doses compared to male *lynx1*^{+/+} mice (**Fig 1a**) (Two-Way Repeated Measures (RM) ANOVA, *Genotype* $F_{(1, 23)}=2.533$, $p=0.1251$; *Dose* $F_{(4, 92)}=21.34$, $p<0.0001$; *Interaction* $F_{(4, 92)}=7.330$, $p<0.0001$). Specifically, male *lynx1*^{-/-} mice earned a significantly greater number of nicotine infusions at the 0.01 mg/kg/infusion ($p=0.0008$) and 0.03 mg/kg/infusion ($p=0.0105$) doses, compared to their wildtype littermates. Female *lynx1*^{-/-} mice also exhibited an enhanced number of nicotine infusions earned, but at fewer doses (**Fig 1b**) (Two-Way RM ANOVA, *Genotype* $F_{(1, 16)}=1.907$, $p=0.1863$; *Dose* $F_{(4, 64)}=31.22$, $p<0.0001$; *Interaction* $F_{(4, 64)}=7.174$, $p<0.0001$). Specifically, female *lynx1*^{-/-} mice earned a significantly greater number of nicotine infusions selectively at the 0.03 mg/kg/infusion ($p<0.0001$) dose, compared to wildtype littermates. We next examined the lever pressing behavior to assess discrimination for the active vs inactive lever across doses and found no deficits in the ability to discriminate between the levers. In males, active lever pressing paralleled the findings with nicotine infusions earned, while low levels of responding was maintained on the inactive lever, for both groups (**Fig 1c**) (Three-Way RM ANOVA, *Lever* $F_{(1, 46)}=113.0$, $p<0.0001$; *Dose* $F_{(4, 184)}=20.67$, $p<0.0001$; *Genotype* $F_{(1, 46)}=0.5222$, $p=0.4736$; *Dose x Lever Interaction* $F_{(4, 184)}=10.50$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 184)}=6.120$, $p=0.0001$; *Lever x Genotype Interaction* $F_{(1, 46)}=2.192$, $p=0.1455$; *Dose x Lever x Genotype Interaction* $F_{(4, 184)}=3.803$, $p=0.0054$). The post-hoc analysis comparing genotypes within each lever for each dose revealed that the male *lynx1*^{-/-} group had significantly higher levels of active lever pressing behavior for the 0.01 ($p=0.0018$) mg/kg/infusion dose compared to the *lynx1*^{+/+} male controls. No statistically significant differences were found in inactive lever pressing at each dose. In females, active lever pressing behavior also paralleled the nicotine infusion findings, in addition to low responding levels being evident on the inactive lever (**Fig 1d**) (Three-Way RM ANOVA, *Lever* $F_{(1, 16)}=113.0$, $p<0.0001$; *Dose* $F_{(4, 64)}=31.22$, $p<0.0001$; *Genotype* $F_{(1, 16)}=1.907$, $p=0.1863$; *Dose x Lever Interaction* $F_{(4, 64)}=7.174$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 64)}=7.174$, $p<0.0001$; *Lever x Genotype Interaction* $F_{(1, 16)}=1.907$, $p=0.1863$; *Dose x Lever x Genotype Interaction* $F_{(4, 64)}=7.174$, $p<0.0001$).

$_{32}=54.66$, $p<0.0001$; *Dose* $F_{(4, 128)}=38.48$, $p<0.0001$; *Genotype* $F_{(1, 32)}=2.791$, $p=0.1045$; *Dose x*
Lever Interaction $F_{(4, 128)}=16.66$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 128)}=9.514$, $p<0.0001$;
Lever x Genotype Interaction $F_{(1, 32)}=1.559$, $p=0.2208$; *Dose x Lever x Genotype Interaction* $F_{(4, 128)}=4.577$, $p=0.0017$). The post-hoc analysis comparing genotypes within each lever for each
dose revealed that the female *lynx1*^{-/-} mice had significantly higher levels of active lever pressing
behavior at the 0.03 mg/kg/infusion dose ($p<0.0001$), compared to the *lynx1*^{+/+} controls, with no
differences in inactive lever pressing behavior at each dose. The male and female *lynx1*^{-/-} and
lynx1^{+/+} groups also did not differ in their responding for saline infusions (0 dose), thereby
demonstrating that the absence of *lynx1* does not alter general, non-contingent lever pressing
behavior.

Lynx1 knockout did not alter operant food reinforcement learning

As a secondary reinforcement measure, we next examined operant food self-administration in
male and female *lynx1*^{-/-} and *lynx1*^{+/+} mice. When analyzing the number of food rewards earned
across training sessions, *lynx1*^{-/-} and *lynx1*^{+/+} mice did not differ for both males (**Fig 2a**) (Two-Way
RM ANOVA, *Genotype* $F_{(1, 23)}=3.666$, $p=0.0681$; *Session* $F_{(4, 92)}=33.24$, $p<0.0001$; *Interaction* $F_{(4, 92)}=0.5226$, $p=0.7193$) and females (**Fig 2b**) (Two-Way RM ANOVA, *Genotype* $F_{(1, 16)}=0.6352$,
 $p=0.4371$; *Session* $F_{(4, 64)}=33.68$, $p<0.0001$; *Interaction* $F_{(4, 64)}=1.428$, $p=0.2349$). Lever pressing
on the active and inactive levers were next examined. In males, both the *lynx1*^{-/-} and *lynx1*^{+/+} mice
established a statistically significant discrimination between the active and inactive levers, with
lynx1^{-/-} mice beginning on training session 2 and then both genotypes significantly discriminating
across sessions 3, 4 and 5 (**Fig 2c**) (Three-Way RM ANOVA, *Lever* $F_{(1, 46)}=382.2$, $p<0.0001$; *Dose*
 $F_{(4, 184)}=86.14$, $p<0.0001$; *Genotype* $F_{(1, 46)}=7.403$, $p=0.0092$; *Dose x Lever Interaction* $F_{(4, 184)}=70.83$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 184)}=1.428$, $p=0.2265$; *Lever x Genotype*
Interaction $F_{(1, 46)}=7.942$, $p=0.0071$; *Dose x Lever x Genotype Interaction* $F_{(4, 184)}=1.347$,

p=0.2543; post-hoc, Active vs. Inactive, lynx1^{-/-} session 2 p=0.0001, both lynx1^{-/-} and lynx1^{+/+} session 3 p<0.0001, session 4 p<0.0001, session 5 p<0.0001). Female lynx1^{-/-} and lynx1^{+/+} mice also exhibited similar food training acquisition, with a statistically significant difference in lever responding for both groups across sessions 3-5 (**Fig 2d**) (Three-Way RM ANOVA, *Lever* $F_{(1, 32)}=108.5$, p<0.0001; *Dose* $F_{(4, 128)}=71.07$, p<0.0001; *Genotype* $F_{(1, 32)}=0.0915$, p=0.7642; *Dose x Lever Interaction* $F_{(4, 128)}=55.48$, p<0.0001; *Dose x Genotype Interaction* $F_{(4, 128)}=0.5368$, p=0.7089; *Lever x Genotype Interaction* $F_{(1, 32)}=0.0300$, p=0.8635; *Dose x Lever x Genotype Interaction* $F_{(4, 128)}=0.4272$, p=0.7888; post-hoc, Active vs. Inactive within genotype: session 3 p<0.0001, session 4 p<0.0001, session 5 p<0.0001). These data reveal that, in both male and female mice, lynx1 does not induce significant effects to mitigate learning and memory function in a cue-paired operant task with a non-drug reinforcer.

Lynx1 mediates nicotine's actions in the VTA

Given that lynx1^{-/-} mice exhibited increased responding for low, reinforcing nicotine doses and nicotine-mediated activation of nAChRs in the VTA is necessary for the formation and maintenance of chronic nicotine use (Picciotto et al., 1998), we first examined baseline levels of *Fos* mRNA expression in the VTA using RT-qPCR. No differences in *Fos* mRNA levels were found between lynx1^{+/+} and lynx1^{-/-} mice (lynx1^{+/+}: n = 11 (6 males, 5 females), mean 3.080 ± SEM 0.2485; lynx1^{-/-}: n = 10 (7 males, 3 females), mean 2.843 ± SEM 0.2854; *unpaired two-tailed t-test*, $t_{(19)}=0.6290$, p=0.5368). Next, we performed immunohistochemistry for c-fos and employed unbiased stereology to quantify cell density in the VTA of control lynx1^{+/+} and lynx1^{-/-} mice injected with saline vehicle. No differences in c-fos cell density in the VTA were found between genotypes (lynx1^{+/+}: n = 7 (4 males, 3 females), mean (cells/μm³ × 100,000) 0.5621 ± SEM 0.0863; lynx1^{-/-}: n = 8 (4 males, 4 females), mean 0.7550 ± SEM 0.1662; *unpaired two-tailed t-test*, $t_{(13)}=0.9856$,

p=0.3423). Thus, these findings confirm no baseline differences in c-fos expression in the VTA with lynx1 knockout conditions.

Next, we assessed whether the absence of lynx1 alters nicotine-mediated cellular activation. Given the established role of VTA nAChRs in nicotine reinforcement (Picciotto et al., 1998), we predicted that the absence of lynx1 would lead to enhanced nicotine-induced activation of nAChRs, thereby increasing the number of cells expressing the immediate early gene c-fos. To explore this possibility, mice were injected with 0.125 mg/kg nicotine, a subthreshold dose that does not have an effect on brain reward thresholds, as previously demonstrated with intracranial self-stimulation in wildtype mice (Fowler et al., 2011). Increased c-fos immunoreactivity was found in the VTA of lynx1^{-/-} mice compared to lynx1^{+/+} littermates (**Fig 3a-b**). Stereological quantification of the density of c-fos positive cells revealed that administration of the low nicotine dose was sufficient to increase cellular activation in the lynx1^{-/-} males (**Fig 3c**) (Unpaired two-tailed t-test, $t_{(9)}=3.085$, $p=0.0130$) and females (**Fig 3d**) (Unpaired two-tailed t-test, $t_{(8)}=8.764$, $p<0.0001$), as compared to their respective sex lynx1^{+/+} mice, thereby confirming that lynx1 acts as a molecular inhibitor of nicotine's actions in the VTA.

To further understand cellular expression patterns of lynx1 with nAChRs in the VTA, we employed multiple approaches. First, RNAscope was used to initially visualize the cell-specific colocalization of lynx1 with $\alpha 4$ and $\beta 2$ nAChR subunits, since lynx1 has been shown to interact with the $\alpha 4\beta 2$ nAChR subtype *in vitro* (Miwa, 2021; Pisapati et al., 2021). We observed co-expression of lynx1 mRNA with both $\alpha 4$ (**Fig 4a**) and $\beta 2$ (**Fig 4b**) nAChR subunit mRNA. Therefore, we next used a bioinformatic approach to quantify the colocalization of *Lynx1* with multiple nAChR subunits. The raw single cell data were derived from an open-source dataset published by Macosko and

colleagues, in which they analyzed the VTA dissected from male and female wildtype mice (Langlieb et al., 2023). Single cell mRNA expression in the VTA of mice then quantified by determining the percentage of *Lynx1*-positive cells that co-express specific nAChR subunits. Interestingly, *Lynx1* was co-expressed with multiple nAChR subunits in the VTA, including $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$ and $\beta 3$. In males (**Fig 4c**), the statistical analysis of *lynx1* co-localization revealed significantly higher expression in cells containing $\alpha 4$, $\alpha 7$ and $\beta 2$ (*One-way ANOVA*, $F_{(8, 36)}=52.85$, $p<0.0001$; Post-hoc: $\alpha 4$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 5$ ($p=0.0002$), $\alpha 6$ ($p=0.0038$), $\alpha 7$ ($p<0.0001$), $\beta 3$ ($p=0.0039$), and $\beta 4$ ($p<0.0001$); $\alpha 7$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 4$ ($p<0.0001$), $\alpha 5$ ($p<0.0001$), $\alpha 6$ ($p<0.0001$), $\beta 2$ ($p<0.0001$), $\beta 3$ ($p<0.0001$), and $\beta 4$ ($p<0.0001$); $\beta 2$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 5$ ($p<0.0001$), $\alpha 6$ ($p=0.0007$), $\alpha 7$ ($p<0.0001$), $\beta 3$ ($p=0.0007$) and $\beta 4$ ($p<0.0001$)). In females (**Fig 4d**), significantly higher *lynx1* co-expression was also in cells containing $\alpha 4$, $\alpha 6$, $\alpha 7$, $\beta 2$ and $\beta 3$ (*One-way ANOVA*, $F_{(8, 45)}=105.5$, $p<0.0001$; Post-hoc: $\alpha 4$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 5$ ($p<0.0001$), $\alpha 6$ ($p<0.0001$), $\alpha 7$ ($p<0.0001$), $\beta 3$ ($p<0.0001$) and $\beta 4$ ($p<0.0001$); $\alpha 6$ compared to $\alpha 4$ ($p<0.0001$), $\alpha 7$ ($p<0.0001$), $\beta 2$ ($p<0.0001$) and $\beta 4$ ($p=0.0432$); $\alpha 7$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 4$ ($p<0.0001$), $\alpha 5$ ($p<0.0001$), $\alpha 6$ ($p<0.0001$), $\beta 2$ ($p<0.0001$), $\beta 3$ ($p<0.0001$), and $\beta 4$ ($p<0.0001$); $\beta 2$ compared to $\alpha 2$ ($p<0.0001$), $\alpha 3$ ($p<0.0001$), $\alpha 5$ ($p<0.0001$), $\alpha 6$ ($p<0.0001$), $\alpha 7$ ($p<0.0001$), $\beta 3$ ($p<0.0001$) and $\beta 4$ ($p<0.0001$); $\beta 3$ compared to $\alpha 2$ ($p=0.0481$), $\alpha 4$ ($p<0.0001$), $\alpha 7$ ($p<0.0001$), $\beta 2$ ($p<0.0001$) and $\beta 4$ ($p=0.0336$)). These findings reveal that *Lynx1* co-localizes with a variety of nAChR subunits, which can form diverse functional nAChR subtypes. Finally, we assessed the baseline expression of $\alpha 4$ and $\alpha 7$ nAChR subunits in the VTA, given their high levels of co-expression with *lynx1*. In males, increased expression of $\alpha 4$ mRNA was found in the *lynx1*^{-/-} mice compared to *lynx1*^{+/+} controls (**Fig 4e**) (Unpaired two-tailed t-test, $t_{(13)}=2.226$, $p=0.0443$), whereas no differences were found in $\alpha 7$ mRNA expression between genotypes (**Fig 4f**) (Unpaired two-

tailed t-test, $t_{(13)}=0.4354$, $p=0.6704$). In females, no differences between $lynx1^{+/+}$ and $lynx1^{-/-}$ mice were found for either $\alpha 4$ (**Fig 4g**) (Unpaired two-tailed t-test, $t_{(12)}=1.721$, $p=0.1108$) or $\alpha 7$ (**Fig 4h**) (Unpaired two-tailed t-test, $t_{(12)}=1.259$, $p=0.2318$) nAChR subunit mRNA.

To further investigate the relevance of $lynx1$ in the VTA, we then assessed whether VTA $lynx1$ knockdown would recapitulate the behavioral phenotype found in the constitutive $lynx1$ knockout mice. A shRNA viral vector targeting *Lynx1* was designed and validated for $lynx1$ mRNA knockdown in the VTA (**Fig 5a-c**) (Unpaired two-tailed t-test, $t_{(12)}=10.68$, $p<0.0001$). Male and female mice were then bilaterally microinjected with the validated AAV-shRNA $Lynx1$ -GFP knockdown vector (VTA $Lynx1^{KD}$) or scrambled control vector (VTA $Control$), and subjects were then examined for differences in nicotine intake across the dose response function. Notably, knockdown of $lynx1$ selectively in the VTA resulted in a similar behavioral phenotype as that found with the full knockout mice (**Fig 5d**) (Two-Way RM ANOVA, *Virus* $F_{(1, 18)}=10.41$, $p=0.0047$; *Dose* $F_{(4, 72)}=31.82$, $p<0.0001$; *Interaction* $F_{(4, 72)}=8.423$, $p<0.0001$). Specifically, the male VTA $Lynx1^{KD}$ mice earned a greater number of infusions at the lower reinforcing nicotine doses of 0.01 ($p<0.0001$) and 0.03 ($p<0.0001$) mg/kg/infusion compared to the VTA $Control$ mice. For the females, VTA $Lynx1^{KD}$ led to an increase in self-administration (**Fig 5e**) (Two-Way RM ANOVA, *Virus* $F_{(1, 16)}=9.673$, $p=0.0067$; *Dose* $F_{(4, 64)}=43.15$, $p<0.0001$; *Interaction* $F_{(4, 64)}=6.721$, $p<0.0001$). The post-hoc revealed that female VTA $Lynx1^{KD}$ resulted in increased responding for the nicotine doses of 0.03 ($p=0.0078$) and 0.1 ($p<0.0001$) mg/kg/infusion compared to the control group. Lever pressing behavior was next examined across the dose response function. For the males, subjects discriminated between the active and inactive lever across doses (**Fig 5f**) (Three-Way RM ANOVA, *Lever* $F_{(1, 36)}=81.80$, $p<0.0001$; *Dose* $F_{(4, 144)}=23.52$, $p<0.0001$; *Genotype* $F_{(1, 36)}=11.39$, $p=0.0018$; *Dose x Lever Interaction* $F_{(4, 144)}=16.95$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 144)}=6.348$, $p<0.0001$; *Lever x Genotype Interaction* $F_{(1, 36)}=11.24$, $p=0.0019$; *Dose x Lever x*

Genotype Interaction $F_{(4, 144)}=5.802$, $p=0.0002$). The post-hoc analysis comparing genotypes within each lever for each dose revealed a significant increase in active lever pressing for the male $VTA^{Lynx1\text{ KD}}$ mice at the doses of 0.01 ($p<0.0001$) and 0.03 ($p<0.0001$) mg/kg/infusion, as compared to the VTA^{Control} . No differences were found in the number of inactive lever presses between groups at any dose. For the females, the active lever was again preferred with low responding on the inactive lever across doses (**Fig 5g**) (Three-Way RM ANOVA, *Lever* $F_{(1, 32)}=102.4$, $p<0.0001$; *Dose* $F_{(4, 128)}=45.08$, $p<0.0001$; *Genotype* $F_{(1, 32)}=13.56$, $p=0.0008$; *Dose x Lever Interaction* $F_{(4, 128)}=23.75$, $p<0.0001$; *Dose x Genotype Interaction* $F_{(4, 128)}=9.174$, $p<0.0001$; *Lever x Genotype Interaction* $F_{(1, 32)}=7.583$, $p=0.0096$; *Dose x Lever x Genotype Interaction* $F_{(4, 128)}=3.858$, $p=0.0054$). The post-hoc analysis comparing genotypes within each lever for each dose found a significant increase in active lever pressing for the 0.03 ($p=0.0043$) and 0.1 ($p<0.0001$) mg/kg/infusion doses with the female $VTA^{Lynx1\text{ KD}}$ group compared to the VTA^{Control} . No differences in the number of inactive lever presses were found between groups at any dose. Moreover, neither group differed in their responding for saline infusions, thereby providing evidence that knockdown of *lynx1* in the VTA during adulthood did not affect general, non-contingent lever pressing behavior. Finally, to ensure that the differences found were specific to nicotine, VTA^{Control} and $VTA^{Lynx1\text{ KD}}$ mice were also examined for their ability to respond for food reward. No significant differences were found between groups in the number of food rewards earned in males (**Fig 5h**) (Two-Way RM ANOVA, *Virus* $F_{(1, 18)}=0.6995$, $p=0.4139$; *Session* $F_{(4, 72)}=47.10$, $p<0.0001$; *Interaction* $F_{(4, 72)}=1.752$, $p=0.1481$). A main effect between groups was found in females (**Fig 5i**) (Two-Way RM ANOVA, *Virus* $F_{(1, 16)}=7.082$, $p=0.0171$; *Session* $F_{(4, 64)}=38.38$, $p<0.0001$; *Interaction* $F_{(4, 64)}=0.0901$, $p=0.9852$), in which $VTA^{Lynx1\text{ KD}}$ mice earned fewer food pellets across all food training sessions together. However, no statistically significant differences were found in the post-hoc when examining for group differences within each session ($p>0.05$). These food training findings support the contention that the differences in nicotine intake were not due to altered learning capabilities nor baseline activity patterns within a session.

DISCUSSION

The current studies revealed a distinct functional role of the endogenous negative allosteric modulator, lynx1, in mediating nicotine reinforcement. Based on the known function of lynx1 from *in vitro* studies and expression of lynx1 in the VTA (Nichols et al., 2014; Parker et al., 2017), we predicted that the absence of the lynx1 protein would lead to an increase in nicotinic receptor signaling in the reward circuit in the presence of the agonist, nicotine. In line with this prediction, our data reveal that lynx1 in the VTA limits nicotine reinforcement at lower, reinforcing doses, as either global lynx1 knockout or VTA-specific knockdown increased nicotine intake. As such, inhibiting lynx1's function serves to increase the reinforcing value of nicotine, resulting in increased nicotine consumption.

Lynx1 as a nAChR modulator of cholinergic behaviors

Lynx1 has been characterized as a 'molecular brake' of cholinergic signaling, with its negative modulating effects documented both *in vivo* and *in vitro* (Miwa et al., 2006). Lynx1 is thought to be selective in this modulatory capacity, as there is no evidence that lynx1 associates with other receptors. In preclinical animal studies, lynx1 has been shown to affect associative and motor learning, sensorimotor gating, and the anti-nociceptive effects of nicotine (Miwa et al., 2006; Miwa and Walz, 2012; Nissen et al., 2018; Sherafat et al., 2021). The specific lynx1-mediated associative and motor learning effects may be in part due to cholinergic action at the $\alpha 7$ nAChR, as intranasal administration of water soluble lynx1 reversed learning deficits induced by an $\alpha 7$ nAChR antagonist (Shenkarev et al., 2020). Interestingly, we found that constitutive knockout or VTA-specific knockdown of lynx1 in mice did not grossly alter learning in the operant food self-administration paradigm, although a minor difference was found in the female VTA-specific lynx1

knockout condition overall even though no differences were evident on individual testing sessions. Thus, these data suggest that lynx1 may specifically regulate only some facets of learning and memory processes. This contention is further supported by previous findings, which revealed that lynx1 did not alter cognitive flexibility in a reversal task for food reward (Sherafat et al., 2021) or contextual fear learning (Miwa et al., 2006) under constitutive knockout conditions. Given the use of a constitutive knockout mouse model, it is necessary to consider that the protein's absence throughout development may alter endogenous signaling mechanisms, potentially leading to compensatory mechanisms across the lifespan. Adult lynx1^{-/-} mice have been shown to exhibit subtle visual acuity improvements compared to their wildtype counterparts (Morishita et al., 2010). Despite subtle effects on visual acuity, the lynx1^{-/-} mice exhibit normal dendritic complexity and spine density of primary visual cortex neurons (Sajo et al., 2016). Thus, the differences in nicotine self-administration in the current study are not likely confounded by any differences in visual cue processing. Lynx1 has also been linked to neuroprotection, as aged lynx1^{-/-} mice have significant dorsal striatum degeneration (Kobayashi et al., 2014), which could have critical implications for reinforcement and drug seeking behaviors. However, this neuroprotective role of lynx1 is evident only after mice reach at least 13 months of age (Kobayashi et al., 2014), which is older than the age of the mice used in this study. Prior studies have also shown that lynx1 does not alter general baseline behavior; for instance, phenotypic differences were not found in the elevated plus maze or open field test (Miwa et al., 2006). Together, these findings indicate that lynx1 shapes cholinergic neural signaling to impact behaviors associated with specific facets of neuropsychiatric symptomology.

Interaction of lynx1 with nAChRs for nicotine reinforcement

In our studies, we found that lynx1 co-localizes with multiple nAChR subunits in the VTA. Interestingly, the highest percentage of co-localization was observed with the $\alpha 7$ nAChR subunits, and prior *in vitro* studies have shown that lynx1 exhibits a binding preference for $\alpha 7$ nAChRs compared to $\alpha 3\beta 4$ nAChRs (Pisapati et al., 2021) and mediate $\alpha 7$ -based calcium levels in cortical neurons (Miwa et al., 2006). Although $\alpha 7$ nAChRs have been implicated in glutamatergic synaptic strength onto dopaminergic neurons in the VTA (Mansvelder and McGehee, 2000; Jin et al., 2011), $\alpha 7$ knockout mice do not show deficits in acquiring nicotine self-administration (Pons et al., 2008), which suggests that our observed differences in nicotine intake were not likely due to $\alpha 7$ nAChR-mediated signaling. Although it is still possible that removal of lynx1 could lead to an $\alpha 7$ -dependent component of nicotine's actions that would otherwise be difficult to detect when suppressed by lynx1. Rather, an extensive literature has implicated the $\alpha 4\beta 2$ -containing nAChRs in mediating nicotine reinforcement in the VTA (Picciotto et al., 1998; Pons et al., 2008; Gotti et al., 2010; Liu et al., 2012), and as previously noted, lynx1 interacts with $\alpha 4\beta 2$ nAChRs to suppress membrane expression and to shift the stoichiometry to the lower affinity subtype (Nichols et al., 2014). This is consistent with our current findings with lynx1 modulation being evident at low to moderate reinforcing nicotine doses. At higher nicotine doses, we have previously shown that the medial habenulo-interpeduncular pathway becomes engaged, which serves to limit nicotine intake across the descending limb of the nicotine dose-response function (Fowler et al., 2011), and lynx1 has been shown to suppress nicotine-evoked currents in the medial habenula (Miwa et al., 2006). Therefore, the lack of an effect of lynx1 at higher nicotine doses is likely due to the integration of signals from both the rewarding and aversive pathways, which together determine drug consummatory behaviors.

Sex-Specific Effects

While both male and female mice lacking lynx1 exhibited an increase in nicotine self-administration, the effect in females was limited to the more moderate dose of nicotine, whereas

males exhibited increased nicotine intake at both the low and moderate doses. The potential reasons for such sex-specific effects are likely multifaceted. Interestingly, sex hormones such as estradiol have been shown to act as positive allosteric modulators of the nAChRs, and controlling for the estrous cycle has been proposed to result in more stable conditions to assess operant behaviors (Curtis et al., 2002). However, our experiments did not control for the estrous phase to allow for more translationally applicable conditions. Thus, it is hypothetically possible that lynx1 and estradiol counteracted one another, with competing negative and positive allosteric modulation, respectively, and this was most evident at the lower nicotine dose, which would be expected to induce relatively less agonist-induced activation of the nAChRs. Another potential mechanism underlying the sex-specific effects observed in self-administration could be the increased $\alpha 4$ nAChR subunit gene expression found in lynx1^{-/-} males, which was not observed in lynx1^{-/-} females. *In vitro*, lynx1 has been shown to skew $\alpha 4\beta 2$ nAChRs to the low sensitivity, ($\alpha 4$)₃($\beta 2$)₂, stoichiometry (Nichols et al., 2014), and as such, lynx1 removal in vivo could potentially lead to greater expression of the higher sensitivity ($\alpha 4$)₂($\beta 2$)₃ stoichiometry, which is more sensitive to upregulation in response to nicotine exposure. Since the $\alpha 4$ nAChR subunit in the VTA plays a critical role in the reinforcing properties of nicotine, these effects at the nAChR receptor level could potentially explain why males had increased nicotine intake at more nicotine doses (Picciotto et al., 1998; Pons et al., 2008). Thus, additional studies are necessary to more clearly disentangle the sex-specific effects found at specific self-administered nicotine doses.

Considerations and limitations

It is relevant to note that while we examined the acute effects of nicotine in the c-fos study, the actions of nicotine under chronic self-administration conditions may differ consistent with changes in nAChR expression in the VTA (Akers et al., 2020), a contention that will need to be investigated in future studies. Another consideration is that we did not distinguish the lynx1-positive neuronal

populations activated by nicotine within the VTA. Nicotinic acetylcholine receptors are expressed on both dopaminergic and GABAergic neurons, where they differentially regulate reward-related circuit activity. In particular, $\alpha 4\beta 2$ - and $\alpha 6$ -containing receptors on dopamine neurons contribute substantially to nicotine-evoked dopamine release and reinforcement (Picciotto et al., 1998; Pons et al., 2008; Gotti et al., 2010; Liu et al., 2012). However, nAChRs are also expressed on VTA GABAergic neurons, where nicotine can alter inhibitory tone and indirectly regulate dopaminergic output (Mansvelder and McGehee, 2000; Jin et al., 2011; Akers et al., 2020). Because *Lynx1* functions as a negative allosteric modulator of multiple nAChR subtypes, including $\alpha 4\beta 2$ -, $\alpha 6$ -, and $\alpha 7$ -containing receptors (Miwa, 2021; Pisapati et al., 2021), the enhanced nicotine-induced *c-fos* expression observed in *Lynx1* knockout mice could reflect increased nicotinic signaling in one or both neuronal populations. Therefore, while our findings demonstrate enhanced VTA activation following *Lynx1* deletion, additional studies assessing *c-fos* expression in a cell-specific manner will be necessary to define the precise neuronal population(s) mitigating these effects.

Conclusions

These findings elucidate a precise functional impact for *lynx1* in the VTA, in which the presence of *lynx1* was found to dampen nicotine reinforcement. Notably, both global knockout and VTA-specific knockdown of *lynx1* impacted the dose response curve, leading to lower doses of nicotine being more reinforcing. This suggests a previously unappreciated endogenous mechanism that regulates nicotine sensitivity at the behavioral level. These results provide a more nuanced understanding of the underlying mechanisms contributing to nicotine addiction. Thus, understanding the cell-specific and nAChR subtype-specific roles of *lynx1* in regulating cognitive processes and reinforcement-related behaviors may lead to the development of approaches that leverage allosteric modulation of cholinergic signaling, with promising implications for future therapeutic approaches.

569

570 Data Availability Statement

571 The data that support the findings of this study will be openly available at OSF
572 (<https://osf.io/dashboard>), upon manuscript acceptance.

573

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579

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581 J.M.M. is the founder and holds shares in Ophidion, Inc., a biotechnology company. C.D.F. serves
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586

587 Author Contributions

588 C.D.F. oversaw the project administration, resources, and experimental methodology. Y.S., M.B.,
589 K.N.L., J.P.F., V.L., and C.D.F. conducted the formal analysis, investigation, data curation, data
590 validation, data visualization, and writing of the original draft. A.R.Z. and J.M.M provided technical

591 and conceptual guidance for experimental interpretation. All authors (Y.S., M.B., K.N.L., J.P.F.,
592 V.L., H.M., A.R.Z., J.M.M., C.D.F.) participated in writing and revising the manuscript and
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FIGURE LEGENDS

Figure 1. Lynx1 mediates the reinforcing effects of lower nicotine doses. (a) Male $lynx1^{-/-}$ mice earned a greater number of nicotine infusions at the 0.01 and 0.03 mg/kg/infusion doses compared to wildtype $lynx1^{+/+}$ littermates (n=12-13/genotype). Groups did not differ at the control (0 mg/kg/infusion) dose. (b) Female $lynx1^{-/-}$ mice earned a greater number of nicotine infusions only at the 0.03 mg/kg/infusion dose compared to wildtype $lynx1^{+/+}$ littermates (n=9/genotype). Groups did not differ at the control vehicle (0 mg/kg/infusion) dose. (c) In analyzing active lever presses, male $lynx1^{-/-}$ mice maintained a significantly higher level of active lever responses at the 0.01 mg/kg/infusion dose than male $lynx1^{+/+}$ mice, whereas no differences were found in inactive lever pressing across doses. (d) Female $lynx1^{-/-}$ mice exhibited a greater number of active lever presses only at the 0.03 mg/kg/infusion nicotine dose, with no differences between genotypes for inactive lever presses, compared to the female $lynx1^{+/+}$ mice. Data represent mean $\pm$ standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Lynx1 does not alter operant learning for a food reinforcer. (a) Male $lynx1^{+/+}$ and $lynx1^{-/-}$ mice exhibit similar operant self-administration for a food reward across training sessions (n=12-13/genotype). (b) Female $lynx1^{+/+}$ and $lynx1^{-/-}$ mice exhibit similar operant food self-administration across training sessions (n=9/genotype). (c) Male $lynx1^{+/+}$ and $lynx1^{-/-}$ mice exhibit similar food training behavior with preferential responding on the active lever and low responding on the inactive lever across sessions. (d) Female $lynx1^{+/+}$ and $lynx1^{-/-}$ mice also exhibit preferential responding on the active lever and low responding on the inactive lever across sessions. Data represent mean $\pm$ SEM. *** $p < 0.001$ active vs. inactive for $lynx1^{-/-}$, **** $p < 0.0001$ active vs. inactive within genotype for both $lynx1^{+/+}$ and $lynx1^{-/-}$.

Figure 3. *Lynx1* mediates cellular activation in response to nicotine in the ventral tegmental area (VTA). (a-b) Representative images of c-fos immunoreactivity in the VTA from *lynx1*^{+/+} (top) and *lynx1*^{-/-} (bottom) mice following a low dose of nicotine (0.125 mg/kg). Males are shown in panels (a) and females in (b). Scale bars=50μm. (c-d) Cell density quantified using unbiased stereology reveals increased c-fos expression in the VTA of *lynx1*^{-/-} mice compared to *lynx1*^{+/+} mice for males (c; n=5-6/genotype) and females (d; n=5/genotype). Data represent mean ± SEM. *p<0.05, ****p<0.0001.

Figure 4. *Lynx1* colocalizes with nicotinic acetylcholine receptor (nAChR) subunits in the VTA. (a-b) Fluorescent photomicrographs show RNAscope colocalization of *lynx1* mRNA with nicotinic acetylcholine receptor (nAChR) subunit mRNA. Scale bars=50μm. (a) Expression of *lynx1* (green, left), α4 nAChR subunit (red, center), and merged (co-expression, right) in the VTA. (b) Expression of *lynx1* (green, left), β2 nAChR subunit (red, center), and merged (co-expression, right) in the VTA. (c) In males (n = 5), single cell data demonstrate colocalization of nAChR subunits in *lynx1* cells of the VTA. # denotes α4 compared to α2 (p<0.0001), α3 (p<0.0001), α5 (p<0.001), α6 (p<0.01), α7 (p<0.0001), β3 (p<0.01), and β4 (p<0.0001). ^ denotes α7 compared to α2 (p<0.0001), α3 (p<0.0001), α4 (p<0.0001), α5 (p<0.0001), α6 (p<0.0001), β2 (p<0.0001), β3 (p<0.0001), and β4 (p<0.0001). @ denotes β2 compared to α2 (p<0.0001), α3 (p<0.0001), α5 (p<0.0001), α6 (p<0.001), α7 (p<0.0001), β3 (p<0.001) and β4 (p<0.0001). (d) In females (n=6), *lynx1* expression was evident in cells co-expressing various nAChRs in the VTA. # denotes α4 compared to α2 (p<0.0001), α3 (p<0.0001), α5 (p<0.001), α6 (p<0.0001), and α7 (p<0.0001). & denotes α6 compared to α4 (p<0.0001), α7 (p<0.0001), β2 (p<0.0001) and β4 (p<0.05). ^ denotes α7 compared to α2 (p<0.0001), α3 (p<0.0001), α4 (p<0.0001), α5 (p<0.0001), α6 (p<0.0001), β2 (p<0.0001), β3 (p<0.0001), and β4 (p<0.0001). @ denotes β2 compared to α2 (p<0.0001), α3

($p < 0.0001$), $\alpha 5$ ($p < 0.0001$), $\alpha 6$ ($p < 0.0001$), $\alpha 7$ ($p < 0.0001$), $\beta 3$ ($p < 0.0001$) and $\beta 4$ ($p < 0.0001$). + denotes $\beta 3$ compared to $\alpha 2$ ($p < 0.05$), $\alpha 7$ ($p < 0.0001$), $\beta 2$ ($p < 0.0001$) and $\beta 4$ ($p < 0.05$). **(e-h)** RT-qPCR of $\alpha 4$ and $\alpha 7$ nAChRs in $lynx1^{+/+}$ and $lynx1^{-/-}$ VTA, with expression normalized to β -actin. In males ($n=6-9$ /genotype), $\alpha 4$ nAChR subunit expression was found to be significantly increased in the $lynx1^{-/-}$ mice compared to the $lynx1^{+/+}$ controls **(e)**, whereas no differences were found in $\alpha 7$ nAChR subunit expression **(f)**. In females ($n=5-9$ /genotype), no phenotype differences were found with expression of either $\alpha 4$ **(g)** or $\alpha 7$ **(h)** nAChR subunits. Data represent mean $\pm$ SEM. * $p < 0.05$.

Figure 5. Site-specific knockdown of *Lynx1* in the VTA increases nicotine reinforcement in a sex-specific manner. **(a)** Mice were bilaterally injected with a *Lynx1* shRNA knockdown virus or a scrambled control vector into the VTA. Illustration created with Biorender.com. **(b)** Representative image of virus expression (GFP, green) in the VTA. DAPI: blue. IPN: interpeduncular nucleus. Scale bar=50 μ m **(c)** Injection of the *Lynx1* shRNA knockdown virus resulted in a significant decrease in *Lynx1* mRNA expression in the VTA ($n=6-8$ /group). **(d-e)** The number of nicotine infusions was examined across a full dose-response function. **(d)** Male VTA^{*Lynx1* KD} mice self-administered higher levels of nicotine at the 0.01 and 0.03 mg/kg/infusion doses compared to the VTA^{Control} group ($n=9-11$ /group). No differences were found with saline control (0 mg/kg/infusion) between groups. **(e)** Female VTA^{*Lynx1* KD} mice self-administered higher levels of nicotine at the 0.03 and 0.1 mg/kg/infusion doses compared to the VTA^{Control} group ($n=8-10$ /group). No group differences were found with saline control (0 mg/kg/infusion). **(f-g)** Active and inactive lever pressing was examined across the nicotine dose response function. **(f)** Male VTA^{*Lynx1* KD} mice exhibited a significant increase in active lever pressing compared to the control group at the 0.01 and 0.03 mg/kg/infusion nicotine doses. No group differences were found in inactive lever presses at any dose. **(g)** Female VTA^{*Lynx1* KD} mice significantly pressed the active lever more than the control group at the 0.03 and 0.1 mg/kg/infusion nicotine doses. No group

differences were found in inactive lever presses at any dose. (h) Knockdown of lynx1 in the VTA did not alter operant responding for food rewards in males. (i) In females, knockdown of lynx1 in the VTA led to an overall main effect with decreased responding compared to the control virus, but no session-specific differences were evident in the post-hoc test. Data represent mean $\pm$ SEM. ** $p < 0.01$; **** $p < 0.0001$.

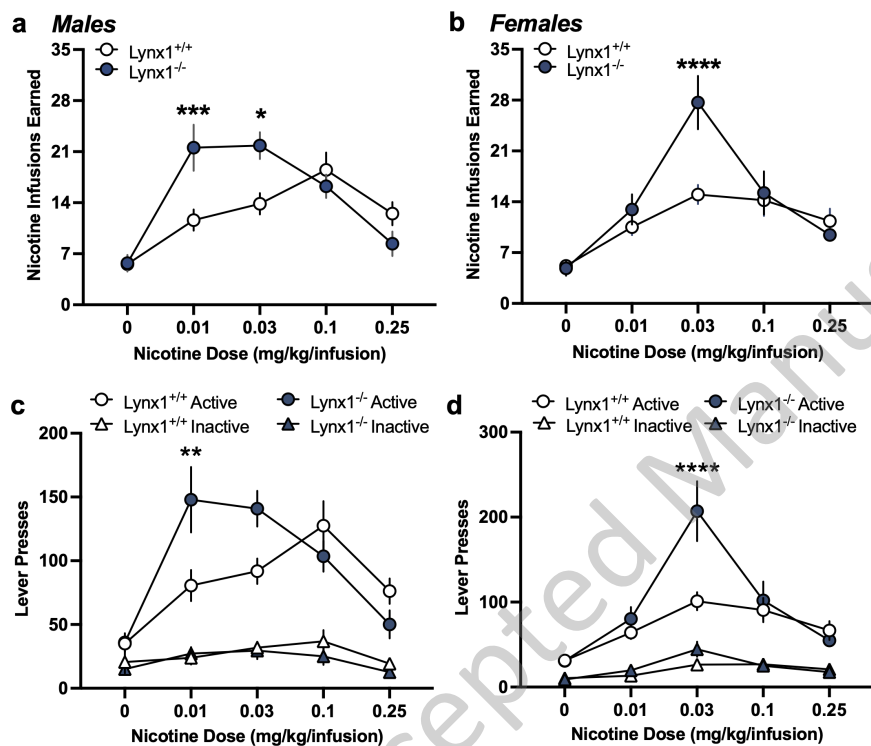


Figure 1

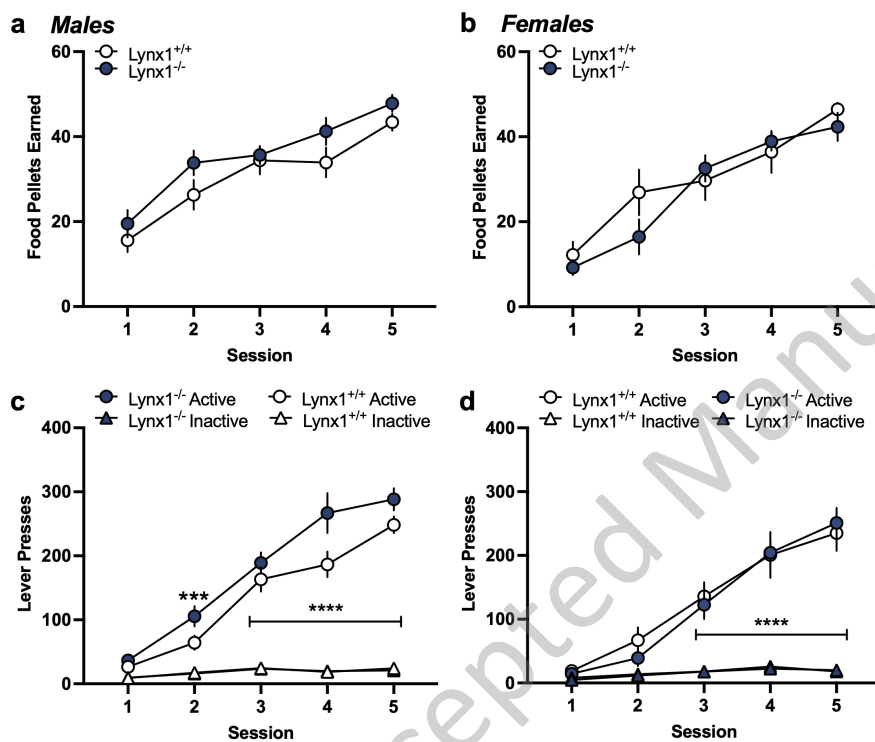


Figure 2

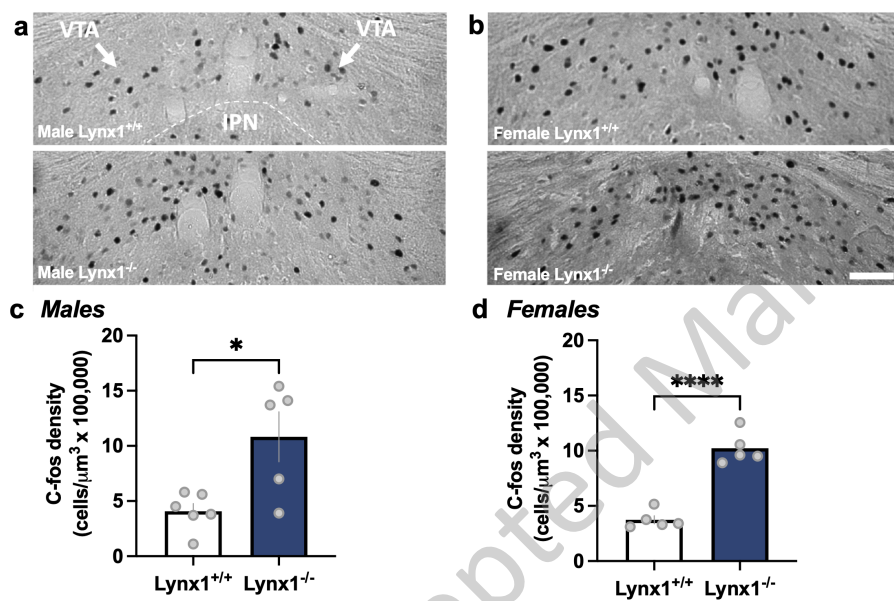
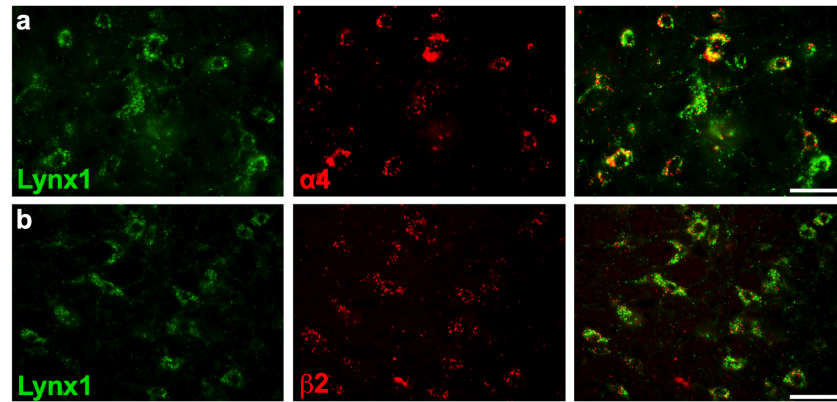
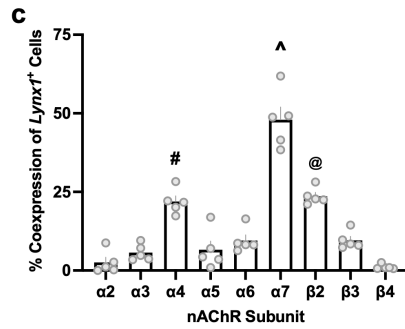


Figure 3



Males



Females

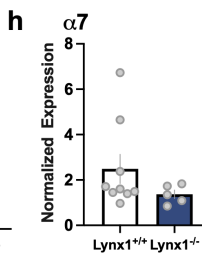
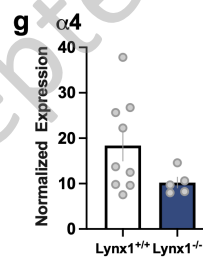
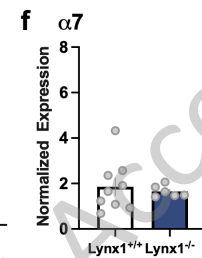
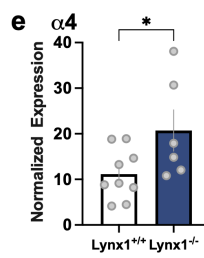
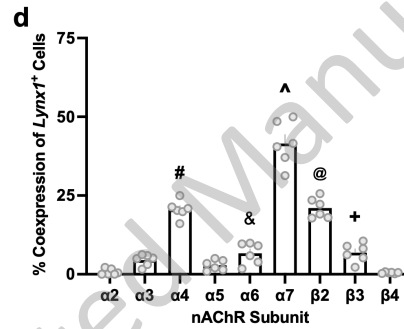


Figure 4

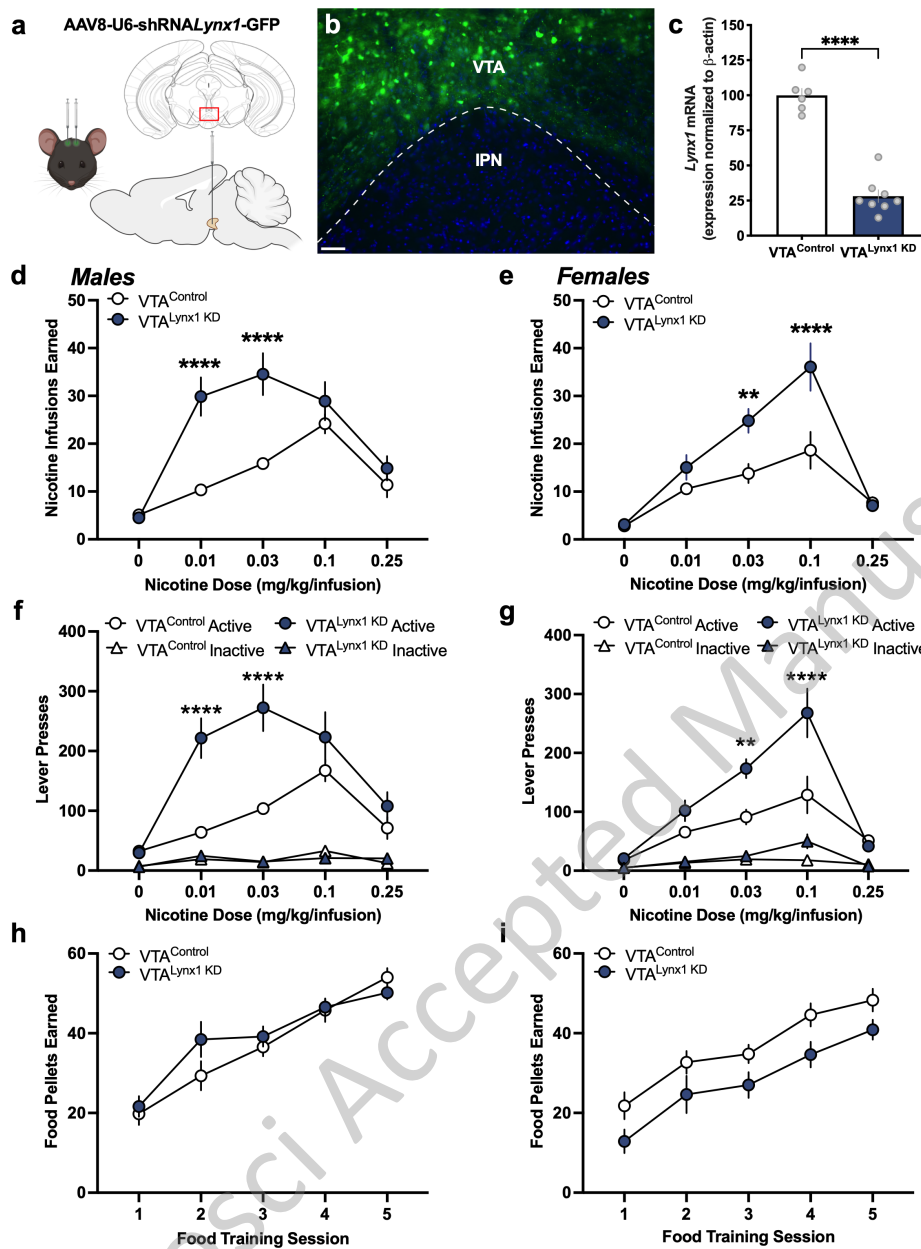


Figure 5