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Considerations when using Cre-driver rodent lines for studying ventral tegmental area circuitry

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SUMMARY

The use of Cre-driver rodent lines for targeting ventral tegmental area (VTA) cell types has generated important and novel insights into how precise neurocircuits regulate physiology and behavior. While this approach generally results in enhanced cellular specificity, an important issue has recently emerged related to the selectivity and penetrance of viral targeting of VTA neurons using several Cre-driver transgenic mouse lines. Here, we highlight several considerations when utilizing these tools to study the function of genetically defined neurocircuits. While VTA dopaminergic neurons have previously been targeted and defined by the expression of single genes important for aspects of dopamine neurotransmission, many VTA and neighboring cells display dynamic gene expression phenotypes that are partially consistent with both classically described dopaminergic and non-dopaminergic neurons. Thus, in addition to varying degrees of selectivity and penetrance, distinct Cre lines likely permit targeting of partially overlapping, but not identical VTA cell populations.

Accurately defining and studying the function of distinct neural subtypes and circuits represents one of the major challenges in neuroscience today. Cell types can be defined by a variety of means such as anatomical location, afferent and efferent connectivity, electrophysiological properties, behavioral functions, or by their molecular characteristic such as their gene expression profiles. Given the intense heterogeneity of nervous system

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- AMS performed experiments and analyzed data.
- PAK performed experiments.

tissue, many recent studies have utilized genetic tools, such as rodent lines, that express Cre-recombinase under selected gene promoters (Gerfen et al., 2013; Taniguchi et al., 2011; Witten et al., 2011) in order to selectively monitor or manipulate neuronal subpopulations. Furthermore, genetically defined neurons that express Cre-recombinase can reliably be targeted for optogenetic or chemogenetic manipulation in anatomically defined brain regions in adult animals by utilizing cre-inducible viral constructs (Atasoy et al., 2008; Sohal et al., 2009) that are stereotaxically delivered to the brain region of interest. Overall, this combined approach has generated tremendous insight into the organization and function of genetically defined neural circuits. These tools have widely been adopted and are now commonly used throughout many distinct subfields of neuroscience. Below we discuss some important aspects related to the use of these approaches within the context of the ventral tegmental area (VTA), a heterogeneous brain region critical for motivated behavior.

The VTA contains many distinct cell types, the two largest classically described populations being dopaminergic and GABAergic neurons that are largely intermixed. A smaller population of VTA glutamatergic neurons has also been identified (Yamaguchi et al., 2007, 2011). VTA dopaminergic neurons have been targeted for manipulation by utilizing mouse lines that express Cre recombinase under the control of gene promoters thought to define a dopaminergic phenotype. The two most commonly used lines drive expression under the tyrosine hydroxylase promoter (TH-Cre; (Lindeberg et al., 2004; Savitt et al., 2005) or the dopamine transporter promoter (DAT-Cre; (Bäckman et al., 2006; Zhuang et al., 2005)). While these are excellent genetic tools to gain further insight into VTA circuits, the presence of a single gene, such as TH or DAT, alone within a neuron contributes to, but does not solely define its phenotype. Consistent with this, dopaminergic neurons have previously been shown to be quite heterogeneous with respect to their projection targets, gene expression profiles, and physiological properties (Lammel et al., 2008; Margolis et al., 2006, 2008).

Lammel et al., report that TH-Cre or GFP mouse lines display ectopic targeting of non-dopaminergic VTA neurons. Below we highlight important conceptual and technical caveats for the use of Cre driver lines to target VTA circuitry. In addition, we discuss the idea that individual neurons are more accurately described by a phenotypic spectrum as opposed to a digital classification system, especially when defining cell populations based on the expression patterns of single genes. This is largely based on evidence that individual VTA cells can co-express genes or immunostain for markers associated with the biosynthesis of multiple neurotransmitters, and that the expression levels of TH and DAT can vary dramatically across individual VTA neurons or animal strain, suggesting that TH-Cre and DAT-Cre transgenic mouse lines label partially overlapping, but not identical midbrain cell populations.

Response to the Lammel et al paper

Lammel et al. report that many midbrain cells targeted in TH-Cre or TH-GFP lines are not TH-positive and that targeted neurons are located outside of the VTA/SNc. They conclude that appreciable ectopic expression exists in these TH-driven transgenic lines, but not in the DAT-cre mouse line that was also tested. In addition, they conclude that VTA neurons that

project to the lateral habenula (LHb) are largely GABAergic. Based on the data and conclusions presented in their paper, there are three points that we think merit further discussion.

1) Selectivity and penetrance of transgene expression in TH-Cre lines and DAT-Cre mouse lines

Lammel et al., report that following Cre-inducible virus injection into the VTA of TH-Cre or DAT-Cre mice, there is appreciable viral-mediated expression in both TH positive and TH negative neurons in and around the VTA in the TH-Cre line, while viral expression is restricted to VTA TH positive neurons in the DAT-Cre line. In addition, in the lateral VTA there is high co-localization between TH and targeted neurons in the TH-Cre line, but co-localization is significantly less in the medial VTA in this line. These results may be due to ectopic cre expression in the medial VTA in the TH-Cre lines (cre expression in cells that are TH negative) or due to sufficient cre expression in neurons only weakly expressing TH mRNA or protein. Consistent with the latter, TH expression and immunofluorescence can vary widely from cell-to-cell and along the medial-lateral axis (Harris and Nestler, 1996; Weiss-Wunder and Chesselet, 1991, Fig. 1), and thus it is possible that immunofluorescent staining may not reliably label neurons that produce low levels of TH protein. This may be less of an issue with the DAT-Cre mouse line since DAT is enriched in cells that also express high levels of TH (Lammel et al., 2008; Stamatakis et al., 2013). It is important to note that Cre-mediated recombination can likely occur in cells with even weakly active promoters and thus low levels of Cre, since virally mediated Cre-inducible transgene expression is decoupled from the levels of expression at the endogenous genetic locus (Atasoy et al., 2008; Sohal et al., 2009). Moreover, it is unclear whether viral titre or injection volume may also influence targeted cell selectivity since Cre-inducible expression systems likely depends not only on the amount of Cre present in a cell, but also on the number of virally delivered transgenes that undergo recombination. Therefore, the discrepancy between the percentage of overlap between eYFP+ and TH+ neurons reported in Lammel et al. and previous studies (Chaudhury et al., 2013; Stamatakis et al., 2013; Tsai et al., 2009; Tye et al., 2013) could be due to differences in immunohistochemical techniques and confocal imaging parameters in addition to the quantification of particular VTA subregions as they discussed.

In wild type animals TH positive neurons are also distributed in areas surrounding the VTA/SNc including the retrorubral field and periaquaductal gray (Nair-Roberts et al., 2008), and to a lesser extent, the substantia nigra pars reticulata (Stork et al., 1994). In fact, the A10 dopaminergic cell group has been previously described to contain a dense extension of TH positive neurons that reside in the tuberomamillary nucleus and the medial supramammiliary region (Swanson, 1982). Therefore, it is not particularly surprising that Lammel et al. report the presence eYFP+ neurons in areas surrounding the VTA in the TH-Cre line, especially considering the large volumes of virus injected in their study. In contrast, DAT expression is largely restricted to TH positive neurons in VTA/Sn, but does not co-localize completely with TH positive neurons in the VTA (Blanchard et al., 1995; Li et al., 2013). Thus, it is likely that DAT-Cre and TH-Cre mouse lines permit targeting of overlapping, but not identical, populations of VTA neurons. The TH-Cre lines thus may display reduced

selectivity for dopaminergic neurons (as defined by expressing high levels of both TH and DAT protein), especially in the medial VTA compared to the DAT-Cre line as reported by Lammel et al. However, the DAT-Cre line may have reduced penetrance for targeting dopaminergic neurons compared to the TH-Cre lines as non-striatal projecting TH+ VTA cells display reduced levels of DAT compared to nucleus accumbens projecting cells (Lammel et al., 2008). Both selectivity and penetrance of transgene expression are important factors to consider, and should be evaluated collectively based on the purpose of the experiment.

2) Cellular Phenotype of LHb projecting VTA neurons

In the manuscript by Lammel et al., they report that LHb projecting VTA neurons are GABAergic or glutamatergic, and that these LHb projections act to inhibit or excite LHb postsynaptic neurons respectively, which in turn promotes reward or aversive-related behavior. These results are consistent with our previous work (Stamatakis et al., 2013) as well the work of others (Hnasko et al., 2012; Root et al., 2014a, 2014b). Moreover, In our previous paper (Stamatakis et al., 2013) we reported that virally targeting of VTA neurons in the TH-Cre line resulted in labeled fibers in the LHb that release GABA to inhibit LHb neurons to promote reward. In addition, retrograde labeling of LHb projecting VTA neurons in mice and rats demonstrates that this projection arises from a mixture of TH positive and TH negative VTA neurons (Gruber et al., 2007; Skagerberg et al., 1984; Stamatakis et al., 2013; Swanson, 1982). Additionally, *in situ* hybridization experiments revealed that over 20% of LHb projecting VTA neurons co-express *TH*, *GAD*, and *VGlut2* (Root et al., 2014b). Thus, LHb projecting VTA neurons are genetically diverse and express markers consistent with multiple neurotransmitter systems, which likely regulates the functionality of this circuit.

We have previously referred to LHb projecting VTA neurons as having a hybrid phenotype, which is consistent with gene expression data from these cells (Root et al., 2014b). However, as we previously demonstrated (and was confirmed by Lammel et al.) the targeted VTA-to-LHb projection in the TH-Cre line is largely functionally GABAergic and acts to inhibit LHb neurons. Interestingly, while Lammel et al. did not observe significant eYFP fibers in the LHb from the DAT-Cre mice, we observed appreciable eYFP fibers in this line following virus injection in the VTA (Fig. 2). We also observed a qualitatively different expression pattern from Vgat-Cre mice (Fig. 2). Collectively, these data (as well as work by Morales and colleagues (Root et al., 2014a, 2014b), demonstrate that LHb-projecting VTA neurons are genetically heterogeneous, and cannot be simply classified as strictly dopaminergic, GABAergic or glutamatergic based on single genetic markers as proposed by Lammel et al. Instead, we argue that the most accurate description of VTA neurons that project to the LHb would be a mixture of both functional GABAergic and glutamatergic neurons, some of which also express genetic markers for dopaminergic neurons. This assessment is based on experiments conducted in both wild type and transgenic animals and is not dependent on any single Cre driver line.

3) Electrophysiological properties of LHb projecting VTA neurons

Lammel et al. recorded from eYFP+/TH+ and eYFP+/TH− VTA neurons in the TH-Cre line (which may have low to no TH expression and protein, as discussed above) and report that eYFP+/TH− neurons have electrophysiological characteristics that are similar to GABAergic neurons and thus these cells are likely ectopically labeled GABAergic neurons and not dopaminergic neurons. However, the electrophysiological phenotype of these neurons is also consistent with medial dopaminergic neurons that show electrophysiological properties similar to GABAergic neurons. Indeed, Lammel and colleagues have previously reported that cortical-projecting dopaminergic neurons, which are enriched in the medial VTA, show elevated basal firing rates, narrow action potentials, lack functional D2 autoreceptors, and lack of an inward hyperpolarizing current (Lammel et al., 2008). Thus the electrophysiological phenotypes of VTA neurons may be more related to their projection target and location within the VTA, than whether they immunostain for TH.

Co-localization and heterogeneity of dopaminergic, glutamatergic, and GABAergic cell markers within VTA neurons: Implications for utilizing single-gene Cre driver lines to study neurocircuitry

As discussed above, some VTA neurons express markers consistent with the synthesis and release of multiple small molecule neurotransmitters. For example up to 20% of VTA TH positive neurons also express the gene coding for the vesicular glutamate transporter 2 (VGLUT2), a marker for putative glutamatergic neurons (Hnasko et al., 2010, 2012; Kawano et al., 2006; Li et al., 2013; Mendez et al., 2008; Root et al., 2014b; Yamaguchi et al., 2011) Consistent with this, optical stimulation of dopaminergic fibers that innervate striatal subregions results in the release of dopamine, glutamate, and GABA (Stuber et al., 2010; Tecuapetla et al., 2010; Tritsch et al., 2012). Thus, there is evidence in the literature that previously defined midbrain dopaminergic neurons can express genes and display physiological properties that are consistent with multiple modalities of neurotransmission. Furthermore, there is emerging evidence that some individual midbrain neurons may express dopaminergic and GABAergic gene markers (Root et al., 2014b). In the lateral VTA and substantia nigra pars compacta (SNc) TH expressing neurons and those that express of the GABA synthetic enzymes (GAD65 and GAD67) are almost completely segregated (Nair-Roberts et al., 2008; Tritsch et al., 2014; Fig. 3). However, in the anterior medial VTA and surrounding areas including the tuberomamillary nucleus, TH or DAT and GAD are co-expressed in a number of cells ((Olson and Nestler, 2007; Root et al., 2014b; Tritsch et al., 2014; Fig. 3). Finally, a recent study has demonstrated that the majority of LHb-projecting VTA neurons, some of which express TH, release both glutamate and GABA (Root et al., 2014b). Collectively, these emerging findings suggest that single gene marker such as TH, DAT, VGLUT2, or GAD65/67 cannot fully define the molecular phenotype of VTA neuronal subpopulations. This is especially important to consider when targeting VTA circuitry with a rodent line that expresses Cre under the control of a single gene promoter.

Additional considerations and future directions

Accurately defining and manipulating exact cellular phenotypes in the nervous system requires comprehensive information on the connectivity, physiological function, anatomical

location, and genetic landscape of numerous individual neurons sampled from a larger population. Applying automated and non-biased cluster analysis approaches to these integrated, but diverse datasets may uncover more specific cellular phenotypes. Future studies that implement these approaches promise to provide a more accurate definition of the cellular diversity within the VTA and other brain structures, and also permit for the implementation of intersectional genetic approaches (Dymecki et al., 2010; Robertson et al., 2013) for targeted manipulation of cell types based on combinatorial gene expression patterns (Fenno et al., 2014). Finally, it is important to consider that neuronal phenotypes are likely dynamically regulated and not static. Developmental, environmental, activity-dependent, and epigenetic regulation likely produce pronounced alterations in neuronal phenotypes and neurocircuit function (Andersson et al., 2006; Dulcis et al., 2013; Gómez-Lira et al., 2005; Guemez-Gamboa et al., 2014; Matsushita et al., 2002; Stott et al., 2013; Telese et al., 2013). Genetically informed neuronal targeting strategies continue to generate new insights. Corroborating these findings with a multifaceted approach will provide a more holistic understanding of the functional cellular architecture of the nervous system.

Methods

Methods were adapted as previously described (Stamatakis et al., 2013). Briefly, **Vgat-ires-Cre:** (*Slc32a1tm2(cre)Lowl*/J, Jackson Laboratory stock number: 016962, **Dat-ires-Cre:** (B6.SJL-*Slc6a3tm1.1(cre)Bkmn*/J, Jackson Laboratory stock number: 006660, and **TH-ires-Cre:** (B6.129X1-Thtm1(cre)Te/Kieg, European Mouse Mutant Archive stock number: *EM: 00254*) were used. Mice were injected bilaterally with AAV-DIO-ChR2-eYFP, (AAV5 ~10^{12} infectious units/mL, packaged and titered by the UNC Vector Core Facility).

Immunohistochemistry

Mice were anesthetized with pentobarbital, and transcardially perfused with PBS followed by 4% (w/v) paraformaldehyde in PBS. Brains were then removed and submerged for 24 hr in 4% paraformaldehyde and transferred to 30% sucrose in ddH2O for 48hr. 40 um brain slices were obtained and subjected to immunohistochemical staining for neuronal cell bodies (NeuroTrace Invitrogen; 435-nm excitation/455-nm emission), and/or tyrosine hydoylase (Pel Freeze; made in sheep, 1:500). Brain sections were mounted, and z-stack and tiled images were captured on a Zeiss LSM 710 confocal microscope using a 20× or 63× objective. For fluorescent intensity analysis, images were acquired using identical pinhole, gain, and laser settings for all brain regions.

In situ hybridization

A 950 bp riboprobe complimentary to GAD67 sense cDNA was inserted into the pcrII-TOPO vector (Life Technologies, Carlsbad, CA). Plasmid DNA was cut with either EcoRV or Asp718 in order to create template for *in vitro* transcription. All probes were created using Fluorescein-labeled nucleotides. The EcoRV template was transcribed with Sp6 RNA Polymerase to generate the sense riboprobe and the Asp718 template was transcribed with T7 RNA polymerase to generate the antisense riboprobe. For detection of TH expression, we used a 1000 bp riboprobe complimentary to tyrosine hydroxylase sense cDNA that was inserted into the pGEM-4Z vector (Promega, Madison, WI). Plasmid DNA was cut with

either EcoRI or HindIII in order to create template for *in vitro* transcription. All probes were created using DIG-labeled nucleotides. The HindIII template was transcribed with Sp6 RNA Polymerase to generate the sense riboprobe and the EcoRI template was transcribed with T7 RNA polymerase to generate the antisense riboprobe.

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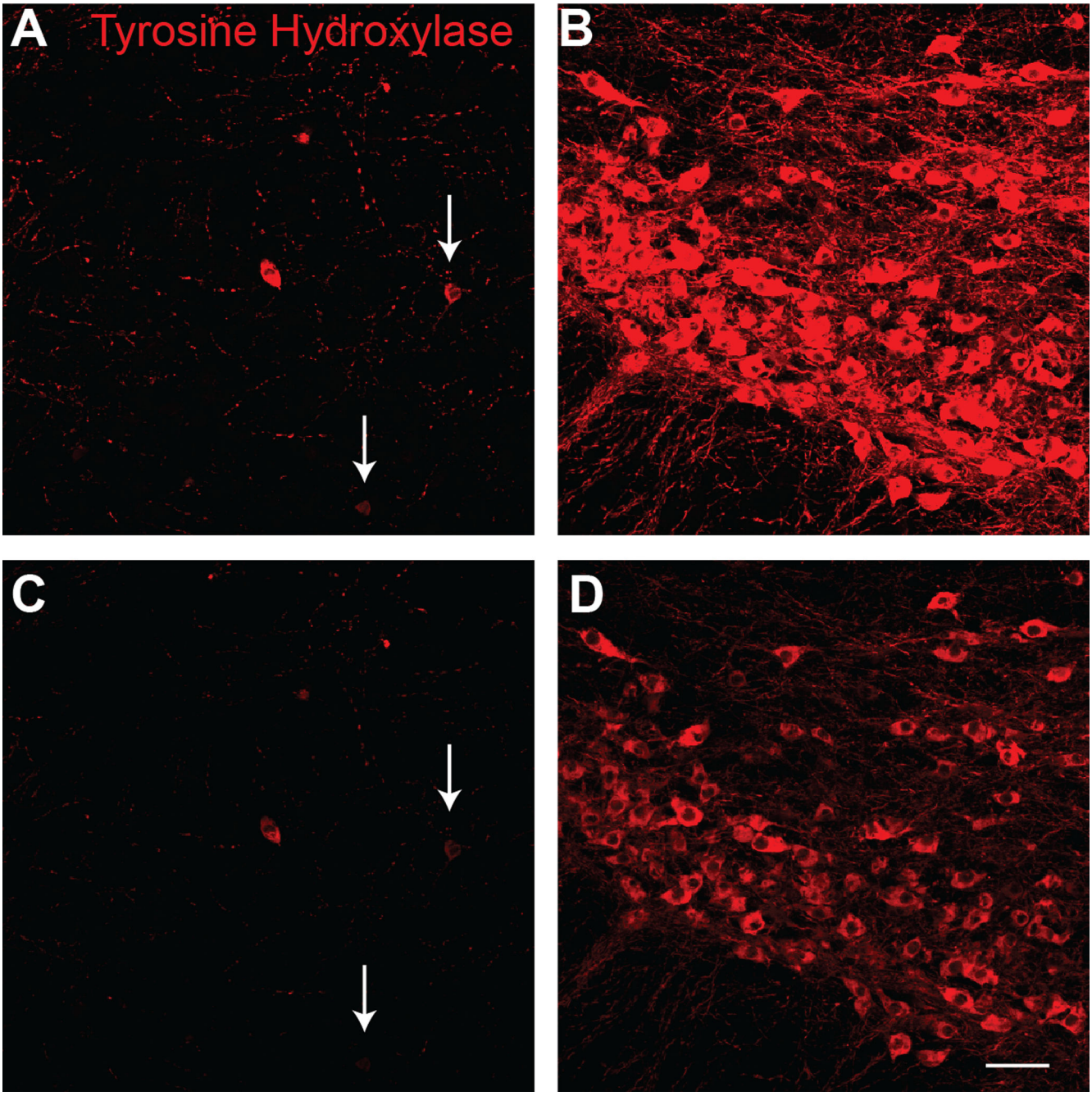

Figure 1. Differences in TH immunofluoresence intesity across the medial-lateral VTA axis

(A) Confocal image of medial TH+ neurons with optimal gain (B) Confocal image of lateral TH+ neurons with gain optomized for the medial portion. Note that most neurons are oversaturated. (C) Confocal image of medial VTA TH+ neurons with gain optimized for lateral VTA TH+ neurons. Note that neurons that were previously visible (A, white arrows) are now difficult to discern. (D) Confocal image of lateral TH+ neurons with gain optimized for lateral portion.

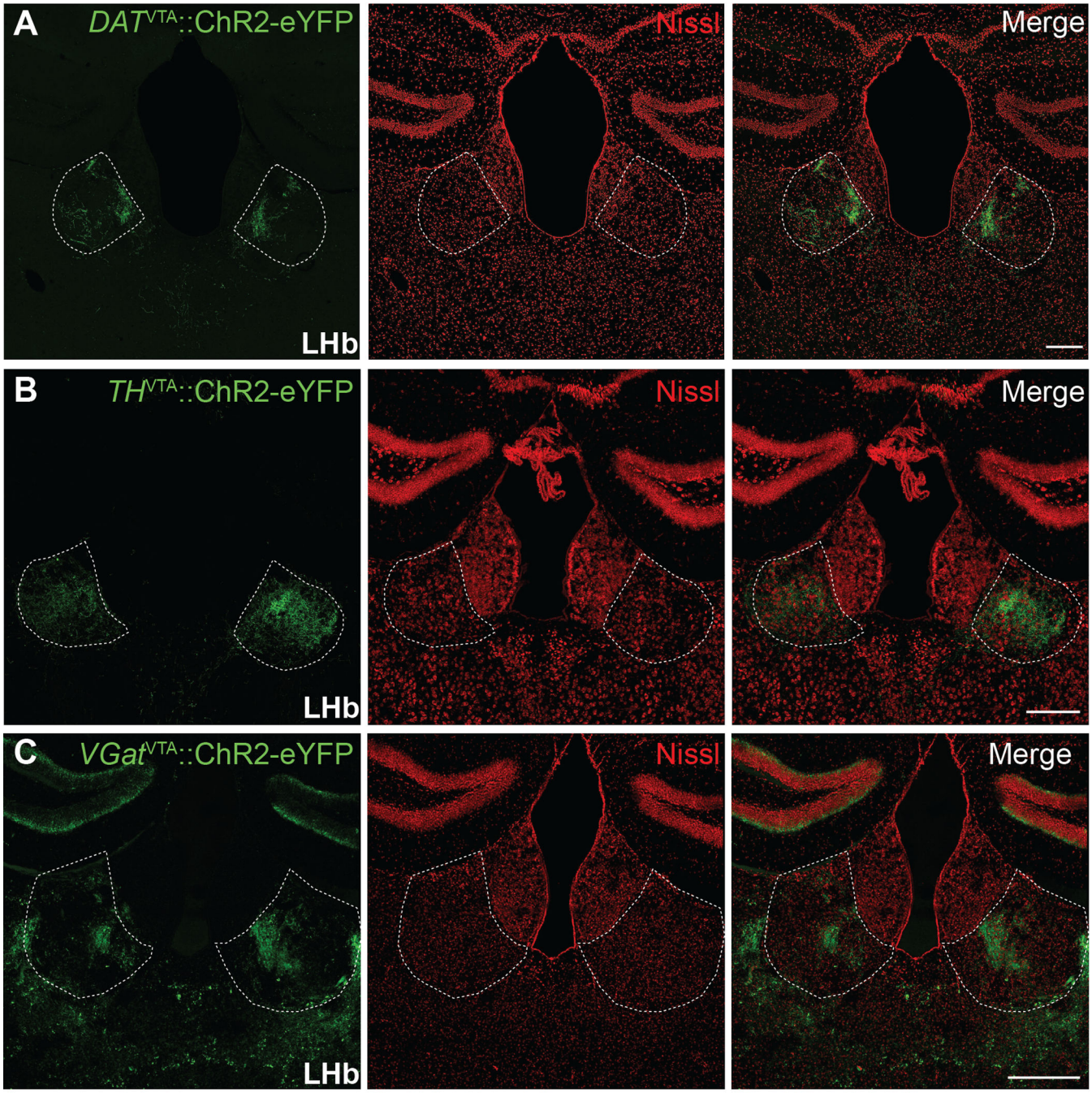

Figure 2. DAT-Cre, TH-Cre, and VGat-Cre targeted neurons project to the LHb

Confocal image of a coronal LHb section (~1.7 posterior) showing expression of ChR2-eYFP fibers in the LHb following injection of cre-inducible ChR2-eYFP into the VTA of a DAT-Cre mouse (A), TH-Cre mouse (B), and VGat-Cre mouse (C). Scale bar = 200 μm.

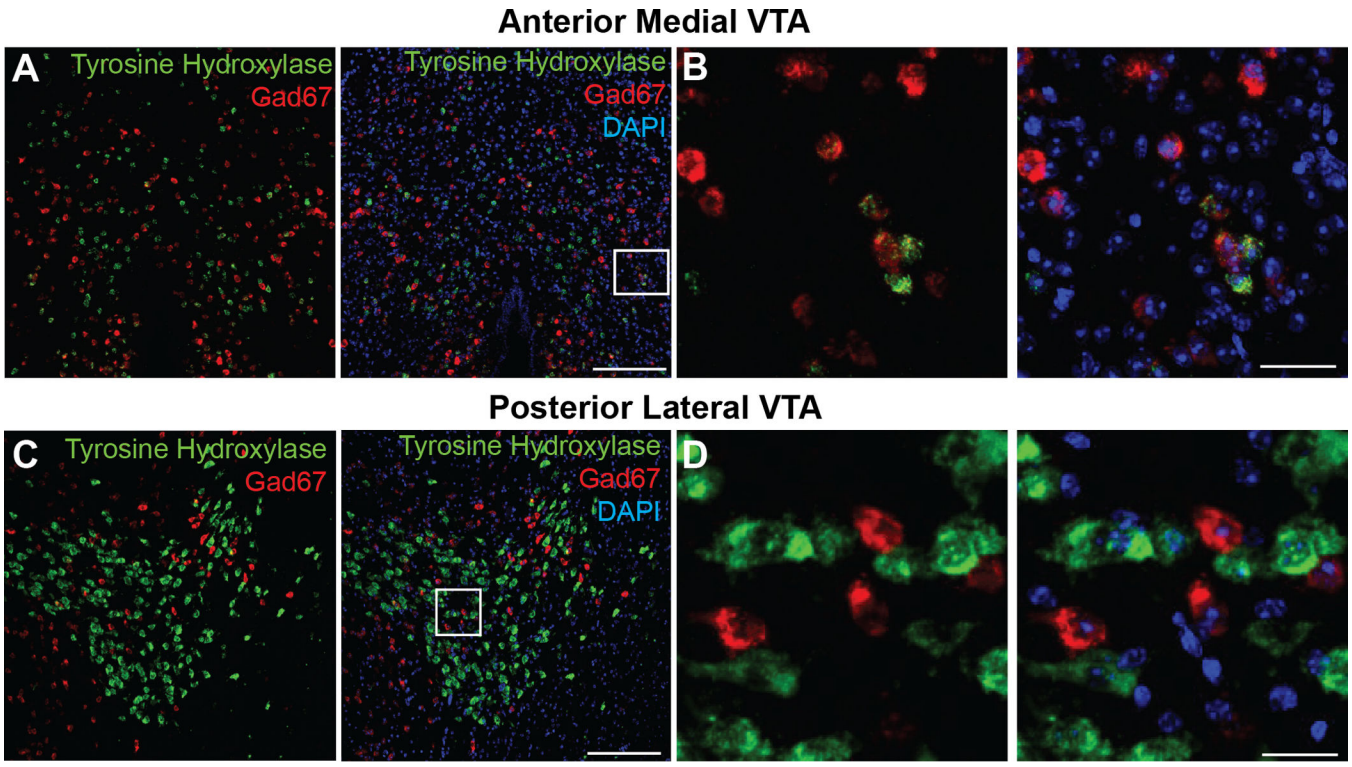

Figure 3. Dual fluorescent in situ hybridization for TH and GAD67 in the VTA

(**A,B**) Confocal image of the anterior medial VTA (~2.8 posterior) from a wild type mouse showing appreciable co-localization between TH mRNA (green) and GAD67 mRNA (red). Overlap = 32/191 neurons (16.7%). Scale bar (A) = 200 μm. Scale bar (B) = 50 μm. (**C,D**) Confocal image of the posterior lateral VTA (~3.2 posterior) from a wild type mouse showing minimal co-localization between TH mRNA and GAD67 mRNA. Overlap = 1/263 neurons (0.38%) Scale bar (C) = 200 μm. Scale bar (D) = 50 μm.