HHS Public Access

Author manuscript

Eur J Neurosci. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Eur J Neurosci. 2015 March ; 41(6): 760–772. doi:10.1111/ejn.12818.

Glutamatergic and Dopaminergic Neurons in the Mouse Ventral Tegmental Area

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Abstract

The ventral tegmental area (VTA) comprises dopamine (DA), GABA and glutamate (Glu) neurons. Some rat VTA Glu neurons, expressing vesicular glutamate transporter 2 (VGluT2), co-express tyrosine hydroxylase (TH). While transgenic mice are now being used in attempts to determine the role of VGluT2/TH neurons in reward and neuronal signaling, such neurons have not been characterized in mouse tissue. By cellular detection of VGluT2-mRNA and TH-immunoreactivity (TH-IR), we determined the cellular expression of VGluT2-mRNA within VTA TH-IR neurons in the mouse. We found that some mouse VGluT2 neurons co-expressed TH-IR, but their frequency was lower than in the rat. To determine whether low expression of TH mRNA or TH-IR accounts for this low frequency, we evaluated VTA cellular co-expression of TH-transcripts and TH-protein. Within the medial aspects of the VTA, some neurons expressed TH mRNA but lacked TH-IR; among them a subset co-expressed VGluT2 mRNA. To determine if lack of VTA TH-IR was due to TH trafficking, we tagged VTA TH neurons by cre-inducible expression of mCherry in TH::Cre mice. By dual immunofluorescence, we detected axons containing mCherry, but lacking TH-IR, in the lateral habenula, indicating that mouse low frequency of VGluT2 mRNA (+)/TH-IR (+) neurons is due to lack of synthesis of TH protein, rather than TH-protein trafficking. In conclusion, VGluT2 neurons are present in the rat and mouse VTA, but they differ in the populations of VGluT2/TH and TH neurons. We reveal that under normal conditions, the translation of TH protein is suppressed in the mouse mesohabenular TH neurons.

Keywords

VTA; lateral habenula; reward; interpeduncular nucleus; optogenetics and VGluT2

INTRODUCTION

The A10 area has dopamine (DA) neurons highly concentrated within three midline nuclei [caudal linear nucleus (CLi), interfascicular nucleus (IF), and rostral linear nucleus of the

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raphe (RLi)] and two more lateral midbrain nuclei [parabrachial pigmented (PBP) and paranigral nuclei (PN)] that lie just medial to the substantia nigra. The PBP and PN have been classically considered the major nuclei of the ventral tegmental area (VTA) (Swanson, 1982). In addition to DA neurons, GABA and glutamate neurons are found in the rat A10 area (Hur & Zaborszky, 2005; Kawano *et al.*, 2006; Yamaguchi *et al.*, 2007; Nair-Roberts *et al.*, 2008; Yamaguchi *et al.*, 2011; Li *et al.*, 2013; Morales & Root, 2014).

Glutamatergic neurons within the rat A10 area selectively express transcripts encoding vesicular glutamate transporter-2 (VGluT2). Whereas most of these VGluT2 neurons lack expression of both protein and mRNA for tyrosine hydroxylase (TH, limiting enzyme for the production of DA) (Yamaguchi *et al.*, 2007), there are some VGluT2 neurons located in the medial PBP, medial PN, CLi, RLi and IF of the rat that co-express VGluT2 mRNA and TH-protein (VGluT2/TH neurons) (Kawano *et al.*, 2006; Yamaguchi *et al.*, 2011). Recent findings from combination of neuronal tract tracing, *in situ* hybridization and immunohistochemistry show that in the rat the VGluT2-only neurons and VGluT2/TH neurons each innervate the medial prefrontal cortex and the nucleus accumbens, leading to the suggestion that in addition to the well-recognized mesocorticolimbic DA and GABA pathways, there exists a parallel mesocorticolimbic glutamatergic pathway from VGluT2-only and VGluT2/TH neurons (Yamaguchi *et al.*, 2011).

Whereas our current understanding of the molecular characteristics of VGluT2 and VGluT2/TH neurons has been obtained from rat brain tissue analysis (Yamaguchi *et al.*, 2011; Li *et al.*, 2013); mice are increasingly used to determine the role of midbrain glutamate and glutamate-dopamine neurons in brain function (Birgner *et al.*, 2010; Hnasko *et al.*, 2010; Stuber *et al.*, 2010; Tecuapetla *et al.*, 2010; Alsio *et al.*, 2011; Fortin *et al.*, 2012; Hnasko *et al.*, 2012; Adrover *et al.*, 2014; Chuhma *et al.*, 2014; Nordenankar *et al.*, 2014; Root *et al.*, 2014a; Root *et al.*, 2014b). However, the VGluT2 and VGluT2/TH neurons in the mouse VTA are not well characterized, and the extent to which these neurons compare with those in the rat VTA is not known. In here, we found that in common with the rat, the mouse VTA has neurons expressing VGluT2 mRNA intermixed with neurons expressing TH-protein. In contrast to the rat VTA, however, most of the neurons in the mouse RLi and IF lack detectable levels of TH-protein despite expressing both TH-mRNA along with VGluT2 mRNA. We caution that these mouse RLi and IF neurons expressing TH-mRNA could be erroneously classified as non-TH neuron due to their undetectable levels of TH immunoreactivity. However, these RLi and IF neurons can be manipulated in TH::Cre mice because they express the TH promoter, as such leading to the erroneous assumption that DA releasing neurons are being manipulated. In addition, we detected in the mouse interpeduncular nucleus (IP), but not the rat, many neurons that lack detectable levels of TH-protein but contain TH-mRNA.

MATERIALS AND METHODS

Surgical procedure for viral injection

VGluT2::Cre mice [from Dr. Kiehn's laboratory (Borgius *et al.*, 2010); background: C57BL/6 J mouse, breeding at least five generations, 25-30 g body weight] and TH::Cre mice [from the Jackson Laboratory, strain name: B6.Cg-Tg(Th-cre)1Tmd; background:

C57BL/6NJ inbred mouse, 25-30 g body weight] were injected into the VTA with Cre-inducible recombinant AAV encoding ChR2 tethered to mCherry (VGluT2-mCherry or TH-mCherry mice). Mice were anesthetized with 3% isoflurane (Butler Schein, Dublin, OH). The deeply anesthetized mice were fixed in a stereotaxic apparatus and 0.5 μl (0.1 μl/min) of pAAV-ChR2-mCherry viral vector [AAV-EF1a-DIO-hChR2-mCherry, 3×10^{12} genomes/ml, UNC Vector Core Facility, Chapel Hill, NC] was injected bilaterally into the VTA (Bregma in mm: AP −3.4, ML ± 0.3, DV −4.3) using a NanoFil syringe with 35 gauge needle (NANOFIL, WPI, Sarasota, FL). The needle was left in place for 10 minutes after each injection. Six weeks after the virus injection, the mice were perfused. Mice were housed in groups of up to four animals per cage in an animal room at 22°C under a 12 h light/dark cycle (light on at 7 am), with ad libitum access to food and water. Each animal procedure was approved by the NIH/NIDA Animal Care and Use Committee, and experiments were carried out in accordance with the guidelines laid down by the NIH regarding the care and use of animals for experimental procedures.

Tissue preparation

Male Sprague-Dawley rats (260-280 g body weight, n = 3), C57BL/6 mice (25-30 g body weight, n = 10), male VGluT2-mCherry mice (n = 3), and male TH-mCherry mice (n = 3) were anesthetized with chloral hydrate (35 mg/100 g) and perfused transcardially with 4% (W/V) paraformaldehyde (PF) in 0.1 M phosphate buffer (PB), pH 7.3. The brains were left in 4% PF for 2 h at 4°C, rinsed with PB and transferred sequentially to 12%, 14% and 18% sucrose solutions in PB. Coronal serial cryosections of different thickness were prepared, 5 μm (4 mice), 10 μm (3 mice, 3 rats) or 12 μm (3 mice), and for VGluT2-mCherry and TH-mCherry 16-μm thick.

Combination of *in situ* hybridization and immunolabeling

Coronal free-floating cryosections (10, 12 or 16 μm in thickness) or sections collected on glass slides (5 μm in thickness) were processed as described previously [(Wang & Morales, 2008) for free floating sections, and (Morales & Wang, 2002)for sections on glass slides]. Different thicknesses were used to maximize detection of signal in individual neurons. The sections were incubated for 10 min in PB containing 0.5% Triton X-100, rinsed 2 × 5 min with PB, treated with 0.2 N HCl for 10 min, rinsed 2 × 5 min with PB and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. The sections were rinsed 2 × 5 min with PB, post-fixed with 4% PF for 10 min. Prior to hybridization and after a final rinse with PB, the free-floating sections were incubated in hybridization buffer (50% formamide; 10% dextran sulfate; 5× Denhardt's solution; 0.62 M NaCl; 50 mM DTT; 10 mM EDTA; 20 mM PIPES, pH 6.8; 0.2% SDS; 250 μg/ml salmon sperm DNA; 250 μg/ml tRNA) for 2 h at 55°C. The Sections collected on glass slides were dehydrated through a series of graded ethanol solutions (50%, 70% and 95%, 5 min for each concentration). The sections were hybridized for 16 h at 55°C in hybridization buffer containing [^{35}S]- and [^{33}P]-labeled single-stranded antisense or sense TH (nucleotide 14-1165, Accession #NM_012740), VGluT1 (nucleotides 53-2077, Accession # NM_053859.1), VGluT2 (nucleotides 317-2357, Accession # NM_053427), VGluT3 (nucleotides 1-1729, Accession # BC117229.1) probes at 10^7 cpm/ml. Plasmids that contained the VGluT1 or VGluT2 were provided by Dr. Robert H Edwards (University of California, San Francisco). The sections

were treated with 4 μg/ml RNase A at 37°C for 1 h, washed with 1 × SSC, 50% formamide at 55°C for 1 h, and with 0.1 × SSC at 68°C for 1 h. After the last SSC wash, the sections were rinsed with PB–and incubated for 1 h in PB supplemented with 4% bovine serum albumin and 0.3% Triton X-100. This was followed by the overnight incubation at 4°C with mouse anti-TH monoclonal antibody (1: 500, MAB 318, Millipore, Billerica, MA) for which specificity has been documented (Tagliaferro & Morales, 2008) or mouse anti-mCherry antibody (632543; Clontech Laboratories Inc., Mountain View, CA, 1:500 dilution). After rinsing 3 × 10 min in PB, sections were processed with an ABC kit (Vector Laboratories, Burlingame, CA). The material was incubated for 1 h at RT in a 1:200 dilution of the biotinylated secondary antibody, rinsed with PB, and incubated with avidin-biotinylated horseradish peroxidase for 1 h. Sections were rinsed and the peroxidase reaction was then developed with 0.05% 3, 3-diaminobenzidine-4 HCl (DAB) and 0.03% hydrogen peroxide (H_2O_2). Free-floating sections were mounted on coated slides. All slides were dipped in Ilford K.5 nuclear tract emulsion (Polysciences, Inc., Warrington; 1:1 dilution in double distilled water) and exposed in the dark at 4°C for four weeks prior to development.

Immunofluorescence and confocal microscopy

Free floating coronal sections from the nucleus accumbens (nAcc) and the lateral habenula (LHb) (30 μm in thickness) of TH-mCherry mice were incubated for 1 h in PB supplemented with 4% BSA and 0.3% Triton X-100. Sections were then incubated with a cocktail of two primary antibodies [mouse anti-mCherry antibody (1:500, 632543, Clontech Laborotories) + rabbit anti-TH antibody (1:1000, AB152, Millipore)] overnight at 4°C. After rinsing 3 × 10 min in PB, sections were incubated in a cocktail of two corresponding fluorescence secondary antibodies [Alexa Fluor 594-AffiniPure donkey anti-mouse (715-585-151; Jackson Immunoresearch Laboratories, 1:100 dilution) + Alexa Fluor 488-AffiniPure donkey anti-rabbit (711-545-152; Jackson Immunoresearch Laboratories, 1:100 dilution)] for 2 h at room temperature. After rinsing, sections were mounted on slides. Fluorescent images were collected with an Olympus FV1000 Confocal System (Olympus). Images were taken sequentially with different lasers with 20× or 100× oil immersion objectives and z-axis stacks were collected at 0.2 μm.

Data Analysis of cellular subpopulations

Sections were viewed, analyzed, and photographed with bright field or epiluminescence microscopy using a Nikon Eclipse E 800 microscope fitted with 4× and 20× objective lenses. Single and double-labeled neurons were observed within each traced region at high power (20× objective lens) and marked electronically. Subdivisions of the midbrain dopamine system were traced according to Swanson (Swanson, 1982), Phillipson (Phillipson, 1979b; a), Halliday and Törk (Halliday & Tork, 1986), German and Manaye (German & Manaye, 1993), Paxinos and Watson (Paxinos & Watson, 2007), and Paxinos and Franklin (Paxinos & Franklin, 2001). VGluT2/TH double-labeled material was analyzed using epiluminescence to increase the contrast of silver grains (neither dark-field nor bright field optics allow clear visualization of silver grains when co-localized with high concentration of immunoproducts). A cell was considered to express VGluT2 mRNA when its soma contained concentric aggregates of silver particles above background level. A neuron was considered to express TH immunoreactivity when its soma was clearly labeled

as brown. A TH immunolabeled neuron was included in the calculation of total population of TH cells when the stained cell was at least 5 μm in diameter. The cells expressing VGluT2 mRNA, TH immunoreactivity, or both markers were counted separately. To determine cellular coexistence of VGluT2 mRNA and TH immunolabel, (a) silver grains corresponding to VGluT2 expression were focused under epiluminescence microscopy, (b) the path of epiluminescence light was blocked without changing the focus, (c) bright field light was used to determine if a brown neuron, expressing TH in focus, contained the aggregates of silver grains seen under epiluminescence. The same method and criteria were applied to determine cellular coexistence of TH mRNA and mCherry immunolabel in VGluT2::Cre mice, as well as VGluT2 mRNA and mCherry immunolabel in TH::Cre mice. Labeled cells were counted 3 times, each time by a different observer. The background was evaluated from slides hybridized with sense probes. Pictures were adjusted to match contrast and brightness by using the program Adobe Photoshop (Adobe Systems Incorporated, Seattle, WA).

RESULTS

Cellular expression of VGluT2 mRNA, but not VGluT1- or VGluT3-mRNA, in the A10 area

To determine the presence of glutamatergic neurons within the mouse A10 area, we used a combination of radioactive *in situ* hybridization to identify cellular expression of transcripts encoding VGluT1, VGluT2 or VGluT3, and immunodetection for TH protein (Fig. 1). We detected cells expressing VGluT2 mRNA (but not VGluT1 or VGluT3) intermingled with TH immunoreactive (TH-IR) cells in the lateral [parabrachial pigmented (PBP), paranigral (PN) nuclei] and midline nuclei of the A10 area [caudal linear nucleus (CLi), interfascicular nucleus (IF), and rostral linear nucleus of the raphe (RLi)] (Fig. 1A-A”). The specificity of the detection of VGluT2 mRNA was confirmed by the lack of signal when sections were hybridized with the VGluT2 radioactive sense riboprobe (Fig. 1B-B”).

Within the mouse A10 area, neurons expressing VGluT2 mRNA were detected throughout the rostro-caudal levels of the A10 area with a medio-lateral decreasing gradient in concentration (Fig. 2A’-B’). This mouse VGluT2 cellular pattern of distribution was similar to the one observed in the rat (Fig. 2C’-D’).

The majority of mouse neurons expressing VGluT2 mRNA lacked TH-immunoreactivity in the A10 area

To determine the degree of overlapping between neurons expressing VGluT2 mRNA and those expressing TH-IR in the mouse A10 area, coronal sections were processed for dual detection of VGluT2 mRNA and TH-IR. The latero-medial portions of the PBP and PN contained neurons expressing VGluT2 mRNA (Figs. 3-6); about 10% of them co-expressed TH-IR [55 VGluT2 mRNA (+)/TH-IR (−) neurons from a total population of 460 VGluT2 mRNA (+) neurons, Fig. 5]. These VGluT2 mRNA (+)/TH-IR (+) neurons represented as little as 4% of the PBP-PN TH-IR (+) neurons [55 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 1265 TH-IR (+) neurons, Fig. 5]. As observed in the PBP-PN, the majority of VGluT2 mRNA (+) neurons within the midline nuclei also lacked detectable levels of TH-IR (Figs. 5-6). Within the RLi only one percent of the VGluT2 mRNA (+)

neurons were found to co-express TH-IR [3 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 286 VGluT2 (+) neurons]. These RLi dual neurons represented near to 10% of the TH-IR (+) neurons [3 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 33 TH-IR (+) neurons]. Within the IF about 7% of the VGluT2 mRNA neurons co-expressed TH-IR [7 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 95 VGluT2 mRNA (+) neurons]. The IF VGluT2 mRNA (+)/TH-IR (+) neurons represented about 17% of those containing TH-IR [7 VGluT2 mRNA (+)/TH-IR (+) neurons from 42 TH-IR (+) neurons]. Among the different nuclei of the A10 area, the CLi had the highest proportion of VGluT2 mRNA neurons co-expressing TH-IR in which near to 20% of the VGluT2-mRNA neurons contained TH-IR [17 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 82 VGluT2 mRNA (+) neurons]. The VGluT2 mRNA (+)/TH-IR (+) neurons within the CLi represented near to 15% of the TH-IR (+) neurons [17 VGluT2 mRNA (+)/TH-IR (+) neurons from a total population of 111 TH-IR (+) neurons].

A subset of VTA mouse neurons expressed TH mRNA but lacked detectable levels of TH-IR in their cells bodies and processes

In the mouse, TH-IR (+) neurons were numerous in the lateral aspects of the PBP and PN (Fig. 2B), but infrequent in the RLi and IF (Fig. 2B). In contrast, TH-IR (+) neurons were numerous in all subdivisions of the rat A10 area, including the RLi and IF (Fig. 2D). To examine whether the low numbers of mouse VGluT2 mRNA (+)/TH-IR (−) neurons and mouse TH-IR (−) neurons reflect lack of TH protein or lack of TH mRNA, we next determined the degree of cellular co-expression of TH-mRNA and TH-protein in the mouse A10 area. We found that more than 90% of the neurons expressing TH mRNA co-expressed TH-IR in the PBP and PN [1727 TH mRNA (+)/TH-IR (+) neurons from a total of 1842 TH mRNA (+) neurons] and in the CLi [71 TH mRNA (+)/TH-IR (+) neurons from a total of 76 TH mRNA (+) neurons] (Figs. 7-10). In contrast, a large population of neurons in the RLi and IF containing TH mRNA did not have TH-IR (Figs. 7, 9-10). We found that near to 10% of the neurons expressing TH mRNA co-expressed TH-IR in the RLi [16 TH mRNA (+)/TH-IR (+) neurons from a total of 145 TH-mRNA (+) neurons], and about 50% of the neurons expressing TH mRNA co-expressed TH-IR in the IF [59 TH mRNA (+)/TH-IR (+) neurons from a total of 115 TH mRNA (+) neurons] (Figs. 9-10). Many neurons expressing TH mRNA without detectable levels of TH-IR were also found within the interpeduncular nucleus (IP), a ventral structure to the IF, (Figs. 8C-D' and 10A-B').

To determine whether the observed lack of TH-IR in the soma is due to anterograde transport, we looked for TH-IR in fibers derived from VTA TH neurons that were tagged *in vivo* with mCherry by viral vector transduction. We injected into the VTA of TH-Cre transgenic mice a cre-inducible adeno-associated virus (AAV) with double-floxed inverted open reading frame (DIO) expressing genetically engineered channelrhodopsin-2 (ChR2) fused with mCherry, as ChR2 promotes the axonal transfer of mCherry. Because the TH neurons located within the anteromedial VTA provide TH-containing fiber to the lateral habenula (LHb) in the rat (Swanson, 1982), and because the TH mRNA (+)/TH-IR (−) neurons in the mouse are in the anteromedial VTA, we determined whether mesohabenular fibers expressing mCherry under the TH promoter co-expressed TH-IR. By double immunofluorescence, we found mesohabenular fibers containing high levels of mCherry

signal (Fig. 11 A'), but lacking detectable levels of TH-IR (Fig. 11 A''), indicating that the LHb is indeed targeted by TH mRNA (+)/TH-IR (−) neurons. In contrast, the VTA innervations to the nucleus accumbens (nAcc) containing mCherry (Fig. 11B, B') also contained TH-IR, which cell bodies were distributed within the lateral VTA in which TH mRNA (+)/TH-IR (+) neurons, rather than TH mRNA (+)/TH-IR (−) neurons, were present. While we consistently found mCherry labeled axons without TH-IR in the LHb, we found a great variability in the expression of TH-IR within the mCherry labeled neurons located in the medial aspects of the VTA, where TH mRNA (+)/TH-IR (−) were consistently detected in the wild type mice. The expression of TH-IR within mCherry-labeled cell bodies located in the midline nuclei may reflect induced translation of TH protein due to the genetic manipulation of the mice or due to the viral infectivity or synthesis of the complex mCherry-ChR2.

Within the RLi, the majority of neurons expressing TH mRNA co-expressed VGluT2 mRNA

As detailed above, the vast majority of RLi neurons that expressed TH-mRNA (≈ 90%) lacked detectable levels of TH-IR. Thus the lack of TH-IR in neurons that express TH-mRNA may explain, in part, the low numbers of VGluT2 mRNA (+)/TH-IR (+) neurons found in the RLi. To explore this possibility, we used a non-radioactive riboprobe to detect TH mRNA together with a radioactive VGluT2 probe. However, the non-radioactive probe allowed identification of many neurons containing TH mRNA in the PBP, PN and CLi, but none in the RLi. Because neurons expressing TH mRNA were found in the RLi when radioactive riboprobes were used (Fig. 7), we concluded that low levels of TH mRNA expression in the RLi were below the detectable levels permitted by non-radioactive probes. To overcome this limitation, we identified TH neurons by detecting Cre-induced mCherry in the VTA of TH::Cre mice. We confirmed by radioactive *in situ* hybridization that almost all mCherry expressing neurons with the A10 area co-expressed TH mRNA [99.2%; 473 TH mRNA (+) neurons from a total of 477 mCherry-IR (+) neurons]. By combination of mCherry immunolabeling and detection of VGluT2 mRNA, we found that in the RLi near to 68% of mCherry immunoreactive (mCherry-IR) neurons expressed VGluT2-mRNA [124 mCherry-IR (+)/VGluT2 (+) neurons from a total of 182 mCherry-IR (+) neurons]. These findings indicate that the majority of RLi neurons expressing TH mRNA have VGluT2 mRNA, but lack detectable levels of TH-protein.

Within the RLi, half of the neurons expressing VGluT2 mRNA co-express TH mRNA

To further characterize the VGluT2/TH neurons in the RLi within the total population of VGluT2 neurons, we identified VGluT2 expressing neurons by detecting Cre-induced mCherry in the VTA of VGluT2::Cre mice. By a combination of mCherry immunolabeling and detection of TH mRNA, we found that in the RLi about one half of the mCherry-IR neurons co-expressed TH-mRNA [20 TH mRNA (+)/mCherry-IR (+) neurons from a total of 51 mCherry-IR (+) neurons].

DISCUSSION

Most of our knowledge on cellular composition, neuronal connectivity and synaptic interactions of the neurons of the A10 area has been derived from studies in the rat

(Phillipson, 1979a; Swanson, 1982; German & Manaye, 1993; Sesack *et al.*, 2003; Margolis *et al.*, 2006; Geisler *et al.*, 2007; Ikemoto, 2007; Sesack & Grace, 2010; Morales & Pickel, 2012; Morales & Root, 2014; Yetnikoff *et al.*, 2014). However, with the increasing availability of tools for specific cellular targeting in genetically modified mice, the mouse is becoming the species of choice for studying the role of specific neuronal phenotypes in reward or aversion (Alsio *et al.*, 2011; Tan *et al.*, 2012; van Zessen *et al.*, 2012; Stamatakis *et al.*, 2013; Ilango *et al.*, 2014; Lammel *et al.*, 2014). Genetically modified mice (Cre recombinase-dependent) are also being used to determine VTA efferents by viral tracing methodologies (Hnasko *et al.*, 2012; Taylor *et al.*, 2014). The present findings reveal that expression of reporter genes under the control of the TH promoter in the mouse does not guarantee the selective manipulation or mapping of DA projections. This adds to rat evidence that TH, once the standard marker for DA neurons, can identify false positive neurons that express TH-protein, but do not package DA in vesicles (Li *et al.*, 2013) for synaptic or parasynaptic release.

The main difference between the rat and mouse A10 areas was found within the population of neurons expressing TH-protein. While all rat VTA neurons expressing TH-mRNA co-express detectable levels of TH-protein, many neurons in the mouse RLi and IF express TH-mRNA without detectable levels of TH-protein, some of which express VGluT2. As such, the mouse TH-mRNA (+)/TH-IR (−) neurons and the VGluT2-mRNA (+)/TH-mRNA (+)/TH-IR (−) can be genetically manipulated, and leading to the erroneous conclusion that DA is released from these neurons. Likewise, results from genetic manipulations under the control of the VGluT2-promoter maybe erroneously ascribed to glutamatergic-only neurons when in truth they should be ascribed to VGluT2-TH neurons. At present the target sites of the mouse VGluT2-mRNA (+)/TH-mRNA (+)/TH-protein (−) neurons of the RLi and IF are unknown. However, a recent study has shown that RLi and IF neurons expressing mCherry under the VGluT2 promoter and lacking TH-immunolabeling project to the ventral pallidum, bed nucleus of the stria terminalis, the magnocellular preoptic nucleus and the olfactory tubercle (Taylor *et al.*, 2014). Thus, these structures could potentially be targeted by both VGluT2-only (which make half of the VGluT2 neurons in the RLi) and VGluT2-mRNA (+)/TH-mRNA (+)/TH-protein (−) neurons (which make ≈70% of the TH-mRNA neurons in the RLi).

VGluT2-only and VGluT2-TH neurons in mouse and rat A10 area

The rat and the mouse contain VGluT2 mRNA expressing neurons in the lateral and medial nuclei of the A10 area. The mouse VGluT2 neurons, like those of the rat, have a major subpopulation of neurons expressing VGluT2 mRNA but lacking TH-IR (VGluT2-only neurons) and a smaller subpopulation of neurons co-expressing VGluT2 mRNA and TH-IR [VGluT2/TH-IR neurons].

The rat and mouse VGluT2 mRNA expressing neurons each have a decreasing lateral-to-medial density gradient in which the lateral aspects of PBP and PN contain VGluT2-only neurons, and the medial aspects (in addition to the VGluT2-only neurons) contain VGluT2 /TH-IR neurons. Despite the qualitative similarity, the proportions of VGluT2/TH-IR neurons within the VGluT2 population in the rat and mouse are different. In the rat the

subpopulation of VGluT2/TH-IR neurons represents ≈25% of the neurons expressing VGluT2 mRNA in the lateral PBP, lateral PN and RLi; ≈ 30% in the IF; and ≈20% in the CLi (Yamaguchi *et al.*, 2011). In contrast, the mouse subpopulation of VGluT2 /TH-IR neurons, with exception of the CLi, is much smaller than that found in the rat. The mouse VGluT2/TH-IR neurons represent ≈10% of the neurons expressing VGluT2 in the lateral PBP-PN; ≈ 1% in the RLi; ≈ 7% in the IF. The only VTA subregion in which the rat and mouse proportion of VGluT2 /TH-IR neurons is similar is the CLi, where in both cases the VGluT2/TH-IR neurons represent ≈ 20% of the neurons expressing VGluT2.

Within the subdivisions of the A10 area in which VGluT2 neurons are present, the proportion of VGluT2/TH-IR neurons with the TH-IR population also differs between the rat and mouse. We have found by both *in situ* hybridization and single cell qRT-PCR analysis that almost half of the TH-IR neurons in the rat co-express VGluT2 mRNA in the medial PBP, medial PN, RLi and rostral IF (Yamaguchi *et al.*, 2011). In contrast, the frequency of VGluT2/TH-IR neurons is much lower (≈ 4% in the lateral PBP-PN; ≈ 10% in the RLi; ≈17% in the IF; and ≈ 15% in the CLi). Thus we conclude that under normal conditions, most of VGluT2 neurons in the A10 area of the adult rat and adult mouse do not have the capability to co-release dopamine, and that the proportion of A10 neurons with the capability of this dual co-release is lower in the mouse than in the rat.

TH-immunoreactive neurons in the mouse and rat A10 areas

TH immunoreactivity has been used over the last 40 years as the primary marker for the identification of DA neurons in the midbrain (Morales & Pickel, 2012). Recent studies indicate that in the rat A10 area all neurons expressing TH mRNA have TH-immunoreactivity and have the capability to synthesize DA, as all of them co-express aromatic L-amino acid decarboxylase (Li *et al.*, 2013). However some of these neurons do not have detectable levels of proteins or transcripts encoding molecules for the vesicular transport of DA (vesicular monoamine transporter 2, VMAT2) or its reuptake (DAT). The DA neurons lacking both VMAT2 and DAT signals are restricted to the midline nuclei (CLi, RLi and IF), nuclei for which signaling properties and projection targets remain to be determined (Li *et al.*, 2013). While all TH-mRNA neurons in the rat A10 area have detectable levels of TH-protein (detected by immunolabeling), the mouse CLi and IF nuclei have many neurons expressing TH-mRNA that lack detectable levels of TH-protein. The lack of TH-protein in the mouse neurons may reflect the presence of a repression mechanism that limits its translation. In any case, the TH neurons within the midline nuclei of the A10 area in both the rat and the mouse are heterogeneous in their molecular composition. In contrast to the midline nuclei, DA neurons of the lateral aspects of the A10 area and neighboring substantia nigra appear to be less heterogeneous (Li *et al.*, 2013).

In summary, we found that, in common with the rat, the mouse A10 area contains VGluT2, TH and VGluT2/TH neurons. However, unlike the A10 area of the rat, the mouse A10 area contains a group of TH and VGluT2/TH neurons that do not seem to synthesize DA in the adult under normal conditions, as they lack detectable levels of TH protein. Future studies are necessary to determine the extent to which the differences in TH neuronal composition between the rat and mouse A10 areas have functional significance.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute on Drug Abuse. Research was planned and carried by Yamaguchi, Qi and Morales. Yamaguchi, Qi, Wang, Zhang and Morales analyzed data. Morales wrote the paper with the contribution of all the authors. The authors declare that they do not have any conflicts of interest related to the data presented in this manuscript. We thank Bing Liu for her help in processing brain tissue.

Abbreviations

CLi	caudal linear nucleus
DA	dopamine
fr	fasciculus retroflexus
IF	interfascicular nucleus
IP	interpeduncular nucleus
IR	immunoreactive
ml	medial lemniscus
mp	mammillary peduncle
MT	medial terminal nucleus of the accessory optic tract
PB	phosphate buffer
PBP	parabrachial pigmented
PF	paraformaldehyde
PN	paranigral nuclei
RLi	rostral linear nucleus of the raphe
RMC	red nucleus magnocellular part
RT	room temperature
scp	superior cerebellar peduncle
SNC	substantia nigra compacta
TH	tyrosine hydroxylase
VGluT1	vesicular glutamate transporter type 1
VGluT2	vesicular glutamate transporter type 2
VGluT3	vesicular glutamate transporter type 3
VTA	ventral tegmental area

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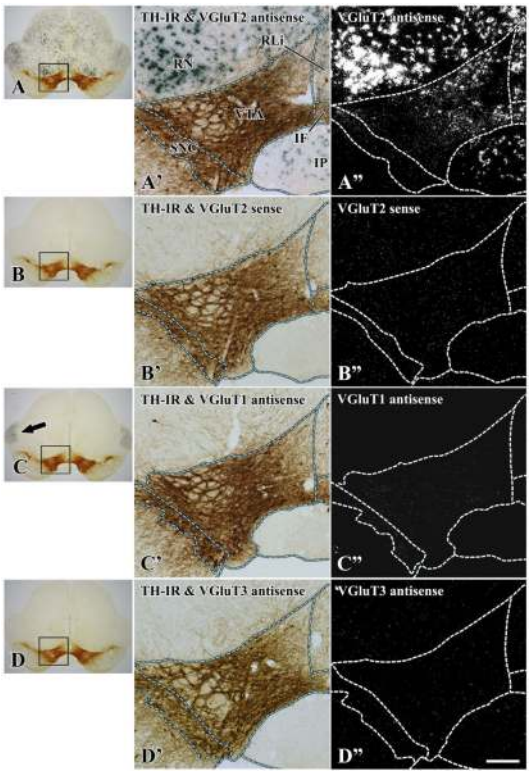

Figure 1. Cellular expression of VGluT2 mRNA in the mouse A10 area, but lack of expression of either VGluT1 or VGluT3 mRNAs (radioactive *in situ* hybridization)

Coronal sections incubated with anti-TH antibodies and hybridized with either VGluT2 antisense (A-A"), VGluT2 sense (B-B"), VGluT1 antisense (C-C") or VGluT3 antisense (D-D") radioactive riboprobes. A-D, Coronal sections at low magnification under bright-field microscopy showing TH immunoreactivity (TH-IR, dark brown label). Delimited areas in A to D are shown at higher magnification in A'-D' under bright-field microscopy for visualization of TH-IR in the A9 (substantia nigra compacta, SNC) and the A10 areas [ventral tegmental area (VTA), rostral linear nucleus of the raphe (RLi) and interfascicular nucleus (IF)]. The same delimited areas are shown under epiluminescence microscopy for visualization of cells expressing transcripts encoding VGluT2 (A''), VGluT1 (C'') or VGluT3 (D"). A", Antisense VGluT2 riboprobe detected cells expressing VGluT2 mRNA (silver white grains) in the VTA, RLi, IF and neighboring structures (red nucleus; RN and interpeduncular nucleus; IP). Lack of signal in the A10 area in sections hybridized with either sense VGluT2 (B"), antisense VGluT1 (C") or antisense VGluT3 (D"). High expression of VGluT1 mRNA is seen in the medial geniculate nucleus (arrow in C). Bregma −3.52 mm. Scale bar shown in D" is 1200 μm for A-D, and 175 μm for A'-D".

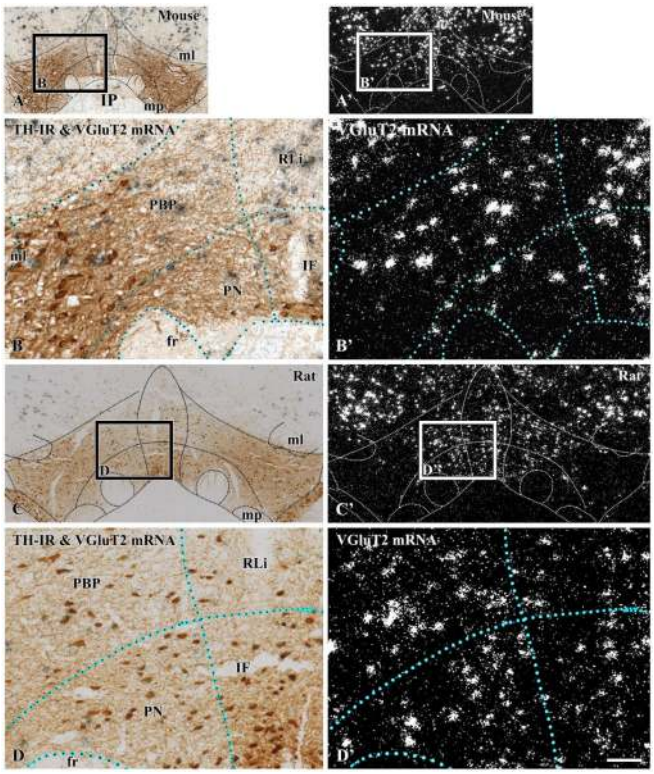

Figure 2. Comparative cellular expression of VGluT2 mRNA and TH-IR in the mouse and the rat A10 areas

Coronal section of mouse (A-B', bregma −3.16 mm) or rat (C-D', bregma −5.40 mm) were incubated with anti-TH antibodies and hybridized with a VGluT2 antisense radioactive probe. B, Mouse TH-IR neurons are mostly detected in the PBP and IF, but rarely in the PN or RLi. B', Mouse neurons expressing VGluT2 mRNA are more prominent in the medial aspects of the A10 area. D, In contrast to the mouse, TH-IR neurons are presents in all aspects of the A10 area, including the RLi and PN. D', The rat VGluT2 neurons, as the mouse VGluT2, are more concentrated in the medial aspects of the A10 area. fr, fasciculus retroflexus; ml, medial lemniscus; mp, mammillary peduncle. Scale bar shown in D' is 300 μm for A, A', C, C' and 62.5 μm for B, B', D, D'.

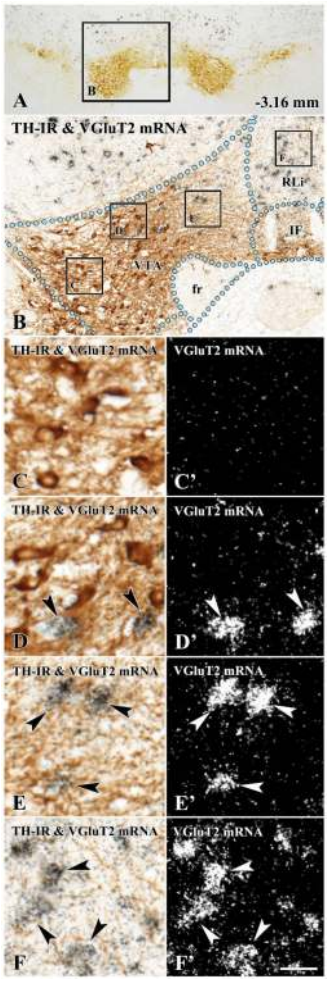

Figure 3. Medio-lateral gradient of distribution of neurons expressing VGluT2 mRNA or TH-IR in the mouse A10 area

A-B, Low (A) and higher magnification (B) of a coronal section with TH-IR. The squares in B delimit areas shown at higher magnification (C-F'). Lack of detection of neurons expressing VGluT2 mRNA in the lateral VTA (C-C'). Neurons expressing VGluT2 mRNA (arrowheads) are seen within the medial VTA (D-E') and RLi (F-F'). Note in the RLi and adjacent medial VTA the presence of TH-IR fibers, but lack of TH-IR cell bodies. Bregma −3.16 mm. Scale bar shown in F' is 560 μm for A, 110 μm for B, and 25 μm for C-F'.

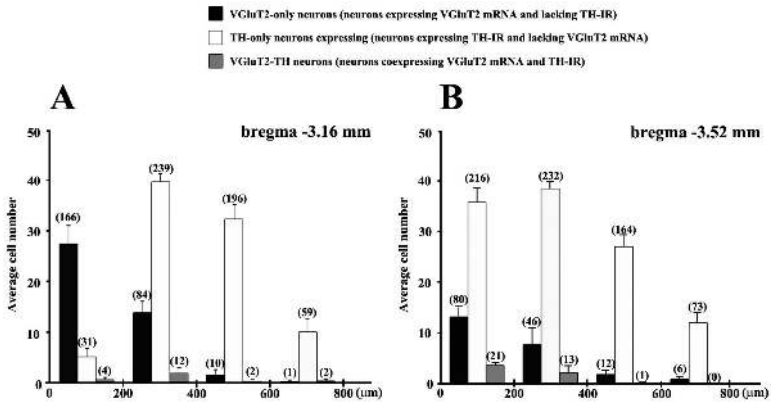

Figure 4. Medio-lateral gradient of distribution of VGluT2, TH-IR and VGluT2 (+)/TH-IR (+) neurons in the mouse A10 area

Bar graphs represent the average number (mean + SEM) of three different classes of neurons: VGluT2-only neurons (expressing VGluT2 mRNA and lacking TH-IR), TH-only neurons (expressing TH-IR and lacking VGluT2 mRNA), and VGluT2 (+)/TH-IR (+) neurons (co-expressing VGluT2 mRNA and TH-IR). The 3 different classes of neurons were counted from the medial (border between the VTA and RLi or between the VTA and IF) to the lateral aspects of the VTA within 4 segments (0-200 μm, 200-400 μm, 400-600 μm, and 600-800μm) from 3 mice at bregma −3.16 mm (A) and −3.52 mm (B). Most of the VGluT2 expressing neurons lack TH-IR and have a decreasing medio-lateral distribution. Total counted neurons are indicated between parentheses.

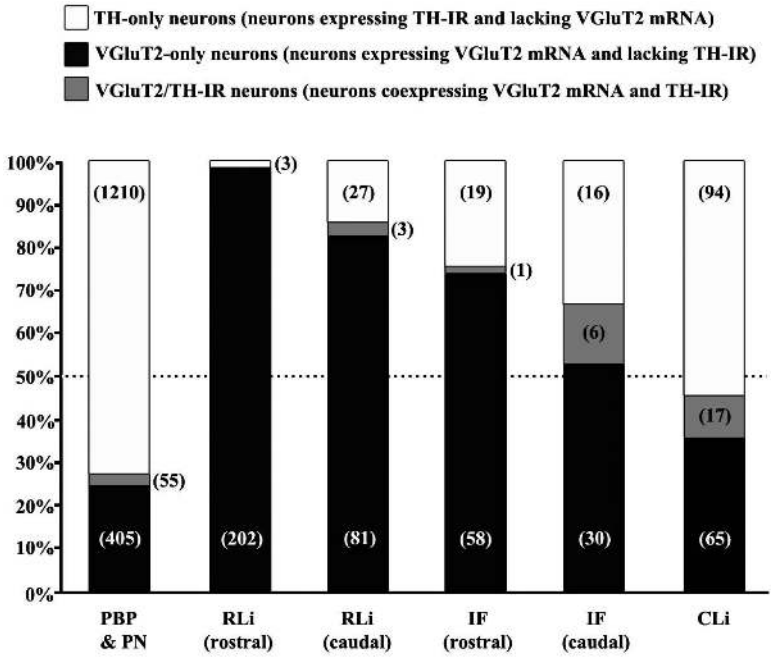

Figure 5. Distribution of neurons expressing VGluT2 mRNA and their degree of TH-IR co-expression in the mouse A10 area

The VGluT2-only (black bars) and the TH-only (white bars) neurons were found in all subdivisions of the mouse A10 area, but with different ratio. In the PBP-PN, the TH-only neurons were about 3 times more abundant than the VGluT2-only neurons. In contrast, the TH-only neurons were fewer than the VGluT2-only neurons in the RLi and IF. In the CLi, the TH-only were about 1.5 times more abundant than the VGluT2-only. Within the PBP/PN, RLi and IF rostral, the subpopulation of VGluT2 (+)/TH-IR (+) neurons (grey bars) was a small fraction of the total subpopulation of neurons that expressed either VGluT2 mRNA or TH-IR. However, about one fifth of the VGluT2 neurons co-expressed TH-IR in the CLi.

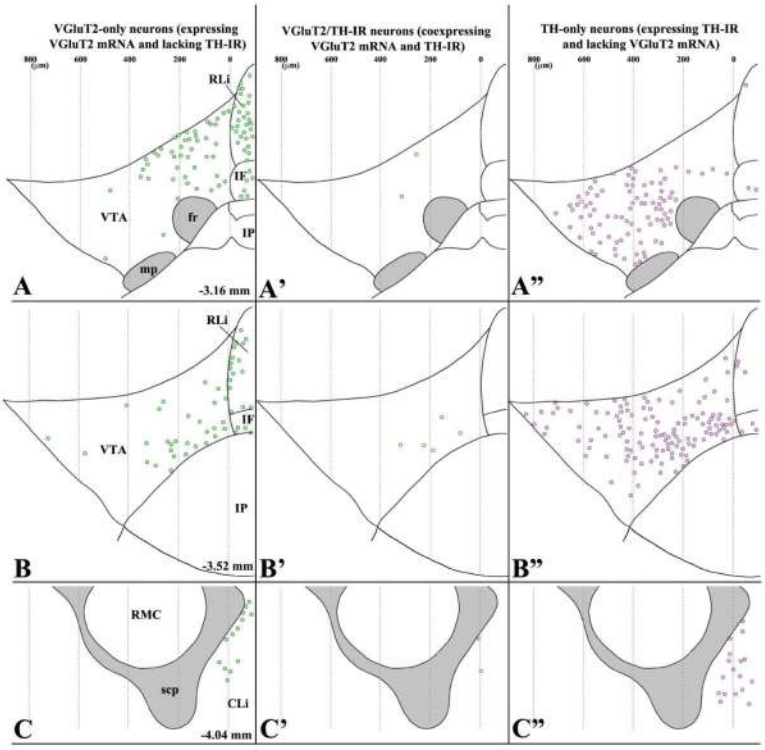

Figure 6. Summary diagram of the distribution of VGluT2-only neurons, TH-only neurons and VGluT2 (+)/TH-IR (+) neurons within each subdivision of the mouse A10 area

A-C, Distribution of VGluT2-only neurons (neurons expressing VGluT2 mRNA and lacking TH-IR). These VGluT2-only neurons are present throughout the rostro-caudal levels of each subdivision of the A10 area with a latero-medial increasing gradient of concentration. In the most lateral aspects of the VTA, VGluT2-only neurons are infrequent, but TH-only neurons are highly concentrated (see A" and B"). A'-C', Distribution of VGluT2 (+)/TH-IR (+) neurons (neurons co-expressing VGluT2 mRNA and TH-IR). A"-C", Distribution of TH-only neurons (neurons lacking expression of VGluT2 mRNA but containing TH-IR). The TH-only neurons are present throughout the rostro-caudal levels of each subdivision of the A10 area. Each panel represents the average number of labeled neurons found in 3 sections, each section from a different mouse. CLi, caudal linear nucleus; fr, fasciculus retroflexus; IF, interfascicular nucleus; IP, interpeduncular nucleus; mp, mammillary peduncle; RLi, rostral linear nucleus of the raphe; RMC, red nucleus magnocellular part; scp, superior cerebellar peduncle; VTA, ventral tegmental area.

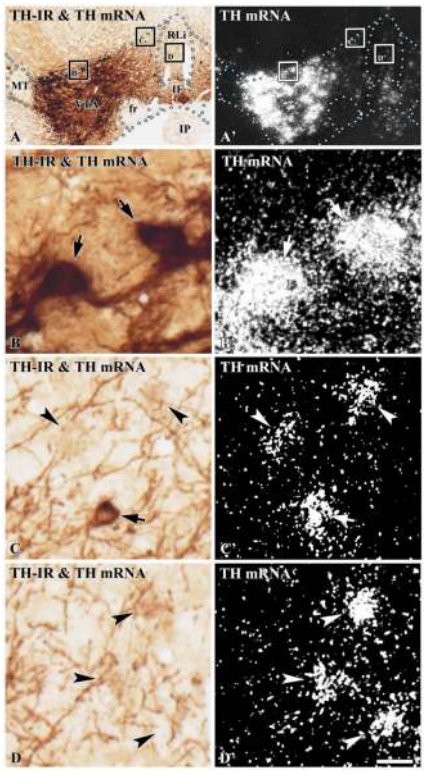

Figure 7. Identification of two distinct subpopulations of neurons expressing TH mRNA in the mouse A10 area

Coronal sections incubated with anti-TH antibodies and hybridized with a TH antisense radioactive riboprobe. A and A', Low magnification of a coronal section is seen under bright field for visualization of TH-IR (A), and under epiluminescence microscopy for visualization of neurons expressing TH mRNA (A'). Squares in A and A' delimit areas shown at higher magnification (B-D'). B-B', Lateral VTA, the majority of TH-IR neurons express high levels of TH mRNA (arrows). C-C', Medial VTA, neurons expressing TH mRNA with (arrows) or without TH-IR (arrowheads). D-D', RLi, neurons expressing TH mRNA lack TH-IR (arrowheads). MT, medial terminal nucleus of the accessory optic tract. Bregma −3.16 mm. Scale bar shown in D' is 200 μm for A-A' and 20 μm for B-D'.

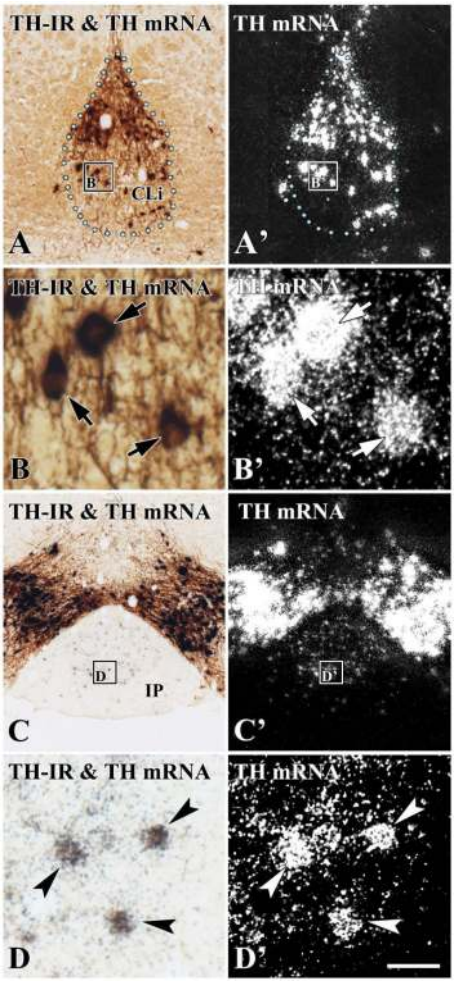

Figure 8. Mouse cellular co-expression of TH mRNA and TH-IR in the CLi, and detection of neurons expressing TH mRNA in the IP

Coronal sections incubated with anti-TH antibodies and hybridized with a TH antisense radioactive riboprobe. A-A' and C-C', Low magnification of coronal sections are seen under bright field for visualization of TH-IR (A and C), and under epiluminescence microscopy for visualization of neurons expressing TH mRNA (A' and C'). Delimited areas in A-A' and C-C' are shown at higher magnification under bright field (B and D) and under epiluminescence microscopy (B' and D'). B-B', CLi has TH-IR neurons co-expressing TH mRNA (arrows) (−4.04 mm from bregma). D-D', In contrast, IP neurons expressing TH mRNA lack TH-IR (arrowheads) (−3. 52 mm from bregma). Scale bar shown in D' is 250 μm for A-A' and C-C', and 25 μm for B-B' and D-D'.

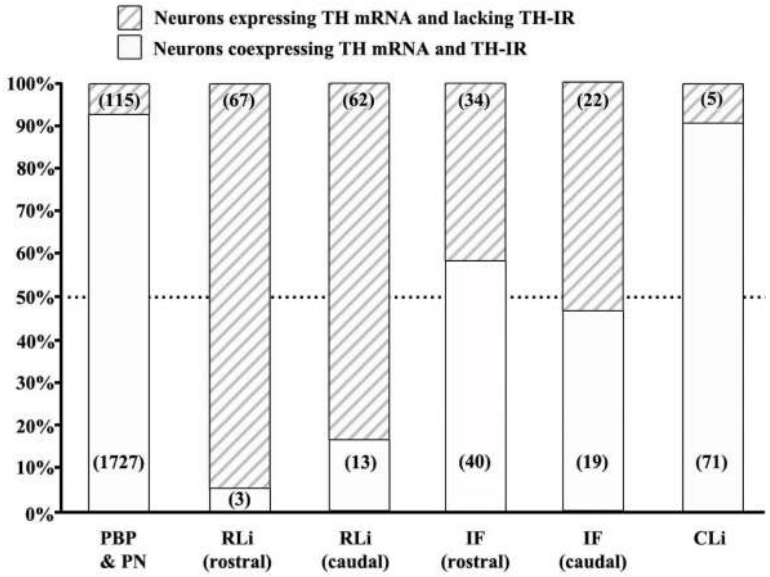

Figure 9. Distribution of neurons expressing TH mRNA and their degree of TH-IR co-expression in the mouse A10 area

Neurons expressing TH mRNA without detectable levels of TH-IR were found in all subdivisions of the mouse A10 area with different ratios. More than 90% of neurons expressing TH mRNA also contained detectable levels of TH-IR in the PBP/PN and CLi. In contrast, the vast majority of neurons expressing TH mRNA in the RLi (83-96%) lacked detectable levels of TH-IR. About half of the neurons expressing TH mRNA in the IF (49%) also lacked TH-IR.

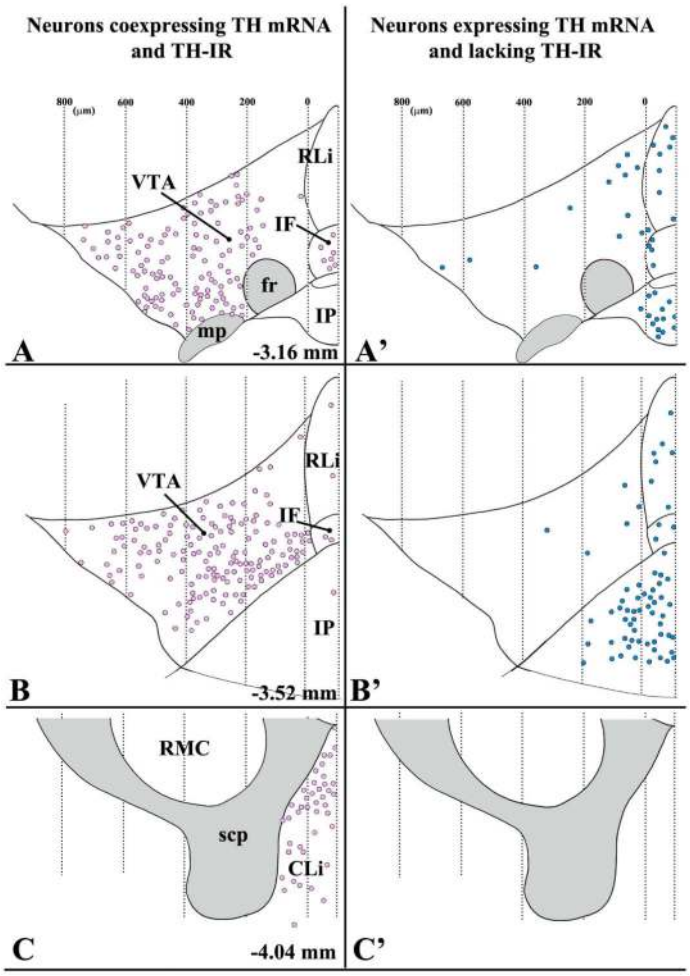

Figure 10. Summary diagram of the distribution of TH mRNA expressing neurons that either co-express or lack TH-IR in the mouse A10 area

A-C, Neurons co-expressing TH-mRNA and TH-IR are frequent in the VTA, IF and CLi, but infrequent in the RLi and IP. A'-C', Neurons expressing TH mRNA without detectable levels of TH-IR are infrequent in the VTA, but prominent in the RLi and IP. Each panel represents the average number of labeled neurons found in 3 sections, each section from a different mouse.

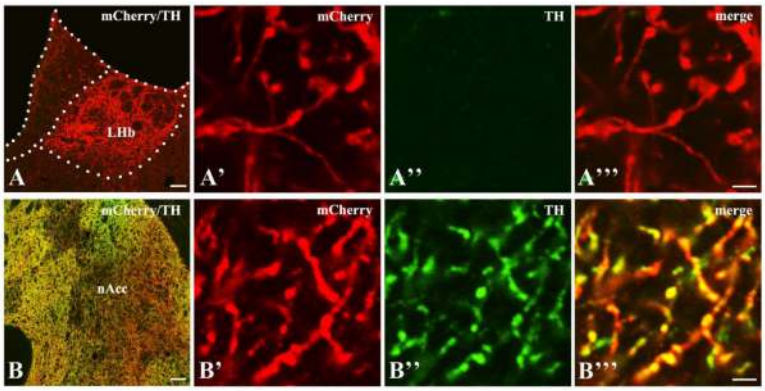

Figure 11. Mesohabenular, but not mesoaccumbens, fibers from TH neurons lack TH-IR

A, Fibers expressing mCherry under the TH promoter lacking TH-IR are present in the lateral habenula (LHb). A'-A''', LHb at high magnification showing mCherry (red) fibers lacking TH-IR (green). B, Fibers expressing mCherry under the TH promoter expressing TH-IR are present in the nucleus accumbens (nAcc). B'-B''', nAcc at high magnification showing mCherry individual fibers co-expressing TH-IR. Scale bars shown in A and B are 50 μm, and in A''' and B''' are 2 μm.