

Histamine H₃ receptors decrease dopamine release in the ventral striatum by reducing the activity of striatal cholinergic interneurons

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Abstract

Histamine H₃ receptors are widely distributed G_i-coupled receptors whose activation reduces neuronal activity and inhibits release of numerous neurotransmitters. Although these receptors are abundantly expressed in the striatum, their modulatory role on activity-dependent dopamine release is not well understood. Here, we observed that histamine H₃ receptor activation indirectly diminishes dopamine overflow in the ventral striatum by reducing cholinergic interneuron activity. Acute brain slices from C57BL/6 or channelrhodopsin-2-transfected DAT-cre mice were obtained, and dopamine transients evoked either electrically or optogenetically were measured by fast-scan cyclic voltammetry. The H₃ agonist α -methylhistamine significantly reduced electrically-evoked dopamine overflow, an effect blocked by the nicotinic acetylcholine receptor antagonist dihydro- β -erythroidine, suggesting involvement of cholinergic interneurons. None of the drug treatments targeting H₃ receptors affected optogenetically evoked dopamine overflow, indicating that direct H₃-modulation of dopaminergic axons is unlikely. Next, we used qPCR and confirmed the expression of histamine H₃ receptor mRNA in cholinergic interneurons, both in ventral and dorsal striatum. Activation of H₃ receptors by α -methylhistamine reduced spontaneous firing of cholinergic interneurons in the ventral, but not in the dorsal striatum. Resting membrane potential and number of spontaneous action potentials in ventral-striatal cholinergic interneurons were significantly reduced by α -methylhistamine. Acetylcholine release from isolated striatal synaptosomes, however, was not altered by α -methylhistamine. Together, these results indicate that histamine H₃ receptors are important modulators of dopamine release, specifically in the ventral striatum, and that they do so by decreasing the firing rate of cholinergic neurons and, consequently, reducing cholinergic tone on dopaminergic axons.

Keywords: Histamine, H3 receptor, dopamine, cholinergic neurons, optogenetics, striatum

Introduction

Histaminergic neurons originate in the tuberomammillary nucleus in the posterior hypothalamus (Wilcox and Seybold, 1982; Panula et al., 1984; Watanabe et al., 1984; Wouterlood et al., 1986) and project throughout most areas of the brain, including the dorsal and ventral striatum (Watanabe et al., 1984; Steinbusch et al., 1986). Of the four histamine receptors known to date, histamine H₁, H₂ and H₃ receptors are densely expressed in the striatum (Palacios et al., 1979; Ruat et al., 1990; Pollard et al., 1993; Pillot et al., 2002), and activation of these receptors has profound regulatory effects on signaling by the diverse components of striatal circuitry (Bolam and Ellender, 2015). Histamine H₃ receptors serve a predominantly inhibitory role by signaling through G_i protein (Arrang et al., 1983), and have a high degree of constitutive activity (Morisset et al., 2000). In the striatum, their activation reduce cortico-thalamic excitatory input onto medium-spiny neurons (Ellender et al., 2011), and may counteract some of the excitatory effects seen with histamine H₁ and H₂ receptor activation (Sittig and Davidowa, 2001).

Dopaminergic neurons are located mostly in the mesencephalon, which is sub-divided in the *substantia nigra* (SN) and ventral tegmental area (VTA). Neurons in the SN densely innervate the dorsal striatum, while those located in the VTA project primarily to the ventral striatum and cortex. Although there is substantial anatomical overlap between the dopaminergic and histaminergic systems, the knowledge on how these systems interact, both at the cellular and behavioral levels, is still incomplete (Ellenbroek, 2013). Histamine H₃ receptors are capable of modulating methamphetamine or amphetamine self-administration (Munzar et al., 2004; Vanhanen et al., 2015), alcohol consumption and reward (Nuutinen et al., 2011), stereotypic behavior (Kitanaka et al., 2011; Mahmood et al., 2012) and L-DOPA-induced dyskinesia (Nowak et al., 2008; but see Papathanou et al., 2014), all of which are thought to be mediated, at least in part, by striatal dopamine release. Studies show that D₁ and H₃ receptors form heteromers in the striatum and this interaction is capable of curbing the levels of phosphorylated MAPK (Moreno et al., 2014), D₁-induced accumulation of cAMP (Sanchez-Lemus and Arias-Montano, 2004) and D₁-induced reduction of dopamine release (Alfaro-Rodriguez et al., 2013). Conversely, histaminergic neurons express D₁ and D₂ receptors, whose activation promotes increased firing activity, histamine release, and wakefulness (Yanovsky et al., 2011). These results denote a concerted interplay between dopamine and histamine, leading to complex physiological and pathophysiological ramifications.

Elsewhere in the brain histamine H₃ receptors have a well described role as pre-synaptic autoreceptors and heteroreceptors, modulating release of not only histamine (Arrang et al., 1985), but also glutamate (Brown and Haas, 1999), GABA (Yamamoto et al., 1997), serotonin (Threlfell et al., 2004) and acetylcholine (Clapham and Kilpatrick, 1992), among others. However, to date, studies that investigated

the effects of histamine H₃ receptors on dopamine release have generated conflicting data, indicating either no modulation (Fox et al., 2005; Giannoni et al., 2010; Aquino-Miranda et al., 2015), or decreased dopamine release upon activation of H₃ receptors (Schlicker et al., 1993). This could be due to regional differences in the effect of H₃ receptors in different parts of the striatum or to the fact that H₃ receptors act indirectly to regulate dopamine release, something that could be missed depending on the methods used. Here, we employed a combination of pharmacological and optogenetics tools, allied to the voltammetric measurement of dopamine to investigate the hypothesis that histamine H₃ receptors modulate dopamine release in the dorsal and ventral striatum of acutely isolated brain slices. We found that activation of H₃ receptors reduces dopamine release, but only in the ventral striatum. We further discovered that this effect is achieved indirectly, via H₃ receptors expressed in cholinergic interneurons, rather than H₃ receptors located on dopaminergic axons. We anticipate that histamine H₃ ligands capable of modulating dopamine release in the ventral striatum could be good candidates for treatment of diseases where dopamine neurotransmission is perturbed, such as substance abuse and anhedonia.

Experimental procedures

Animals and reagents

All procedures were approved by the animal ethics committee (CDEA) of the Université de Montréal. C57BL/6 mice used in the assessment of electrically stimulated dopamine overflow were purchased from Charles River (Senneville, Quebec, Canada). DAT-cre mice (129/ Sv/J background) used in the optogenetics experiments were kindly donated by Dr. Zhuang from the University of Chicago (Zhuang et al., 2005). TH-EGFP/21-31 (TH-GFP) transgenic mice used for purification of dopaminergic neurons were kindly donated by Dr. Katzuto Kobayashi from Fukushima **Medical** University, Japan (Sawamoto et al., 2001). C57BL/6JRccHsd mice used for synaptosomes preparations were purchased from Envigo (Horst, Netherlands). Vglut3-cre transgenic mice (Tg(Slc17a8-icre)1Edw/SealJ, Jax strain 018147) were originally donated by Drs Robert Edwards and Rebecca Seal, and were crossed with tdTomato^{flx} mice (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, Jax stock number 007914) to generate Vglut3-tdTomato mice (homozygous for tdTomato). Animals were group-housed in Plexiglas cages with environment controlled at 23 °C and 50% humidity, food and water *ad libitum* and a light cycle of 12 hours (lights on at 6:00 am). All reagents and drugs were purchased from Sigma-Aldrich (St.-Louis, MO, USA) except when otherwise noted.

Mesencephalic virus injection to deliver Channelrhodopsin-2

Two-month old male DAT-cre heterozygous mice were anesthetized with isoflurane (AErrane™) and fixed on a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The fur on the top of the head was removed and the surgical area disinfected with iodine alcohol. Throughout the entire procedure, artificial tears (Equate™) were applied to the eyes and a heat pad was placed under the animal and kept at 37 °C. Next, 0.05 ml of bupivacaine (Marcaine™) was subcutaneously injected at the surgical site, an incision of about 1 cm made with a scalpel blade and the cranial bones exposed. Using a dental burr, two holes of approximately 1 mm diameter were drilled above the VTA (AP: - 3.3 mm, ML: ± 0.4 mm) (Franklin and Paxinos, 2008). Next, a blunt 34 G needle coupled to a water-filled polyethylene tube and a Hamilton syringe (Reno, NV, USA) was loaded with 0.9 % NaCl solution containing 1×10^{11} VGP of AAV-2 EF1 α -DIO-hChR2(H134R)-EYFP-WPRE-pA (University of North Carolina Gene Therapy Center Vector Core, Chapel Hill, NC, USA). A small air bubble (0.1 μ L) was kept at the interface between water and virus solution. The needle was slowly inserted in the VTA (DV: - 4.7 mm) and 0.4 μ L of virus solution was injected at a rate of 0.1 μ L per min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). The needle was then withdrawn 0.2 mm and the injection repeated. After the second injection, the needle

was left in its place for 10 min to allow virus diffusion. Successful injections were confirmed by the movement of the air bubble in the polyethylene tube. Then, the needle was slowly withdrawn and the procedure repeated in the contralateral side. Once bilateral injections were completed, the surgery wound was sutured and a subcutaneous injection of the anti-inflammatory carprofen (Rimadyl™, 4.4 mg/kg) was given. Animals were allowed to recover in their home cage and closely monitored for 24 hours. A second dose of carprofen was given if deemed necessary. At least three weeks after injection were allowed for virus expression, at which time experiments were performed.

Acute brain slices preparation and solutions.

Brain slicing was performed as described in Ting et al. (2014) with minor modifications. In brief, three to five months old mice were anesthetized with pentobarbital (90 mg/kg i.p.) and transcardially perfused with ice-cold N-Methyl-D-Glucamine-buffer (NMDG-buffer, in mM: NMDG 93, KCl 2.5, NaH₂PO₄ 1.2, NaHCO₃ 30, HEPES 20, Glucose 25, sodium ascorbate 5, thiourea 2, sodium pyruvate 3, MgSO₄ 10 and CaCl₂ 0.5 with pH adjusted to 7.35 with 5N HCl, 300 mOsm/kg and saturated with 95% O₂-5% CO₂). Their brain was quickly removed after gentle opening of the skull and placed in ice-cold continuously oxygenated NMDG-buffer. 300 μm thick coronal brain slices containing the most rostral portion of the striatum (Bregma coordinates: from 1.42 to 0.14 mm) were prepared with a VT1000S vibrating microtome (Leica Microsystems Inc., Nussloch, Germany) in ice-cold NMDG-buffer, and then transferred to 32 °C NMDG-buffer for 15 min. Next, slices were placed at room temperature in a custom-made submerged recovery chamber containing artificial cerebral spinal fluid (aCSF, in mM: NaCl 124, KCl 2.5, NaH₂PO₄ 1.2, NaHCO₃ 24, HEPES 5, Glucose 12.5, MgSO₄ 1.3 and CaCl₂ 2.4 pH adjusted to 7.35 with 5N NaOH, 300 mOsm/kg and saturated with 95% O₂-5% CO₂) for at least 1 hour. Slices were transferred to a custom-made submerged recording chamber superfused with aCSF (approximately 1 mL/min, gravity driven) and maintained at 32 °C with a TC-324B single channel heater controller (Warner Instruments Inc., Hamden, CT, USA). Drugs were reconstituted from stock solutions into aCSF each experimental day.

Electrochemical recordings.

Action potential-induced dopamine overflow transients were evoked either by electrical stimulation with a bipolar electrode (Plastics One, Roanoke, VA, USA) or by a blue light emitting diode (LED, Luxeon, Brantford, Ontario, Canada) connected to a 1 mm diameter optic fiber (Edmond Optics, Barrington, NJ, USA). Transients were detected by fast-scan cyclic voltammetry (FSCV) using a carbon-fiber electrode placed into either the dorsal or ventral striatum, approximately 100 μm below the surface. Carbon-fiber

electrodes were fabricated as previously described (Martel et al., 2011). Briefly, carbon fibers (Cytec Industries Inc., NJ, USA) of approximately 5 μm in diameter were aspirated into ethanol-cleaned glass capillaries (1.2 mm O.D., 60.68 mm I.D., 4 inches long; A-M Systems, WA, USA). The glass capillaries were then shaped using a P-2000 micropipette puller (Sutter Instruments, Novato, CA, USA), dipped into 90°C epoxy for 30 s (Epo-Tek 301, Epoxy Technology, MA, USA) and cleaned in hot acetone for 3 s. The electrodes were heated at 100°C for 12 h and 150°C for 5 days. Before and after usage, electrodes were cleaned with isopropyl alcohol to promote greater sensitivity. Carbon fibers were cut using a scalpel blade under direct visualization to obtain a maximal basal current in aCSF between 100 and 180 nA. Electrodes were finally selected for their sensitivity to dopamine using *in vitro* calibration with 1 μM dopamine in aCSF before each experiment. The potential of the carbon fiber electrode was scanned at a rate of 280 V/s according to a 10 ms triangular voltage waveform (-400 to 1000 mV vs Ag/AgCl reference) with a 10 Hz sampling frequency using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data were acquired using a DigiData 1440A analog to digital board converter (Molecular Devices) connected to a computer running Clampex 10 (Molecular Devices). Electrical stimuli (1 ms long monophasic pulses of 400 μA) were generated by a S-900 stimulator (Dagan Corporation, Minneapolis, MN, USA) every 2 min to evoke dopamine release for a total of 10 min. Recordings in which peak dopamine overflow showed deviation greater than 20% between the first and the fifth response to single stimulation were considered unstable and not used for further analyses (n = 6). Next, single pulse stimuli were intercalated with paired pulses (100 Hz) every 2 min, and a baseline recorded for 12 min. Slices were then superfused with aCSF containing the H₃ receptor agonist α -methylhistamine (1 μM , Tocris, Bristol, United Kingdom), the H₃ receptor inverse agonist thioperamide (10 μM , Tocris) or vehicle for 48 min. All values obtained thereafter were transformed to the baseline average. A subset of slices was recorded in presence of the nicotinic receptor antagonist dihydro- β -erythroidine (1 μM , Tocris) throughout the experiment. For the optogenetic experiments, 1 ms stimuli of 20 mW/mm² and 480 nm wave length were generated by a home-built LED controller coupled to a 1 W blue-light emitting diode attached to an optic fiber. Optical stimulation was performed near the recording site. Stimulating and recording parameters were the same as above, with the exception of paired pulses were triggered at 10 Hz instead of 100 Hz in order to accommodate the slower kinetics of channelrhodopsin-2. Recordings were done either in the dorsolateral striatum (referred here as dorsal striatum) or in the core portion of the *nucleus accumbens* (referred here as ventral striatum).

Cells preparation and Fluorescence Activated Cell Sorting (FACS)

Transgenic mice expressing the eGFP gene in monoaminergic neurons under control of the TH promoter (TH-GFP mice) and transgenic mice expressing the tdTomato gene in glutamatergic neurons under

control of the VGluT3 promoter (VGluT3-tdTomato mice) were used. Mice were anesthetized with sodium pentobarbital (90 mg/ml, i.p.) and perfused with an oxygenated (100% O₂) NMDG-buffer as described above. The brain was extracted and transferred to a dish containing the NMDG-buffer to dissect the SN and VTA, or the dorsal and ventral striatum. Each piece of tissue was incubated with 0.8 mg/ml trypsin (Gibco, Waltham, MA, USA) in an oxygenated (100% O₂) PIPES-buffered saline (in mM: 115 NaCl, 5 KCl, 4 MgCl₂, 1 CaCl₂, 25 glucose, 20 piperazine-N,N'-bis-[2-ethanesulfonic acid]) at pH 7.0 for 30 min at 30 °C. The tissue was then transferred briefly to the same buffer containing fetal bovine serum (FBS) 10% to arrest the action of trypsin. The tissue was placed for 90 min in the same oxygenated PIPES solution at room temperature, then triturated delicately with a 5 ml glass pipette and centrifuged. After this, cells were recovered by resuspension of the pellet in PBS containing 1% FBS. Finally, eGFP-positive neurons or tdTomato-positive neurons were purified by FACS and directly placed in a Trizol-Reagent solution (Ambion, Waltham, MA, USA). The samples were stored at -80 °C until RNA extraction.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Primers for the transcript variant 1 of *mus musculus*' H3 receptor (H3R, NCBI Reference Sequence: NM_133849.3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with the *Primers 3* and Vector NTI software. Primers were synthesized by Alpha DNA (Montreal, Quebec, Canada). Primers for RT-qPCR in experiments on TH-eGFP and VGluT3-tdTomato mice were as follow: H3R 5'-tgggtgccttctgcatc-3' and 5'-gaggaggcacacagtagtag-3'; (GAPDH) 5'-ggaggaaacctgccaagtatga-3' and 5'-tgaagtgcaggagacaacc-3'. Validation of the primers was carried out by comparing primer sequences to the nucleotide sequence database in GenBank using BLAST (www.ncbi.nlm.nih.gov/BLAST/). RNA extraction was performed with the Rneasy Mini kit (Quiagen, Toronto, Ontario, Canada) according to the manufacturer's instructions. The concentration and the purity of the RNA from dopaminergic and cholinergic neurons were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). Total purified RNA (40 ng) was reverse-transcribed in a total of 20 µl including 1 µl of dNTP, 1 µl of random hexamer, 4 µl of 5X buffer 5X, 2 µl of dithiothreitol (DTT), 1 µl of RNase-Out and 1 µl of the Moloney Murine Leukemia Virus reverse transcriptase enzyme (MML-V). RT-qPCR was carried out in a total of 15 µl, consisting of 3 µl cDNA, 7.5 µl SYBER green PCR master mix (Quanta Biosciences, Gaithersburg, MD, USA), 10 µM of each primer, completed to 15 µl with RNA-free water. RT-qPCR was performed on a Light Cycler 96 (Roche Diagnostics, Laval, Quebec, Canada) using the following protocol: 10 min at 95 °C; 40 cycles of 30 sec at 95 °C, 40 sec at 57 °C and 40 sec at 72 °C; 1 cycle of 15 sec at 95 °C, 15 sec 55 °C and 15 sec 95 °C. The efficiency of the reaction $E=10^{(-1/\text{slope})} - 1$ was calculated from the slope of the linear relationship between the log values of

the RNA quantity and the cycle number (Ct) in a standard curve. Calculation of relative mRNA levels was performed by using the $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001), where the Ct value of the mRNA level for H3R were normalized to the Ct value of GAPDH in the same sample. Ct values used were the averages of triplicate repeats. Melt-curves of tissue homogenate indicated specific products after H3 receptor qPCR mRNA amplification, attesting the adequate quality of the primers chosen (not shown).

Loose cell-attached electrophysiological recordings

For recordings in the ventral striatum, acute brain slices from male and female VgluT3-tdTomato mice were prepared as described above and placed in a recording chamber under an Olympus upright microscope continuously perfused with oxygenated aCSF and kept at 32 °C. In the striatum, the only population of neurons expressing VgluT3 is composed of cholinergic interneurons (Gras et al., 2002). Ventral-striatal cholinergic interneurons were identified by their fluorescence and spontaneous activity. A borosilicate glass-pulled electrode (3-6 M Ω resistance) filled with aCSF was placed nearby the target cell, and gentle negative pressure was applied to facilitate the formation of a membrane seal. Loose cell-attached voltage-clamp recordings were amplified at 10 x gain and low-pass filtered at 2 kHz in a MultiClamp 700B Amplifier, and digitized at 50 kHz in a 1440 DigiData analog-to-digital converter board. Signals were recorded with Clampex 10 and analyzed in Clampfit 10 (all from Molecular Devices). Spontaneous events were detected using a threshold greater than four standard deviations of noise. Cells with average spontaneous activity lower than 0.1 Hz and greater than 12 Hz were deemed unhealthy and discarded (n = 8). After a 5 min baseline, slices were superfused with aCSF containing α -methylhistamine (1 μ M), thioperamide (10 μ M) or vehicle for 15 min. Event frequency was averaged at 1 min intervals and transformed to the average of baseline (first 5 min) to yield the normalized event frequency, an indicator of how frequency changes over time. Instant event frequencies of the last 5 min of the experiment were broken into 0.5 Hz bins to define frequency/activity histograms. These values were then transformed to the total number of events during those 5 min to yield the relative event frequency, an indicator of how the patterns of firing frequencies are affected by each drug treatment.

Recordings in the dorsal striatum were performed in similar conditions, with the exception that slices were obtained from male C57BL/6 mice. Cholinergic interneurons were identified by their morphology (i.e. scarce, evenly distributed, very large neurons) and spontaneous firing when in loose cell-attached configuration.

Whole-cell electrophysiological recordings

Slices were obtained from male C57BL/6 mice and placed in a recording chamber as described above. Cholinergic interneurons were selected by their morphology. A pipette filled with solution containing, in mM; 2.25 KCl, 125.3 KMeSO₃, 10 HEPES, 0.1 EGTA acid, 1 MgCl₂, 2 MgATP, 0.5 Na-GTP, 5 Na₂-phosphocreatine, 2 Na-pyruvate, 2 malate, neurobiotin, pH 7.2 with KOH (295 mOsm adjusted with KMeSO₃) was placed next to the target cell. Gentle negative pressure was applied to the pipette to induce the formation of a tight membrane seal. After obtaining whole-cell configuration, the identity of cholinergic interneurons was confirmed by their electrophysiological characteristics: cell capacitance between 60 and 150 pF, resting membrane potential around -55 mV and occurrence of spontaneous action-potentials followed by large, transient, hyperpolarization periods. Next, about 5-7 min were elapsed to allow cell stabilization. A second pipette connected to a Picospritzer III (Parker Hannifin, Cleveland OH, USA) and containing either aCSF alone or aCSF with α -methylhistamine (10 μ M) was placed in the vicinity of the cell being recorded. Next, resting membrane potential and spontaneous activity were recorded in current clamp (I_0) mode for a period of 1 min. Then, drug treatment was delivered by applying 2 PSI for 30s to the drug-containing pipette. Resting membrane potential and spontaneous activity were measured during the 30s drug ejection and for additional 30s (1 min total). Action potentials were identified using a peak template in Axograph X (Axon Instruments, Foster City, CA, USA). Cell neurochemical identity was confirmed by co-immunostaining the neurobiotin-filled cells for streptavidin and choline acetyltransferase (ChAT).

Immunohistochemistry and imaging

Hemisected brains from the above experiments were immediately fixed in 4% paraformaldehyde solution for at least 7 days at 4 °C. Coronal slices (50 μ m thick) containing the striatum or mesencephalon were prepared in a vibrating microtome (Leica) and placed in PBS. Striatal and mesencephalic slices from virus-injected DAT-cre mice had non-specific sites blocked with goat serum, and were incubated overnight with mouse anti-TH antibody (1:1000, Millipore, Billerica, MA, USA) and rabbit anti-GFP antibody (1:2000, Cedarlane, Burlington, Ontario, Canada). Slices from VGluT3-tdTomato mice had non-specific sites blocked with donkey serum, and were subsequently incubated with rabbit anti-RFP antibody (1:1000, VWR, Radnor PA, USA) and goat anti-ChAT antibody (1:250 Millipore, Etobicoke Ontario, Canada). Slices containing neurobiotin injected cells were blocked with donkey serum and incubated with streptavidin 546 (1:500) and goat anti-ChAT (1:250). Pairs of secondary antibodies (respectively goat Alexa 546 anti-mouse and goat Alexa 488 anti-rabbit, or donkey Alexa 555 anti-rabbit and donkey Alexa 488 anti-goat, or Alexa 488 anti-goat alone, all purchased from Invitrogen, Carlsbad, CA, USA) were incubated for 2 h to reveal specific immunostaining. Images of 5 to 10 μ m depth were obtained using an Olympus FV1000 confocal microscope using a 20 x water immersion objective and 488 and 543 nm laser

excitation. Where appropriate, cells deemed positive for each signal were manually counted using the Cell Counter plug-in in ImageJ software (NIH, Bethesda, MD, USA). Percentages of overlapping counts were used as indication of signal co-localization.

Synaptosome preparation and [³H]-acetylcholine release assay

The synaptosome release assay was performed using the methods of Grady et al. (2001) with minor modifications. Mice were killed by cervical dislocation, the brain was rapidly removed, and the dorsal and ventral striata were dissected on ice using a mouse brain matrix and a punch tool. The dorsal and ventral striata from one mouse were separately homogenized in a glass-teflon homogenizer by 16 strokes by hand in 500 µl of ice-cold 0.32 M sucrose buffered to pH 7.5 with 5 mM HEPES. The homogenate, combined with two 500 µl rinses of the homogenizer, was centrifuged at 12000 g for 20 min at 4 °C and the resulting pellet was used in the release assay. The synaptosome pellet was resuspended in 400 µl of superfusion buffer (in mM: 128 NaCl, 2.4 KCl, 3.2 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 HEPES pH 7.5, 10 glucose, 0.1 % BSA) and incubated for 30 min at 37 °C with 8 µCi [³H]choline (0.25 µM). Aliquots of 40 µl were distributed to parallel filters and superfused in room temperature with superfusion buffer at 0.8 ml/min. Superfusion was continued for ten min, and release was stimulated using an isotonic 20 mM potassium solution (20 mM K⁺). Varying concentrations of the drugs of interest were present in the superfusion buffer after five min of superfusion. The perfusate was collected on 96-well plates in 10 second (~130 µl) fractions, OptiPhase Supermix scintillation cocktail (100 µl/well; PerkinElmer, Boston, MA, USA) was added, and radioactivity was measured with liquid scintillation counting (1 min per well; 1450 MicroBeta TriLux; Wallac, Turku, Finland). Drug effects on basal [³H]acetylcholine release were determined by summing the radioactivity (counts per min, CPM) released during the six fractions (one min) immediately before K⁺ stimulation. Drug effects on K⁺-stimulated [³H]acetylcholine release were determined by summing the radioactivity released during six fractions beginning from the onset of the K⁺ stimulation. The instrument background level of 10 CPM (10–20 % of basal release) was subtracted from all fractions. All radioactivity measurements were normalized to the wet tissue weight of the samples, and results are presented as CPM/mg tissue.

Statistical analysis.

Statistical comparisons were carried out with Prism 6 (GraphPad Software Inc. La Jolla, CA, USA); significance level was set at 0.05. All data samples were tested for normal distribution to choose between parametric versus non parametric statistics and to represent population parameters either as mean ± standard error (SEM) or as median with percentile range; figure legends state the specific test employed in each case. To estimate the rate of dopamine re-uptake, a single exponential model was fitted to the

single pulse evoked dopamine transients from the peak to the end of the signal (Yorgason et al., 2011); the down stroke of the transient is known to represent the clearance of dopamine by concurrent reuptake and diffusion. The model had a good fit to the data [R^2 median with 25 and 75 % percentiles: 0.9837 (0.9606-0.9933)].

Results

We first examined electrically-evoked dopamine overflow in the ventral and dorsal striatum in order to provide a baseline against which to compare the effects of histamine H₃ receptor activation. Representative voltammograms and traces of dopamine overflow peaks evoked by single electrical pulses and paired pulses are depicted in Figure 1 and Table 1. Oxidation and reduction peaks were respectively detected at approximately +345 and -180 mV, and the maximal amplitude and time course of the detected electroactive substance was greatly enhanced by the dopamine transporter blocker nomifensine (5 μM, not shown), used here as a positive control, confirming the identity of the electroactive molecule as dopamine. Average single-pulse evoked dopamine overflow in the dorsal striatum was 0.39 ± 0.04 μM, with a paired-pulse ratio of 0.06 ± 0.01 and peak half-life of 0.43 ± 0.05 s (Table 1, Fig. 1A-1C). In the ventral striatum, dopamine overflow evoked by single pulses averaged 0.31 ± 0.03 μM, with a paired pulse ratio of 0.26 ± 0.03 and peak half-life of 0.66 ± 0.05 s (Table 1, Fig. 1D-1F). Although there were no significant differences in dopamine overflow between dorsal and ventral striatum ($t_{(73)} = 1.573$, $P = 0.1201$, Table 1), the paired-pulse ratio and peak half-life were significantly greater in the ventral striatum (paired pulse ratio $t_{(73)} = 4.679$, $P < 0.0001$ and peak half-life $t_{(70)} = 3.254$, $P = 0.0018$, Table 1). The difference in paired pulse ratio observed here is in line with previously published studies (i.e.: Zhang et al., 2009) and could be due to a lower probability of release in the ventral compared to the dorsal striatum. It should be noted, however, that dopamine overflow levels in dorsal striatum is generally found to be higher than in the ventral striatum (i.e.: Zhang et al., 2009; Calipari et al., 2012) whereas, in the present study, although the tendency was in this direction, there were no significant differences. Our finding of a longer peak half-life in the ventral compared to the dorsal striatum is in line with previous observations of lower uptake rates in the ventral striatum (Calipari et al., 2012).

Histamine H₃ receptor activation reduces dopamine release in the ventral striatum, but not in the dorsal striatum.

We next examined the effects of the histamine H₃ receptor agonist α-methylhistamine (1 μM) and the inverse agonist thioperamide (10 μM) on dopamine overflow evoked by electrical stimulation of dorsal and ventral striata. Dopamine overflow evoked by single electrical pulses were stable through the experiment (Fig 1G and 1H, open circles). In the dorsal striatum, application of either α-methylhistamine or thioperamide failed to alter dopamine overflow evoked by single electrical pulses (two-way RM ANOVA effect of treatment: $F_{(2, 26)} = 1.652$, $P = 0.2112$, Fig 1G). In the ventral striatum, however, α-methylhistamine significantly reduced dopamine overflow evoked by single electrical pulses (two-way RM ANOVA effect of treatment: $F_{(3, 42)} = 2.912$, $P = 0.0454$, Fig 1H, red circles). While thioperamide did

not affect dopamine overflow on its own (Fig 1H, black circles), it blocked the effects of α -methylhistamine (Fig 1H, magenta circles).

Histamine H₃ receptor mRNA is expressed by striatal cholinergic interneurons, but not by mesencephalic dopaminergic neurons.

We considered two possible sites for histamine H₃ receptor-mediated reduction of dopamine release. First, since numerous studies indicate that these receptors are found in axons and act as hetero-receptors capable of decreasing neurotransmitter release (Clapham and Kilpatrick, 1992; Yamamoto et al., 1997; Brown and Haas, 1999; Threlfell et al., 2004), we hypothesized that these receptors are expressed by dopamine neurons and reduce dopamine release by directly inhibiting dopaminergic axon terminals. Second, since electrical stimulation of the striatum causes the release of acetylcholine which, in turn, activates nicotinic receptors present on dopaminergic axon terminals and boosts dopamine release (Cachope et al., 2012; Threlfell et al., 2012), we hypothesized that activation of histamine H₃ receptors on cholinergic interneurons could result in their inhibition and decreased cholinergic tone on dopaminergic axon terminals, hence indirectly reducing dopamine release. We started investigating these hypotheses by quantifying expression of histamine H₃ mRNA in mesencephalic dopaminergic neurons and striatal cholinergic interneurons. Male and female TH-GFP mice were used for FACS purification of dopaminergic neurons. Cholinergic interneurons were purified by FACS from VGluT3-tdTomato mice. RT-qPCR on cell-sorted samples indicated that H₃ receptor mRNA is expressed at similar levels by VGluT3-tdTomato neurons harvested from the dorsal and ventral striatum (Table 2, $t_5 = 1.100$, $P = 0.3315$). In contrast, no amplification products of H₃ receptor mRNA were detected in TH-GFP neurons harvested in the mesencephalon (Table 2). Given that amplification of the reference mRNA, GAPDH, was seen at similar levels in all samples (Kruskal-Wallis = 4.805, $P = 0.1916$) we concluded that H₃ receptor mRNA is absent or expressed at only very low levels by mesencephalic dopaminergic neurons. These data argue against a direct regulation of dopaminergic axon terminals by H₃ receptors.

Histamine H₃ receptors do not modulate dopamine release directly from dopaminergic axons

Electrical stimulation of brain slices causes numerous neurotransmitters to be released simultaneously, and their interaction may act as potential confounding factors on determining the site of action for a particular substance. To avoid this issue, we employed optogenetics to selectively stimulate dopaminergic axons in the ventral striatum. We hypothesized that if histamine H₃ receptors do not reside on dopaminergic axons their effect on dopamine release should be absent when dopamine release is triggered by selective stimulation of these axons. To this end we injected a viral vector containing double-inverted

open reading frame coding for channelrhodopsin-2-eYFP (ChR2-eYFP) into the VTA of DAT-cre male mice. Expression of ChR2-eYFP was limited to the mesencephalon-striatum, and restricted to tyrosine-hydroxylase (TH) containing neurons (Fig 2A, upper panels). On average, 72 ± 3 % of TH positive neurons were found to express ChR2-eYFP protein. As expected, the ventral striatum had enriched expression of ChR2-eYFP signal in dopaminergic axon terminals (Fig 2A, bottom panels). Figure 2B and 2C depict voltammograms after optical stimulation by a single-pulse of blue light (1 ms, 20 mW). Light-evoked dopamine overflow peaks averaged 0.32 ± 0.04 μ M, with a paired pulse ratio of 0.53 ± 0.03 and peak half-life of 0.89 ± 0.09 seconds. Responses were completely blocked by TTX (Fig 2 C-D), confirming they were action-potential dependent. Evoked dopamine overflow was stable for the duration of the experiment (Fig 2E). Neither the histamine H_3 agonist α -methylhistamine nor its inverse-agonist thioperamide altered optogenetically evoked dopamine release triggered by single light pulses (two-way RM ANOVA effect of treatment: $F_{(2, 21)} = 0.5555$, $P = 0.5820$, Fig 2E) In addition, none of the drugs affected the paired-pulse ratio or peak-decay kinetics. These results indicate that histamine H_3 receptors do not modulate dopamine release by acting directly on dopaminergic axon terminals.

Modulation of ventral-striatal dopamine release by histamine H_3 receptors is mediated by acetylcholine

To test if histamine H_3 receptor-inhibition of electrically-evoked dopamine release is mediated by acetylcholine, we electrically stimulated ventral striatal slices in presence of the nicotinic acetylcholine receptor blocker dihydro β -erythroidine (DH β E, 1 μ M). Figures 3A and 3B illustrate voltammograms in absence and presence of DH β E. Blockade of high affinity nicotinic receptors reduced dopamine overflow to about 37 ± 7 % of its initial value (two-way RM ANOVA time and treatment interaction: $F_{(12, 48)} = 10.66$, $P < 0.0001$, Fig 3C-E). Once nicotinic receptor blockade was established, evoked dopamine overflow was stable through the rest of the experiment. The presence of DH β E occluded the effects of α -methylhistamine on dopamine overflow evoked by single electrical pulses (two-way RM ANOVA effect of treatment: $F_{(2, 22)} = 0.7979$, $P = 0.4629$ Fig 3F). This was unlikely to be due to any floor effect, as levels detected in the presence of Dh β E were still quite substantial and with a high signal to noise ratio (Fig 3D). These results are compatible with a role for acetylcholine in mediating the effects of α -methylhistamine on ventral striatal dopamine release.

Histamine H_3 receptor activation decreases spontaneous cholinergic interneuron activity in the ventral, but not in the dorsal striatum.

Striatal cholinergic interneurons were identified by the presence of VGluT3-tdTomato (ventral striatum) or by cell morphology and electrophysiological characteristics (dorsal striatum). In both cases, cells exhibited typical spontaneous activity (representative traces in Fig 4B and 5A).

In the ventral striatum, event frequency was significantly reduced in response to α -methylhistamine (two-way RM ANOVA effect of treatment: $F_{(2, 25)} = 4.071$, $P = 0.0295$ Fig 4C). Post-hoc analysis confirmed a sustained reduction of event frequency by α -methylhistamine, whereas with thioperamide, only a transient increase was observed at 10 min after drug application. Stratification of event frequencies indicated that α -methylhistamine increased the occurrence of time bins without events by about 4-fold (two-way RM ANOVA interaction of treatment and frequency bin: $F_{(22, 275)} = 2.058$, $P = 0.0042$, Fig 4D). These results suggest that the spontaneous activity of cholinergic interneurons in the ventral striatum is modulated by histamine H_3 receptors, and that activation of these receptors changes the activity profile of these interneurons to one that favors lower-frequency firing.

In the dorsal striatum neither application of α -methylhistamine nor thioperamide altered spontaneous firing of cholinergic interneurons (two-way RM ANOVA for treatment: $F_{(2, 23)} = 1.127$, $P = 0.3414$, Fig 5C). These results reinforce the notion that the effects of histamine H_3 modulation of cholinergic interneuron activity are specific to the ventral striatum.

Local activation of histamine H_3 receptors in ventral striatal cholinergic interneurons decreases resting membrane potential and spontaneous firing

We further investigated the effects of H_3 receptor activation on ventral striatal cholinergic interneurons using the whole cell patch-clamp technique. Cells obtained from male C57BL/6 mice were identified by their morphology and the identity was confirmed, *post-hoc*, by filling the cells with neurobiotin and co-immunostaining against ChAT (Fig 6A). Once in whole-cell mode, these cells exhibited capacitance varying from 60 to 150 pF, resting membrane potential of approximately -55 mV and spontaneous action potentials, characteristics typical of striatal cholinergic interneurons. Stable firing activity was monitored for at least five min, after which a 1min baseline was recorded (representative traces in Fig 6B). To improve temporal resolution of the drug effects, we performed application of α -methylhistamine by ejecting the drug through a locally placed pipette over a 30s period. Applying α -methylhistamine in this manner resulted in a significant decrease of resting membrane potential (two-way RM ANOVA, significant time vs. treatment interaction: $F_{(1, 12)} = 182.0$, $P < 0.0001$, Fig 6C) and in the number of

spontaneous action potentials (two-way RM ANOVA significant time vs. treatment interaction: $F_{(1, 12)} = 21.17$, $P = 0.0006$, Fig 6D).

Histamine H₃ receptors activation does not reduce acetylcholine release from isolated striatal nerve endings

Last, we used isolated synaptosome preparations to assess whether histamine H₃ receptors modulate acetylcholine release directly from striatal nerve endings (Fig. 7). We hypothesized that, should these receptors be present in cholinergic axon terminals, H₃ receptor agonist stimulation should reduce [³H]acetylcholine release from synaptosomes, measured as radioactive counts per minute (CPM) normalized to tissue mass. The H₃ receptor agonist α -methylhistamine had no significant effects on either basal [³H]acetylcholine release (one-way ANOVA: ventral striatum $F_{3,28} = 0.04$, $P = 0.990$ Fig 7D; dorsal striatum $F_{3,28} = 0.0671$, $P = 0.9769$, Fig 7A) or K⁺-stimulated [³H]acetylcholine release (one-way ANOVA: ventral striatum $F_{3,28} = 0.0458$, $P = 0.9867$, Fig 7D; dorsal striatum $F_{3,28} = 0.0038$, $P = 0.9997$, Fig 7A). Surprisingly, the highest concentration of the H₃ receptor inverse agonist thioperamide significantly decreased basal [³H]acetylcholine release from ventral striatum synaptosomes (one-way ANOVA followed by Dunnett: $F_{3,24} = 3.108$, $P = 0.0453$, Fig 7E) but not from the dorsal striatal synaptosomes (one-way ANOVA: $F_{3,24} = 0.5148$, $P = 0.6760$, Fig 7B). Thioperamide had no significant effects on K⁺-stimulated [³H]acetylcholine release either in from ventral (one-way ANOVA: $F_{3,24} = 0.9539$, $P = 0.4304$, Fig 7E) or dorsal ($F_{3,24} = 0.0884$, $P = 0.9657$, Fig 7B) striatal synaptosomes. The D₂ receptor agonist apomorphine was used as a control, as dopaminergic agonists have previously been shown to inhibit acetylcholine release from rodent striatal slices and synaptosomes (de Belleruche et al., 1982). Apomorphine (100 μ M) significantly decreased basal [³H]acetylcholine release from both the ventral striatal (Student's "t" test: $t_{(28)} = 5.91$, $P < 0.0001$, Fig 7F) and the dorsal striatal (Student's "t" test: $t_{(28)} = 3.186$, $P < 0.0035$, Fig 7C) synaptosomes, and also significantly inhibited K⁺-stimulated release from ventral striatal (Student's "t" test: $t_{(28)} = 4.547$, $P < 0.0001$, Fig 7F), but not dorsal striatal (Student's "t" test: $t_{(28)} = 1.791$, $P = 0.0841$, Fig 7C) synaptosomes.

Discussion

The central finding of the present study is that activation of histamine H₃ receptors by α -methylhistamine reduced electrically evoked dopamine overflow in the ventral striatum of mice. This effect was completely blocked by simultaneous application of thioperamide, an inverse-agonist of H₃ receptors, confirming that H₃ receptors were selectively activated. Further, α -methylhistamine did not alter dopamine overflow half-life or the paired pulse ratio, indicating that the reduced dopamine overflow was not due to increased uptake/diffusion or to reduced pre-synaptic vesicle mobilization. Our work suggests that regulation of dopamine release by H₃ receptors was caused not by a direct heterosynaptic inhibition of dopaminergic axon terminals, but rather by inhibition of the firing of striatal cholinergic interneurons, leading to reduced nicotinic facilitation of dopamine release. In favor of this model, we found that H3R mRNA is absent from dopamine neurons and that the H₃ agonist had no effect on dopamine overflow evoked by direct optogenetic stimulation of dopaminergic axons. Conversely, we found measurable levels of H3R mRNA in striatal cholinergic interneurons and that the nicotinic acetylcholine receptor antagonist DH β E occluded the effects of α -methylhistamine on electrically evoked dopamine overflow. We also showed that α -methylhistamine dramatically reduced the spontaneous firing of ventral striatal cholinergic interneurons, but did not reduce acetylcholine release from acutely isolated synaptosomes, suggesting that the observed modulation of dopamine overflow is probably mediated by H₃ receptors located in the somatodendritic compartment of cholinergic interneurons, rather than at their axon terminals.

To our knowledge, this is the first study to directly investigate activity-dependent H₃-receptor modulation of dopamine release in the mouse striatum using voltammetric measurements of dopamine overflow. This technique offers key advantages given the fast kinetics of activity-dependent evoked dopamine transients in the striatum that might otherwise be missed by traditional techniques such as microdialysis. Our data are compatible with the increasing evidence suggesting that histamine plays a modulatory role upon dopaminergic neurotransmission, both in mice and other rodents. In an earlier study, Schlicker et al. (1993) demonstrated that histamine causes a discrete reduction of electrically-evoked [³H]dopamine release from mouse striatal slices, an effect mimicked by α -methylhistamine and blocked by thioperamide at the same concentrations of those used here. Subsequent studies indicated that histamine H₃ receptors fail to affect baseline levels of dopamine (Fox et al., 2005; Giannoni et al., 2010; Aquino-Miranda et al., 2015), but reliably modulate extracellular dopamine elevations evoked by pharmacological stimuli (Munzar et al., 2004; Nowak et al., 2008; Alfaro-Rodriguez et al., 2013). In addition, H₃ receptors have been shown to modulate behaviors typically regulated by dopaminergic neurotransmission, such as alcohol consumption reinstatement (Nuutinen et al., 2015), alcohol-mediated reward (Nuutinen et al., 2011; Morais-Silva et al., 2016), amphetamine self-administration (Munzar et al., 2004), amphetamine-

induced locomotor activity (Munzar et al., 2004; Banks et al., 2009), apomorphine-induced turning behavior in mice with unilateral striatal ablation (Garcia-Ramirez et al., 2004), among others. It is important to point out, however, that other uncontrolled parameters perhaps exist influencing how these receptors act to regulate the dopaminergic system under different experimental conditions. At least for studies performed using brain slices, a key parameter may be the viability and excitability of cholinergic interneurons. In the present experiments, we employed a novel protective slicing technique (Peca et al., 2011; Zhao et al., 2011; Ting et al., 2014). Although a systematic comparison between slicing methods was beyond the scope of the present study, we empirically found that experimental reproducibility improved with the new method in comparison with more traditional brain slice preparation methods. We postulate that increased interneuron viability in these slices may have been relevant for the results observed, especially since experiments were performed in adult mice.

Our results suggest that H₃-mediated reduction of dopamine release is achieved indirectly by reducing the firing of cholinergic interneurons and reducing basal cholinergic tone. This could lead to a reduced efficacy of electrically-evoked acetylcholine release to trigger dopamine release, but further experiments would be needed to determine this. The link between acetylcholine release and dopamine release has been the focus of multiple recent studies (Zhou et al., 2001; Cachope et al., 2012; Threlfell et al., 2012; see de Kloet et al., 2015 for a recent review). **Optogenetic activation of striatal cholinergic interneurons can directly trigger dopamine release from dopaminergic axon terminals in both the ventral and dorsal striatum (Threlfell et al., 2012). Furthermore,** acetylcholine released from cholinergic interneurons and other cholinergic terminals in the striatum activates β_2 subunit-containing nicotinic receptors in dopaminergic axons that, in turn, allow influx of Na⁺, leading to depolarization of these axons and exocytosis of dopamine. Therefore, it is logical to hypothesize that drugs capable of regulating cholinergic tone may also affect dopamine release. This view is supported by the fact that local inactivation of H₃ receptors increased acetylcholine release *in vivo* (Prast et al., 1994). Interestingly, our results indicate that although striatal cholinergic neurons contain H3R mRNA, H₃ receptors do not modulate acetylcholine release from isolated striatal synaptosomes, perhaps indicating that these receptors are somatodendritic. Although it is possible that the synaptosome assay we used was not sufficiently sensitive to detect a H₃-mediated inhibition of acetylcholine release, our results with the dopamine receptor agonist apomorphine provide a positive control showing that our experimental conditions allowed for detection of modest levels of acetylcholine release inhibition. Moreover, our observations are compatible with previous work indicating that striatal H₃ receptors are predominantly post-synaptic rather than pre-synaptic (i.e. Ferrada et al., 2009; Moreno et al., 2011; reviewed by Ellenbroek and Ghiabi, 2014). We cannot exclude, however, that reduced cholinergic interneuron activity resulted from inhibition

of glutamatergic inputs onto these neurons. Indeed, previous studies point out that histamine H₃ receptors depress striatal glutamate release (Doreulee et al., 2001; Molina-Hernandez et al., 2001; Ellender et al., 2011), and that glutamatergic tone is pivotal for cholinergic regulation of striatal dopamine release (Kosillo et al., 2016).

Modulation of striatal glutamate has also been shown to regulate dopamine release (Bennett and Gronier, 2005; David et al., 2005; Beurrier et al., 2009), and changes in the recruitment of cholinergic interneurons, although not directly investigated in these studies, cannot be ruled out. Cholinergic interneurons also express VGluT3 and are capable of releasing glutamate (Higley et al., 2011) and may decrease local glutamate release via mGluR activation (Sakae et al., 2015). Yet, given that α -methylhistamine was no longer effective in the presence of Dh β E, we interpret these results as a cholinergic rather than a glutamatergic effect. Clearly, the effects of H₃ receptor activation/inactivation on more detailed electrophysiological parameters of cholinergic interneurons, such as resting membrane potential and excitatory and inhibitory inputs as well as the direct consequence for evoked acetylcholine release and for cholinergic/glutamatergic neurotransmission should be the subject of further investigation.

We found that thioperamide transiently increased the spontaneous activity of cholinergic interneurons in the ventral striatum. Given this effect, one would expect to find a similar outcome when measuring dopamine release evoked by single electrical pulses. However, that was not the case. We speculate that the effects of thioperamide and the related basal level of activation of H₃ receptors that it may block may have been too small to be picked up in this assay, **or that the receptor may be subject to rapid desensitization upon prolonged exposure to the inverse agonist.**

An intriguing observation of the present study is the lack of effect of histamine H₃ drugs in the dorsal striatum. This is particularly surprising in light of previous radioligand binding studies and in-situ hybridization suggesting that the dorsal striatum is one of the brain structures with the highest density of H₃ receptors (Jansen et al., 2000; Pillot et al., 2002). In rats, striatal H₃R mRNA was found to co-localize with markers for cholinergic interneurons and with D₁- and D₂-expressing medium spiny neurons (González-Sepúlveda et al., 2013). Here, we found that H₃R mRNA is present in dorsal-striatal cholinergic interneurons at levels similar to those found in the ventral striatum. Thus, lack of H₃ receptor expression by cholinergic neurons cannot readily explain the failure of histamine H₃ drugs to modulate dopamine release in the dorsal striatum. In addition, blockade of β_2 subunit-containing nicotinic receptors was previously shown to reduce dorsal striatal dopamine overflow to an extent similar to that seen in the ventral striatum (Salinas et al., 2016), thus ruling out a disconnection between cholinergic and dopaminergic systems in this region. It should be taken into account, however, that presence of mRNA

does not warrant quantifiable levels of its respective protein, and nicotinic modulation of dopamine overflow in dorsal striatum will not guarantee that crosstalk between histamine and acetylcholine is as efficient as in the ventral striatum. Therefore, it is still possible that dorsal-striatal cholinergic interneurons have fewer H₃ receptors, or that these receptors couple less efficiently to their signaling effectors. Finally, it remains to be investigated whether activation of H₃ receptors in such interneurons will lead to **membrane hyperpolarization** and reduced firing to a degree similar to that observed here in the ventral striatum.

In rats, evidence shows that dopaminergic neurons express H3R mRNA (González-Sepúlveda et al., 2013). Here, we did not find detectable levels of H3R mRNA in dopaminergic neurons harvested either from the VTA or SN of mice. The lack of any observable effect of H₃ receptor activation on optogenetically evoked dopamine overflow is also compatible with our observation of a lack of expression of H₃ receptors in dopamine neurons in mice. These findings highlight a possible interspecies difference in histamine H₃ receptor brain distribution and function.

Our findings highlight histamine H₃ receptors as critical and region-specific regulators of dopamine release in the ventral but not the dorsal striatum. **Our findings do not exclude other effects of H3Rs in the dorsal striatum, as suggested by receptor work suggesting a role for this receptor in sensorimotor gating (Kononoff Vanhanan et al., 2016). Nonetheless,** considering the major interest in region-specific regulation of dopamine release in mesostriatal versus mesolimbic pathways in the context of diseases such as drug abuse and schizophrenia, our results suggest that this pathway could represent an interesting new target for the development of novel therapeutic approaches for the treatment of these important brain diseases.

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Legends

Figure 1 Effects of histamine H₃ agonist and inverse agonist on dopamine overflow evoked by electrical stimuli in the dorsal and ventral striatum. Electrically evoked dopamine levels were detected by fast-scan cyclic voltammetry in the dorsal and ventral striatum. Heat maps illustrating the current detected as a function of time and applied potential in the dorsal (A) and ventral striatum (D). The vertical dotted lines in the heat map indicate the maximum oxidation/reduction peaks for dorsal and ventral striatum, and the corresponding cyclic voltammograms are illustrated in (B) and (E). The traces illustrate representative dopamine overflow peaks evoked by **single and paired electrical stimulation** in the dorsal (C) and ventral striatum (F). In the dorsal striatum (G) neither the selective H₃ agonist α -methylhistamine (red circles) nor the selective H₃ inverse agonist thioperamide (black circles) affected dopamine overflow evoked by single electrical pulses. In the ventral striatum (H), α -methylhistamine (red circles) significantly reduced dopamine overflow evoked by single electrical pulses, an effect prevented by concomitant application of thioperamide (magenta circles). Thioperamide alone (black circles) did not affect dopamine overflow evoked by single electrical pulses in the ventral striatum. Gray bars indicate the period when drugs were present. Data were analyzed by two-way RM-ANOVA followed by Sidak. * P < 0.05, ** P < 0.01, *** P < 0.001 when compared to controls.

Figure 2 Effects of histamine H₃ agonist and inverse agonist on optogenetically evoked dopamine overflow in the ventral striatum. Coronal sections containing the mesencephalon (A, upper panels) or ventral striatum (A, bottom panels) were obtained 4 weeks after injection of AAV5-DIO-EF1 α -ChR2-(H134R)-EYFP into the VTA of DAT-Cre +/- mice. Sections were stained against the dopamine neuron marker tyrosine hydroxylase (TH, red channel) and the transfected ChR2-eYFP protein (green channel). Merged images indicate selective virus expression in dopamine cell bodies (upper right panels) and their axons (bottom right panels). White calibration bar indicates 50 μ m. Illuminating the slice with a single light-pulse of 1 ms duration caused dopamine overflow to be observed in the fast-scan cyclic voltammetry heat map (B). Cyclic voltammograms are presented in (C), and representative traces of light-evoked dopamine overflow are shown in (D). The sodium channel blocker tetrodotoxin (TTX, red traces) completely blocked dopamine release induced by the optical stimuli (C-D). Next, a baseline was recorded for each drug condition. Each circle or bar represents the mean \pm SEM normalized to that baseline. Neither α -methylhistamine (red circles/bar) nor thioperamide (black circles/bar) affected dopamine overflow evoked either by single light pulses (E) or by 2-s trains (F). Data were analyzed by two-way RM ANOVA (E) or one-way ANOVA (F). No statistically significant effects were observed (P > 0.05).

Figure 3 Effects of nicotinic acetylcholine receptor blockade on histamine H₃-mediated reduction of electrically-evoked dopamine overflow. Fast-scan cyclic voltammetry heat maps illustrate the current detected as a function of time and the applied potential during baseline (A) or under blockade of nicotinic acetylcholine receptors by dihydro- β -erythrodine (DH β E) (B). Cyclic voltammograms detected are represented in (C). Representative traces illustrating peak dopamine overflow are illustrated in (D). DH β E reduced the magnitude of electrically-evoked dopamine overflow to about 37% of its initial value (E) and was stable for the duration of the entire experiment. A baseline was recorded for each condition under nicotinic blockade and all subsequent results were normalized to that baseline (F). No significant effect of α -methylhistamine (red circles) was detected on dopamine overflow evoked by single pulse stimulation in the presence of DH β E. Data were analyzed by two-way RM ANOVA (E-F). No statistically significant effects were observed ($P > 0.05$).

Figure 4 Effects of histamine H₃ receptor agonist and inverse agonist on spontaneous firing activity of ventral striatal cholinergic interneurons. Coronal sections of the ventral striatum of mice expressing the tdTomato fluorescent protein in Vglut3-containing neurons allowed for identification of cholinergic interneurons (A). Sections were stained against tdTomato (red channel, upper panel) and the cholinergic marker choline acetyl transferase (ChAT, green channel, middle panel). Merged images (bottom panel) confirm that Vglut3-Tomato cells are indeed cholinergic interneurons. Spontaneous cell firing measured by loose-cell attached electrophysiology (representative recording in B, upper trace) was reduced by the selective H₃ agonist α -methylhistamine (bottom trace). Event frequency was normalized to a 5 min baseline and binned in 1 min intervals (C). α -Methylhistamine (red circles) significantly reduced the frequency of spontaneous events, while the selective H₃ inverse-agonist only transiently increased event frequency when compared to controls. Stratification of events by their instantaneous frequency during the last 5 min of recording (D) indicates that α -methylhistamine (red bars) increases the proportion of events with frequency between 0.01 and 0.5 Hz, while thioperamide (black bars) tended to increase the predominance of events with frequency greater than 6 Hz ($P = 0.0528$). All data are represented as the mean \pm SEM normalized to the baseline (C) or the total number of events for each category bin (D). All results were analyzed by two-way RM ANOVA followed by Sidak. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ when compared to controls.

Figure 5 Effects of histamine H₃ receptor agonist and inverse agonist on spontaneous firing activity of dorsal striatal cholinergic interneurons. Spontaneous firing was measured by loose-cell attached patch recording (representative traces in A). Event frequency was normalized to a 5 min baseline and binned in 1 min intervals (B). Neither α -Methylhistamine (red circles) nor Thioperamide (black circles)

altered the frequency of spontaneous events (two-way RM ANOVA, $P > 0.05$). The grey bar indicates the period of drug treatment.

Figure 6 Effects of local application of the H₃ receptor agonist α -methylhistamine on resting membrane potential and spontaneous action potentials of ventral striatal cholinergic interneurons.

Cholinergic interneurons were initially identified by cell morphology and electrophysiological properties, and the identity of some cells was confirmed by co-immunostaining neurobiotin-filled cells with a ChAT antibody (A). Recorded neurons exhibited a resting membrane potential of approximately -55 mV and spontaneous action potentials (AP), typical of cholinergic interneurons (representative trace in B). Ejection of α -methylhistamine through a locally placed pipette (duration of drug ejection represented by a gray bar) significantly reduced the resting membrane potential (C) and frequency of spontaneous APs (D). Scale bars in A indicate 20 μ m. All results were analyzed by two-way RM ANOVA followed by Sidak. *** $P < 0.001$ or **** $P < 0.0001$ when compared to pre-drug baseline.

Figure 7 Effects of H₃ and D₂ ligands on the release of [³H]acetylcholine from mouse ventral and dorsal striatum synaptosomes.

Synaptosomes prepared separately from samples of ventral and dorsal striatum were loaded with [³H]choline, incubated for 30 min and superfused for ten minutes prior to stimulating release with 20 mM K⁺. Thioperamide, α -methylhistamine or apomorphine were present after five min of superfusion. Graphs show radioactivity (CPM/mg tissue; mean \pm SEM) released immediately before stimulation (basal) as well as immediately after stimulation (stimulated). The H₃ agonist α -methylhistamine had no effect on basal or stimulated release (A, D). The H₃ inverse agonist thioperamide inhibit basal [³H]acetylcholine release from the ventral striatal synaptosomes, but only at the highest dose tested (E). No effect was detected on dorsal striatal synaptosomes (B). The D₂ agonist apomorphine significantly inhibited basal [³H]acetylcholine release from both dorsal (C) and ventral (F) striatal synaptosomes, and also reduced stimulated release from ventral striatal synaptosomes (F). Results were analyzed by one-way ANOVA followed by Dunnett's test (A-B, D-E) or by Student's t test (C and F). * $P < 0.05$; ** $P < 0.01$ or *** $P < 0.001$ when compared to controls.

Tables

Table 1

Table 1: Summary of electrically-evoked dopamine-overflow in dorsal and ventral striatum

	Dorsal striatum (n)	Ventral striatum (n)	P value
Single-pulse DA peak amplitude (μM)	0.39 ± 0.04 (29)	0.31 ± 0.03 (46)	0.1201
Paired-pulse ratio [(2 nd - 1 st)/1 st]	0.06 ± 0.01 (29)	0.26 ± 0.03 (46)	< 0.0001
Peak half-life (s)	0.43 ± 0.05 (28)	0.66 ± 0.05 (44)	0.0018

Table 2

Table 2: Quantification of H3R mRNA in striatal ACh interneurons and mesencephalic DA neurons

	Striatal ACh interneurons		Mesencephalic DA neurons	
	Dorsal striatum	Ventral striatum	SN	VTA
H3R mRNA Ct	32.07 (30.92, 32.42)	31.42 (30.34, 32.61)	n.d.	n.d.
GAPDH Ct	23.68 (22.8, 24.23)	23.16 (22.76, 25.48)	25.54 (24.23, 26.33)	23.86 (22.79, 25.34)
$2^{-\Delta\Delta Ct}$	1.082 (0.704, 1.313)	1.809 (0.718, 2.947)	n/a	n/a

Values presented as “median (25% percentile, 75% percentile)”

n.d.: Not detected

n/a: Not applicable

Sample sizes: Dorsal striatum = 3; Ventral striatum = 4; SN = 4; VTA = 3.