Diversity of Transgenic Mouse Models for Selective Targeting of Midbrain Dopamine Neurons

Highlights

- Low dopamine specificity of transgene expression in ventral midbrain of TH-Cre mice
- DAT-Cre mice exhibit dopamine-specific Cre expression patterns
- VTA GABA and glutamate neurons bi-directionally modulate lateral habenula cells

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In Brief

Transgenic Cre-driver mouse lines are an important tool for optogenetic manipulations of neural circuit function. Lammel et al. find that a prominent supposedly dopamine-specific transgenic mouse line exhibits dramatic non-dopamine specific expression patterns in the ventral midbrain.
Diversity of Transgenic Mouse Models forSelective Targeting
of Midbrain Dopamine Neurons

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SUMMARY

Ventral tegmental area (VTA) dopamine (DA) neurons have been implicated in reward, aversion, salience, cognition, and several neuropsychiatric disorders. Optogenetic approaches involving transgenic Cre-driver mouse lines provide powerful tools for dissecting DA-specific functions. However, the emerging complexity of VTA circuits requires Cre-driver mouse lines that restrict transgene expression to a precisely defined cell population. Because of recent work reporting that VTA DA neurons projecting to the lateral habenula release GABA, but not DA, we performed an extensive anatomical, molecular, and functional characterization of prominent DA transgenic mouse driver lines. We find that transgenes under control of the tyrosine hydroxylase, but not the dopamine transporter, promoter exhibit dramatic non-DA cell-specific expression patterns within and around VTA nuclei. Our results demonstrate how Cre expression in unintentionally targeted cells in transgenic mouse lines can confound the interpretation of supposedly cell-type-specific experiments. This Matters Arising paper is in response to Stamatakis et al. (2013), published in Neuron. See also the Matters Arising Response paper by Stuber et al. (2015), published concurrently with this Matters Arising in Neuron.

INTRODUCTION

Transgenic rodent models that provide genetic access to specific cell populations in the nervous system have become indispensable in modern neuroscience research. In recent years the use of Cre-driver lines in combination with Cre-dependent optogenetic tools has proven to be a particularly fruitful approach, permitting selective, bidirectional control of neural activity in genetically defined cell populations (Deisseroth, 2014; Yizhar et al., 2011). Many of these studies have focused on midbrain dopamine (DA) neurons, thus far identifying roles for these cells in reward, reinforcement, motivation, aversion, depression, social interaction, and other aspects of normal and maladaptive brain function (Chaudhury et al., 2013; Ilango et al., 2014; Lammel et al., 2012; Rothermel et al., 2013; Stamatakis et al., 2013; Steinberg et al., 2013; Tsai et al., 2009; Tye et al., 2013). The most widely implemented approach for targeting midbrain DA neurons involves the use of transgenic Cre-driver mouse lines where Cre recombinase is expressed under the control of tyrosine hydroxylase (TH) or dopamine transporter (DAT) promoters (Lindeberg et al., 2004; Savitt et al., 2005; Zhuang et al., 2005).

In the ventral midbrain, TH, the rate-limiting enzyme in the synthesis of DA, is considered to be the gold standard for identifying DA neurons and is now the most common genetic “handle” used to drive exogenous gene expression. Accordingly, TH-Cre knock-in mouse driver lines have been used in ~80% of the studies that involved Cre-dependent targeting of midbrain DA neurons (Adamantidis et al., 2011; Chaudhury et al., 2013; Chuang et al., 2011; Danjo et al., 2014; Friedman et al., 2014; Guanaydin et al., 2014; Ilango et al., 2014; Kim et al., 2013; Lammel et al., 2012; Rothermel et al., 2013; Stamatakis et al., 2013; Tan et al., 2012; Tsai et al., 2009; Tye et al., 2013; Walsh et al., 2014). Furthermore, the most widely accepted method of confirming DAergic identity in vivo and ex vivo is to demonstrate co-localization of immunohistochemically detected TH with a cell-filling dye injected during recording (Ungless and Grace, 2012).

Using TH-Cre mice, a recent study described a unique population of lateral habenula (LHb)-projecting VTA DA neurons that release GABA but not DA (Stamatakis et al., 2013). Accurate interpretation of these and other experiments that employ cell-type-specific transgenic animals crucially depends on the degree to which transgene expression faithfully reproduces native gene expression patterns. Insofar as Cre expression extends beyond the cellular population of interest, the effects of manipulating unintentionally targeted cells may be erroneously attributed. This issue is especially significant in brain regions where neighboring cells are functionally diverse, such as the...
Figure 1. Anatomical Characterization of Transgenic Cre Mouse Driver Lines
(A) Regions of the posterior midbrain in which eYFP/TH co-localization was analyzed (blue: midline VTA; green: lateral VTA, red: interpeduncular nucleus, IPN).

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VTA, which in addition to a heterogeneous population of DA neurons (Fields et al., 2007; Lammel et al., 2014; Roeper, 2013) also contains subpopulations of heterogeneous GABAergic and glutamatergic neurons (Brown et al., 2012; Hnasko et al., 2012; Li et al., 2013; Margolis et al., 2012; Nair-Roberts et al., 2008; Olson and Nestler, 2007). Here, we systematically evaluated prominent DA transgenic mouse lines to assess transgene specificity for midbrain DA neurons. We found that lines employing the TH, but not DAT, promoter exhibit substantial transgene expression in non-DA neurons. By analyzing VTA neurons projecting to LHb in these and other Cre drive mouse lines, we identify a previously unappreciated population of LHb-projecting VTA GABA neurons, thereby highlighting the potential for ectopic expression in supposedly DA-specific mouse lines to cloud our understanding of complex midbrain circuits.

RESULTS

Anatomical Characterization of DA Neuron-Targeted Transgenic Mouse Lines

Double-floxed (DIO) AAV-DJ-eYFP (1 μl, ~10^{12} infectious units/ml) was unilaterally injected into the VTA of TH::IRES-Cre knock-in mice (European Mouse Mutant Archive; stock number: EM:00254; backcrossed at least 5 generations with C57Bl/6 mice) (Lindeberg et al., 2004). This injection volume and viral titers are similar to those used by other groups to target midbrain DA neurons with Cre-dependent AAV viruses (Chaudhury et al., 2013; Stamatakis et al., 2013; Tsai et al., 2009; Tye et al., 2013). Surprisingly, TH immunostaining revealed dramatic eYFP expression in TH-immunonegative cells within and adjacent to the VTA, in addition to expression in TH-immunopositive neurons in the VTA and substantia nigra (Figures 1A–1F). Notably, strong eYFP expression was observed in brain areas adjacent to the VTA that typically do not exhibit TH immunostaining, including the interpeduncular nucleus (IPN) and supramammillary (SuM) nucleus. We systematically analyzed co-localization of eYFP expression with immunohistochemically detected TH in five different regions of the posterior (Figures 1A–1C) and anterior (Figures 1D–1F) ventral midbrain. Co-localization was the highest in lateral regions of the posterior and anterior VTA. In contrast, extremely low levels of co-localization were observed in midline VTA regions of the posterior and anterior ventral midbrain. No co-localization was detected in the IPN although we observed strong eYFP expression in this area (Figures 1G and S1A). Importantly, when these five regions were considered collectively, we found similar proportions of eYFP-expressing cells that lacked TH and eYFP-expressing cells that contained TH (eYFP+/TH− 52% ± 3%; eYFP+/TH+ 48% ± 3%; n = 3 mice, Figure 1H).

Given that prior studies that employ TH-Cre mice generally report high levels of TH specificity (> 98%) (e.g., Chaudhury et al., 2013; Stamatakis et al., 2013), we characterized another TH::IRES-Cre knock-in mouse line generated by a different laboratory (Savitt et al., 2005) and obtained from the Jackson Laboratory (stock number: JAX:8601). These mice showed a similar eYFP expression pattern (eYFP+/TH− 41% ± 1%; eYFP+/TH+ 59% ± 1%; n = 3 mice, Figures 1G, 1H, and S1B). The high levels of specificity observed in previous studies of TH-Cre mice were likely observed because only lateral portions of the VTA were examined. However, the standard volume of virus we infused (1 μl) spread well beyond the borders of the lateral VTA, making it difficult to envision how optogenetic manipulations could be exclusively confined to this region in order to circumvent the lack of specificity in transgene expression observed in immediately adjacent areas.

Because of the poor specificity of transgene expression in TH-Cre mice, we conducted the same systematic anatomical analysis of transgene expression in DAT-Cre mice (Jackson Laboratory, stock number: 006660) (Zhuang et al., 2005). In marked contrast to TH-Cre mice, TH immunostaining of brain sections from DAT-Cre mice previously injected with AAV-DJ-DIO-eYFP (1 μl) revealed that the overwhelming majority of eYFP-expressing neurons in all ventral midbrain regions were TH-immunopositive (eYFP+/TH− 4% ± 1%; eYFP+/TH+ 96% ± 1%; n = 3 mice, Figures 1G, 1H, and S1C). We also analyzed transgenic mice that expressed GFP under control of the TH promoter (Sawamoto et al., 2001). Co-localization of GFP with TH was similar to that of TH-Cre mice (GFP+/TH− 31% ± 4%; GFP+/TH+ 69% ± 4%; n = 4 mice, Figures 1G, 1H, and S1D).

Additional results suggest that our measures of TH and eYFP/GFP co-localization in TH-driver lines were accurate and not due to leaky expression of Cre-dependent viral constructs, or non-optimal immunohistochemical and/or imaging techniques. First, VTA injection of either AAV5-DIO-eYFP (1 μl) (Figure S1E) or AAV5-DIO-ChR2-eYFP (1 μl, data not shown) shows similar lack of specificity. Second, we observed almost no eYFP expression following double-floxed AAV injections into the VTA of wild-type C57Bl6 mice (< 10 eYFP-positive cells per mouse, n = 2 mice, data not shown). Third, similar expression patterns were observed when we crossed TH-Cre or DAT-Cre mice with a tdTomato reporter line (Ai14). Analysis of co-localization of TH with tdTomato in 3-week-old TH-Cre/Ai14 or DAT-Cre/Ai14 mice revealed substantial expression of tdTomato in TH-immunonegative cells within and adjacent to VTA nuclei in TH-Cre driver lines but not DAT-Cre-driver lines (data not shown). Fourth, DOPA decarboxylase, another key enzyme required for catecholamine synthesis, was also not detectable.
Figure 2. Electrophysiological and Molecular Properties of eYFP-Expressing Cells in TH-Cre Mice
(A) Experimental approach for characterizing electrophysiological properties of eYFP-expressing cells in TH-Cre mice. Inset shows a neuron expressing eYFP prior to recording. Scale bar: 10 μm.
(B) Anatomical positions of the recorded and neurobiotin-filled cells that are TH immunonegative (green) or TH immunopositive (yellow).

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in eYFP+/TH− cells (Figure S2A). Fifth, in TH-Cre mice that received VTA-targeted injections of AAV-DJ-DIO-eYFP we observed substantial axonal eYFP expression in brain regions (LHb and lateral septum) with extremely low TH immunoreactivity. This pattern was not observed in DAT-Cre mice (Figures S2B and S2C). Sixth, we tested two different TH antibodies (Calbiochem and Pel-Freez), but neither led to detectable cellular TH immunosignals in areas such as the IPN or SuM, which would explain the transgene expression patterns observed in TH-Cre mice (data not shown).

**Different Electrophysiological and Molecular Properties of Cre-Expressing DA and Non-DA Neurons**

Because many midbrain cells in TH-Cre mice expressed eYFP but did not show TH immunostaining, we next determined whether the electrophysiological properties of eYFP+/TH− cells were distinct from those of eYFP+/TH+ cells. To accomplish this, we injected AAV-DJ-DIO-eYFP (1 μl) into the VTA of TH-Cre mice (JAX:86011) and performed whole-cell recordings from eYFP-positive neurons in coronal midbrain slices (Figure 2A). Cells were filled with neurobiotin during recordings and subsequently analyzed for neurochemical phenotype using TH immunohistochemistry. Consistent with our anatomical characterization, we found eYFP+/TH− cells in midline VTA regions of the posterior and anterior midbrain as well as in the IPN. In contrast, most eYFP+ expressing cells in more lateral VTA regions were TH+ (Figures 2B and 2C).

Upon injection of hyperpolarizing currents both eYFP+/TH+ and eYFP+/TH− cells showed large variability in the amplitude of the sag component that is mediated by HCN (hyperpolarization-activated cyclic nucleotide-gated) channels (sag amplitude: TH−, 16.2 ± 3.4 mV, n = 10; TH+, 17.5 ± 3 mV, n = 11; p = 0.7656) (Figures 2D and 2E). However, upon repolarization at the end of the hyperpolarizing step, eYFP+/TH− cells showed a prominent inhibition of spike firing with a slow return to the firing threshold. In contrast, lack of a prominent rebound delay was nearly uniform in eYFP+/TH− cells (TH−, 58.5 ± 12.6 ms, n = 10; TH+, 368.4 ± 68.3 ms, n = 10; p = 0.0004) (Figures 2D and 2E). In addition, the maximal firing frequencies in response to increasing steps of current injections (TH−, 69.3 ± 13.6 Hz, n = 10; TH+, 16.8 ± 2.9 Hz, n = 15; p = 0.0001) (Figures 2F and 2G) as well as the spontaneous discharge rate (TH−, 7.1 ± 1 Hz, n = 6; TH+, 2.3 ± 0.3 Hz, n = 8; p = 0.0002) (Figure 2G) were in most cases higher in eYFP+/TH− cells as compared to eYFP+/TH+ cells. Analysis of fully resolved action potentials (AP) revealed that AP durations in eYFP+/TH− cells were in most cases shorter than those of eYFP+/TH+ cells (Figures 2H and 2I) (AP duration at threshold: TH−, 2.3 ± 0.4 ms, n = 6; TH+, 4.4 ± 0.3 ms, n = 8; p = 0.0016). There were no significant differences in the single AP afterhyperpolarization (AHP: TH−, −59.4 ± 2.2 mV, n = 6; TH+, −56.5 ± 1.1 mV, n = 8; p = 0.2342) (Figure 2I) nor AP threshold (data not shown). Importantly, the electrophysiological properties of eYFP+/TH− cells are consistent with properties previously observed in VTA GABAergic neurons (Chien et al., 2011; Johnson and North, 1992; Korotkova et al., 2004), and are substantially different from identified DA subpopulations, including those with unconventional electrophysiological properties (e.g., mesocortical DA neurons, see Lamml et al., 2008).

We next quantified the gene expression profiles of single eYFP+ neurons in TH-Cre knock in mice (JAX:8601) by extracting their intracellular contents and using parallel quantitative real-time PCR (Fluidigm, BioMark) (Figure 2J). Most eYFP+ cells in the substantia nigra pars compacta (SNc) and lateral VTA expressed genes classically associated with DA synthesis (TH: tyrosine hydroxylase), uptake (DAT: dopamine transporter), and release (VMAT2: vesicular monamine transporter 2) (SNc TH: 87.5%, DAT: 0%, VMAT2: 75%, n = 8; lateral VTA TH: 92.9%, DAT: 78.6%, VMAT2: 78.6%, n = 28). In contrast, in midline VTA regions a much smaller percentage of eYFP+ cells expressed these genes (TH: 25%, DAT: 15%, VMAT2: 10%, n = 20). Even though eYFP+ cells in the IPN did not express detectable TH protein, the vast majority of these cells did exhibit detectable levels of TH mRNA transcripts. However, these cells completely lacked other important DAergic marker genes such as DAT and VMAT2 (TH: 67.7%, DAT: 0%, VMAT2: 0%, n = 15) (Figures 2K and S3). Furthermore, while ~75% of eYFP+ neurons in the SNc and lateral VTA co-expressed TH, DAT, and VMAT2 (72.2%, n = 26/36 cells), only ~6% of the cells in IPN and midline VTA regions co-expressed these genes (5.7%,

(C) Sample confocal images of individual eYFP-expressing cells (488 nm, green) that were filled with neurobiotin (NB; 546 nm, red) via the recording pipette and immunostained for tyrosine-hydroxylase (TH; 647 nm, blue). All scale bars: 10 μm.

(D) Sample whole-cell patch-clamp recording from an eYFP-expressing cell that has been NB-filled and posthoc stained for TH. Left: TH-immunonegative cell; right: TH-immunopositive cell. Corresponding membrane potentials in response to hyperpolarizing currents shows lack of rebound delay in TH-immunonegative cells. Note that both cell types show prominent sags during the hyperpolarizing steps.

(E) Bar graphs showing mean sag amplitudes (left) and mean rebound delays (right) for eYFP+/TH− (green) and eYFP+/TH+ (yellow) cells. **p < 0.001. Data represent means ± SEM.

(F) Corresponding membrane-potential responses to depolarizing current pulses. Note that eYFP+/TH− (left) and eYFP+/TH+ (right) cells show depolarization block of spontaneous electrical activity after discharging at different maximal frequencies.

(G) Bar graphs showing mean maximal firing frequency after reaching depolarizing block (left) and mean spontaneous firing frequencies (no current injections) from eYFP+/TH− (green) and eYFP+/TH+ (yellow) cells. **p < 0.001. Data represent means ± SEM.

(H) Single high-resolution action-potential (AP) waveforms from an eYFP+/TH− (left) or eYFP+/TH+ (right) cell.

(I) Bar graphs showing mean AP duration (left) and mean afterhyperpolarization (AHP) amplitudes (right) for eYFP+/TH− (green) and eYFP+/TH+ (yellow) cells. **p < 0.01. Data represent means ± SEM.

(J) Schematic drawing of the experimental approach to analyze gene expression profiles of eYFP-expressing cells in TH-Cre mice.

(K) Bar graph comparing the percentage of eYFP-expressing cells that express TH, DAT, or VMAT2 genes in the substantia nigra pars compacta (SNc), lateral VTA, midline VTA, and interpeduncular nucleus (IPN).

(L) Pie charts showing eYFP-expressing cells that co-express TH, DAT, and VMAT2 (yellow) or lack co-expression of TH, DAT, and VMAT2 (green) for SNc and lateral VTA as well as midline VTA and IPN regions. Striped area indicates cells expressing GAD1 and/or GAD2 genes.
Figure 3. Optogenetic Dissection of the Mesohabenular Pathway

(A) Schematic showing dual injections of fluorescent beads into the LHB and AAV into the VTA of a TH-Cre mouse (JAX:8601).

(B) Confocal image demonstrating retrogradely labeled (i.e., bead-containing, 546 nm, red) and TH (647 nm, blue)-immunonegative VTA neurons that express eYFP (488 nm, green). Scale bar: 20 μm.

(C) Bar graph showing that all retrogradely labeled cells that also express eYFP do not possess detectable TH immunosignals.

(D) Schematic of AAV injection into the VTA of TH-Cre, DAT-Cre, GAD2-Cre, and VGlut2-Cre mice and ex vivo whole-cell patch-clamp recordings of LHB neurons.

(E) Left: light-evoked IPSCs recorded from LHB neurons in TH-Cre mice are blocked by bath application of picrotoxin (red trace). Scale bar: 100 pA, 10 ms. Inset shows ChR2-eYFP (488 nm, green) expression in the LHB of a TH-Cre mouse. Scale bar: 200 μm. Right: bar graph showing mean IPSC amplitudes before (blue) and after (red) bath application of picrotoxin. Pie chart indicates the percentage of recorded LHB neurons that responded to light stimulation. **p < 0.01. Data represent means ± SEM.

(F) Same experimental approach as in Figure 3E, but for DAT-Cre mice.

(G) Same experimental approach as in Figure 3E, but for GAD2-Cre mice. *p < 0.05. Data represent means ± SEM.

(H) Left: light-evoked EPSCs recorded from LHB neurons in VGlut2-Cre mice are blocked by bath application of CNQX (red trace). Scale bar: 100 pA, 10 ms. Inset shows ChR2-eYFP (488 nm, green) expression in the LHB of a VGlut2-Cre mouse. Scale bar: 200 μm. Right: bar graph showing mean EPSC amplitudes before

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n = 2/35 cells). In contrast, nearly half of the eYFP+ cells in the IPN and midline VTA that lacked co-expression of TH, DAT, and VMAT2 showed detectable levels of GAD1/2 gene expression (48.6%, n = 17/35 cells). Only ~6% of the eYFP+ cells that co-expressed TH, DAT, and VMAT2 expressed GAD1/2 (5.6%, n = 2/36) (Figures 2L and S3).

**Optogenetic Dissection of the Mesohabenular Pathway using Transgenic Mouse Lines**

TH-Cre mice that received VTA-targeted injections of AAV-DJ-DIO-eYFP showed substantial axonal eYFP expression in the LHB while TH-immunolabeled terminals are almost completely absent (Figure S2B; Hnasko et al., 2012; Stamatakis et al., 2013). Because of this inconsistency the existence of a unique population of VTA DA neurons that projects to the LHB has been proposed. Specifically, using TH-Cre knock-in mice (TH::iRES-Cre) it was demonstrated that optogenetic activation of this projection inhibits LHB neurons via synthetically released GABA, but not DA, to promote reward-related behaviors (Stamatakis et al., 2013). Because of the prominent ectopic patterns of transgene expression we observed in TH-Cre mice we re-evaluated this conclusion by first injecting retrobeads into the LHB and AAV-DJ-DIO-eYFP (1 μl) into the VTA of TH-Cre mice (JAX:8601). Consistent with previous reports (Gruber et al., 2007; Skagerberg et al., 1984; Stamatakis et al., 2013; Swanson, 1982), retrogradely labeled neurons were predominately located in midline VTA nuclei of the anterior and posterior midbrain. However, all retrogradely labeled cells that expressed eYFP were also TH-immunonegative (n = 36/36 cells, n = 2 mice) (Figures 3A–3C). Indeed, additional retrograde tracing experiments in C57Bl6 wild-type mice revealed that the vast majority (~97%–99%) of the mesohabenular neurons were TH-immunonegative (Figures S4A–S4H). To determine the neurochemical phenotype of VTA neurons projecting to LHB we injected retrobeads into the LHB of GAD2-Cre and VGlut2-Cre mice that had been crossed with an Ai14 reporter line. Virtually all of the LHB-projecting, retrobead-containing cells expressed GABAergic or glutamatergic markers with few if any TH-immunopositive neurons (Figures S4I and S4J). Importantly, a recent study suggests that the majority of mesohabenular neurons co-express both GABAergic and glutamatergic molecular markers, but do not possess detectable TH immunosignals (Root et al., 2014a).

To further dissect the functional role of mesohabenular projections, we injected AAV5-DIO-ChR2-eYFP (1 μl) into the VTA of TH-Cre (JAX:8601), DAT-Cre, GAD2-Cre, and VGlut2-Cre mice (Figure 3D). We observed strong ChR2 expression in the LHB of all transgenic lines except DAT-Cre mice, which showed extremely sparse ChR2 expression within the LHB (Figures 3E–3H; Figure S2B). Voltage clamp recordings from LHB neurons revealed that light pulses that selectively stimulated TH-Cre or GAD2-Cre ChR2 fibers in the LHB evoked inhibitory postsynaptic currents (IPSCs) that were almost completely blocked by the GABA-A receptor antagonist picrotoxin (50 μM) (TH-Cre: −548.2 ± 85.8 pA, n = 8, in picrotoxin: −48.9 ± 12 pA, n = 4, p = 0.0025; GAD2-Cre: −370.4 ± 94.1 pA, n = 9, in picrotoxin: −25.3 ± 5.6 pA, n = 5, p = 0.02). Of the neurons recorded in the LHB ~67% (n = 8/12 cells) received direct monosynaptic inhibitory input from VTA TH-Cre neurons and 75% (n = 9/12 cells) received direct monosynaptic inhibitory input from VTA GAD2-Cre neurons (Figures 3E and 3G). Light pulses never evoked detectable IPSCs in LHB neurons from DAT-Cre mice (n = 0/15 cells) (Figure 3F). In contrast, light stimulation of VGlut2-Cre ChR2 fibers in the LHB evoked excitatory postsynaptic currents (EPSCs) that were blocked by an AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonist (−382.6 ± 75.8 pA, n = 9, in 10 μM CNQX: −46.6 ± 23.1 pA, n = 7, p = 0.002) in 90% (n = 9/10 cells) of LHB neurons, thus revealing a strong direct excitatory input from VTA VGlut2-Cre neurons to LHB (Figure 3H).

Optogenetic activation of the mesohabenular pathway in TH-Cre mice induced a reward-related phenotype that required GABA release in the LHB (Stamatakis et al., 2013). Our results suggest that we should observe a similar behavioral phenotype in both TH-Cre and GAD2-Cre mice. In contrast, as optogenetic activation of excitatory inputs from the entopeduncular nucleus to LHB as well as from LHB to RMTg/VTA mediates aversive behaviors (Lammel et al., 2012; Shabel et al., 2012; Stamatakis and Stuber, 2012), we hypothesized that activation of excitatory mesohabenula projections in the LHB of VGlut2-Cre mice would generate aversion. These predictions were confirmed using a real-time place preference assay in TH-Cre (JAX:8601) (n = 4), GAD2-Cre (n = 5), and VGlut2-Cre (n = 4) mice that were injected in the VTA with AAV5-DIO-ChR2-eYFP (1 μl) and implanted with bilateral optical fibers above the LHB (Figures 3I–3K). Activation of mesohabenula projections in the LHB of VGlut2-Cre mice caused a robust real-time place aversion (stimulation compartment versus non-stimulation compartment p = 0.003; 2-way repeated-measures ANOVA followed by Student-Newman-Keuls post hoc test; Figure 3J lower panel, and Figure 3K). Indeed, switching the stimulation to the original non-stimulated compartment caused immediate avoidance of that compartment (p < 0.001; Figure 3K inset). In contrast, consistent with previous work (Stamatakis et al., 2013), activation of mesohabenula projections in the LHB of TH-Cre mice and GAD2-Cre mice
caused a more modest rewarding behavioral response. Both groups spent more time in the compartment where they received stimulation as compared to the compartment where they did not receive stimulation (Figure 3J upper panel, and Figure 3K; although this comparison did not reach statistical significance; p = 0.061 for TH-Cre, p = 0.158 for GAD2-Cre). Furthermore, TH-Cre and GAD2-Cre mice spent more time in the compartment where they received stimulation compared to the neutral area in our three-chamber assay (p values < 0.041) whereas there was no significant difference between time spent in the non-stimulated compartment versus the neutral area (p values > 0.255). Importantly, the behaviors of the TH-Cre and GAD2-Cre mice did not differ from each other yet both dramatically differed from the VGlut2-Cre mice (Figure 3K; VGlut2-Cre versus TH-Cre/GAD2-Cre all p values < 0.012 for stimulation/non-stimulation compartments; TH-Cre versus GAD2-Cre all p values > 0.63 on the same measures).

DISCUSSION

We have performed an extensive anatomical, molecular, and functional characterization of transgenic mouse lines commonly used to identify and target midbrain DA neurons (TH-Cre, DAT-Cre and TH-GFP mice) and found that TH-Cre knock-in mouse lines exhibit pronounced transgene expression in non-DAergic, i.e., TH-immunonegative, neurons in the VTA and adjacent nuclei. Previous studies have shown that the expression levels of DA-related genes such as TH, DAT, and VMAT2 vary among VTA DA neurons with DA neurons in medial regions of the VTA tending to express lower levels of TH and DAT (Lammel et al., 2008; Li et al., 2013). If TH protein or mRNA was present but levels fell below our threshold for detection in TH driver lines, this could theoretically account for the reduced fidelity we observed in midline regions. However, these unconventional DA neurons (e.g., mesocortical DA neurons) possess detectable TH immunosignals as well as other key enzymes in the synthesis of catecholamines such as DDC (Lammel et al., 2008), markers that were completely absent in many eYFP+ neurons in this study. Furthermore, the ectopic expression observed in TH-Cre mice extends well beyond typical VTA regions and can be found in areas that either are not known to contain DA neurons (e.g., IPN, supramamillary nucleus) or contain only a few scattered DA neurons (e.g., area between the fasciculus retroflexus in the anterior midbrain). Indeed, transgene-expressing cells in these regions expressed neither detectable TH nor DDC immunosignals. Moreover, our single-cell profiling data show that nearly half of the Cre-expressing cells in the IPN and midline VTA nuclei possess GAD1/2 genes but lack co-expression of DA markers including TH, DAT, and VMAT2. This is consistent with previous studies indicating that GABAergic neurons are particularly prominent in these regions (Nair-Roberts et al., 2008; Olson and Nestler, 2007). These cells also lacked a rebound delay in spiking following a prolonged hyperpolarization and fired at higher frequencies, properties typically found in VTA GABAergic neurons but not in identified DA subpopulations (Chieng et al., 2011; Johnson and North, 1992; Korotkova et al., 2004; Li et al., 2012; Lammel et al., 2006).

Because the ability to selectively target DA neurons is critical for the meaningful interpretation of “DA-specific” experiments, we also demonstrate how this ectopic expression can confound data interpretation by focusing on the identity and function of projections from the VTA to the LHb, which has a central role in the control of motivated behaviors via its reciprocal projections to the VTA and the adjacent rostromedial tegmental nucleus (Bromberg-Martin et al., 2010; Hikosaka, 2010; Hong et al., 11; Lammel et al., 2012; Meye et al., 2013; Stamatakis and Stuber, 2012). Our results demonstrate that < 2% of murine mesohabenular projections arise from DA (i.e., TH-immunopositive) neurons, which may explain the absence of TH- immunolabeled axons in the LHb in mice (Figure S2B) (Hnasko et al., 2012; Stamatakis et al., 2013), the lack of detectable DA release in the LHb of mice (Stamatakis et al., 2013), and that transmitter release in the LHb is not affected by VMAT2-inhibition (Stamatakis et al., 2013). Although in rats the number of VTA DA neurons projecting to LHb seems to be higher (Root et al., 2014a; Gruber et al., 2007; Skagerberg et al., 1984) and terminals expressing both TH as well as functional D2 and D4 receptors have been reported (Good et al., 2013; Jhou et al., 2013; Gruber et al., 2007), it appears that in both species the majority of mesohabenular neurons signal through GABA and/or glutamate (Figures S4I and S4J) (Root et al., 2014a).

Furthermore, we present several lines of evidence that the LHb-projecting VTA neurons that release GABA should not be considered dopaminergic. First, both the majority of transgene-expressing TH- and DDC-immunonegative cells in TH-Cre mice and TH-immunonegative cells projecting to LHb are located in the same VTA region (Figures 1 and S4). Second, in TH-Cre mice, eYFP+ neurons projecting to LHb do not express detectable TH immunosignals (Figures 3A–3C). Third, in TH-Cre mice, eYFP+/TH− cells possess molecular and electrophysiological properties that are characteristic of VTA GABA neurons (Figure 2). Fourth, robust light-evoked IPSCs in LHb neurons were generated in both TH-Cre and GAD2-Cre mice but not DAT-Cre mice when VTA projections were optogenetically activated (Figures 3E–3G). Fifth, in vivo optogenetic activation of VTA projections in the LHb induces a similar behavioral phenotype in both TH-Cre and GAD2-Cre mice (Figure 3K). In addition, consistent with a recent study (Root et al., 2014b) and single-unit recording data reporting that absence of a reward or punishment excites LHb neurons (Hikosaka, 2010; Matsumoto and Hikosaka, 2007, 2009), we also identified a novel role for LHb-projecting VTA glutamate neurons in promoting aversion.

Ectopic expression driven by the TH promoter has been described previously (Lindeberg et al., 2004; Min et al., 1994; Savitt et al., 2005), possibly as a result of inappropriate expression driven by an exogenous promoter, or because of TH promoter activity in precursor cell populations that either never produced TH protein or subsequently lost this ability, potentially as a consequence of post-transcriptional regulation of TH mRNA (Lindeberg et al., 2004). Although we found widespread ectopic transgene expression in TH-Cre mice, virtually all studies that involve optogenetic manipulation of DA neurons in these lines consistently report the presence of TH protein in more than 98% of Cre-expressing cells in midbrain VTA nuclei (e.g., Chaudhury et al., 2013; Ilango et al., 2014; Stamatakis et al., 2013; Tsai et al.,...
2009). This likely occurred because lateral regions of the VTA were considered in isolation (Figure S1). Using common optogenetic experimental configurations, it also seems likely that the inherent difficulty in restricting the spread of viral infection and the light path to a precisely circumscribed area will lead to manipulation of non-DA neurons concurrently with targeted DA neurons. Studies that seek to selectively visualize or manipulate axonal projections of DA neurons in TH-Cre driver lines present a related problem, as the location of the corresponding somata is often not identified and projections of interest may in fact derive from neurons with ectopic transgene expression. Although DAT-Cre mice are likely to suffer from their own limitations for example, low DAT expression in mesocortical DA neurons may make it difficult to target this subpopulation, see Lamml et al., 2008, our results suggest that transgene expression is much more specific in this line. Regardless of the mouse line chosen, validating key assumptions and conclusions with complementary methodologies—such as pharmacological manipulations, careful immunohistochemical studies, or mRNA read-outs—will considerably strengthen conclusions drawn from the use of transgenic mouse lines, which certainly will continue to be a critically important tool for cell-type-specific analyses and manipulations in the mammalian brain.

**EXPERIMENTAL PROCEDURES**

Immunohistochemistry, Optogenetics, and recordings from VTA neurons were performed essentially as previously described (Lamml et al., 2008, 2011, 2012). All experimental procedures are described in detail in Supplemental Information.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.12.036.

**AUTHOR CONTRIBUTIONS**

S.L., E.S., and C.F. contributed equally to performing the experiments in this study. Stereotactic injections were performed by S.L. and E.S. Immunohistochemistry was performed by S.L., E.S., N.W., and K.B. Electrophysiology was performed by S.L. and C.F. Single-cell mRNA profiling was performed by C.F. Behavior experiments were performed by E.S. and S.L. The study was designed by S.L. and R.C.M. Results were analyzed and interpreted by S.L., E.S. and R.C.M with assistance of the other authors. The manuscript was written by S.L., E.S., and R.C.M and edited by all authors.

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Diversity of Transgenic Mouse Models for Selective Targeting of Midbrain Dopamine Neurons

Stephan Lammel, Elizabeth E. Steinberg, Csaba Földy, Nicholas R. Wall, Kevin Beier, Liqun Luo, and Robert C. Malenka
**Fig. S1 Lammel et al.**

**A** TH-cre (EM:00254)

- **Posterior Lateral**
- **Posterior Midline**
- **IPN**
- **Anterior Lateral**
- **Anterior Midline**

**B** TH-cre (JAX:8601)

**C** DAT-cre

**D** TH-GFP

**E** AAV5 → TH-cre (JAX:8601)

**TH** | **EYFP**
Fig. S4 Lammel et al.

A. C57Bl6

B. Posterior Midbrain

C. Anterior Midbrain

D. TH beads

E. IPNissl beads

F. TH beads

G. TH- 99.1%

H. RVtdT

I. Midline VTA

J. Lateral VTA

K. Midline VTA

L. cell/log fluorescence of beads+ cells (a.u.)

M. cell/log fluorescence of beads+ VTA cells (a.u.)

N. cell/log fluorescence of beads+ cells (a.u.)
SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** Anatomical characterization of transgenic mouse lines (is related to Figure 1).

(A) TH-Cre *(EM:00254)* mice injected with 1 µl AAV-DJ-DIO-eYFP into the VTA. Confocal images showing TH-immunostaining (red, 546 nm) and eYFP-expression (green, 488 nm) for the different areas delineated in Figures 1A, 1D. Bar graphs indicate the mean number of eYFP-expressing TH-immunopositive and eYFP-expressing TH-immunonegative neurons. Posterior midbrain: lateral VTA: TH+ 127.7±8.8, TH- 13.3±2.7; midline VTA: TH+ 43±10.5, TH- 79.7±26.4; IPN (Interpeduncular nucleus): TH+ 0.7±0.7, TH- 139.3±10.4. Anterior midbrain: lateral VTA: TH+ 96.3±4.4, TH- 4±2.6; midline VTA: TH+ 16±5.5, TH- 74.3±3.4 (n=3 mice).

(B) Same approach as in Figure S1A, but for TH-Cre *(JAX:8601)* mice. Posterior midbrain: lateral VTA: TH+ 102.3±10.4, TH- 3.7±0.7; midline VTA: TH+ 26±8.5, TH- 61.3±9; IPN: TH+ 0, TH- 18±7.8. Anterior midbrain: lateral VTA: TH+ 84±8, TH- 3.7±1.8; midline VTA: TH+ 8±2.5, TH- 66±6.6 (n=3 mice).

(C) Same approach as in Figure S1A, but for DAT-Cre mice. Posterior midbrain: lateral VTA: TH+ 115±13.9, TH- 3.3±1.9; midline VTA: TH+ 53.7±2.6, TH- 2.7±1.2; IPN: TH+ 0, TH- 0. Anterior midbrain: lateral VTA: TH+ 91.7±5.4, TH- 4.3±2.4; midline VTA: TH+ 22±2.1, TH- 1.7±1.2 (n=3 mice).

(D) Same approach as in Figure S1A, but for TH-GFP mice. Posterior midbrain: lateral VTA: TH+ 126.8±4, TH- 1.8±0.3; midline VTA: TH+ 37.3±7, TH- 53±10.9; IPN: TH+ 0, TH- 3.5±2.2. Anterior midbrain: lateral VTA: TH+ 130.3±2.6, TH- 3.3±0.5; midline VTA: TH+ 18.5±1.8, TH- 81±11.3 (n=4 mice).

(E) Same approach as in Figure S1A, but for TH-Cre *(JAX:8601)* mice injected with 1 µl AAV5-DIO-eYFP into the VTA. Posterior midbrain: lateral VTA: TH+ 142.5±5.5, TH- 12.5±4.5; midline VTA: TH+ 18±2, TH- 69.5±1.5; IPN: TH+ 0, TH- 81±16. Anterior midbrain: lateral VTA: TH+ 118±6, TH- 17±7; midline VTA: TH+ 10±6, TH- 116.5±28.5 (n=3 mice).

(All scale bars 20 µm) (Data in Figures S1A-S1E represent means ± SEM)

**Figure S2.** Key enzymes in the synthesis of catecholamines are not detectable in many eYFP-expressing VTA neurons and axonal projections in TH-Cre mice (is related to Figure 1).
(A) Upper left panel: schematic showing dopamine synthesis (DOPA: 3,4-dihydroxyphenylalanine, TH: tyrosine hydroxylase, DDC: DOPA decarboxylase). Upper right and lower panels: confocal images showing TH (red) and DDC (blue) immunostaining as well as eYFP (green) expressing neurons in different regions of a TH-Cre mouse (JAX:8601) that has been injected previously with 1 µl AAV-DJ-DIO-eYFP into the VTA. Note that all TH and eYFP-immunopositive neurons coexpress DDC (lateral VTA: n=37 of 37 cells; midline VTA: n=14 of 14 cells), while all eYFP-positive and TH-immunonegative neurons lack detectable DDC immunosignals (IPN: n=14 of 14 cells; midline VTA: n=51 of 51 cells). (Scale bars 20 µm)

(B) Fluorescence images showing terminals in the lateral habenula expressing eYFP (green, 488 nm) but lacking TH-immunostaining (red, 546 nm) from a TH-Cre (JAX:8601) mouse which has been injected previously with 1 µl AAV-DJ-DIO-eYFP into the VTA (left). DAT-Cre mice which received an injection of 1 µl AAV-DJ-DIO-eYFP into the VTA show neither notable eYFP expression nor TH-immunosignals in the lateral habenula (right). Arrows indicate lateral habenula. (Scale bars 200 µm)

(C) Fluorescence images showing terminals in the lateral septum expressing eYFP (green, 488 nm) but lacking TH-immunostaining (red, 546 nm) from the same TH-Cre (JAX:8601) mouse brain shown in Figure S1B (left). In DAT-Cre mice there is neither notable eYFP expression nor TH-immunostaining of axon terminals in the lateral septum (right). Arrows indicate lateral septum. (Scale bars 200 µm)

**Figure S3. Molecular profiling of eYFP expressing neurons in TH-Cre mice** (is related to Figure 2).

Results of single-cell transcriptional analysis for eYFP-expressing neurons in (A) substantia nigra pars compacta (SNc), (B) lateral VTA, (C) interpeduncular nucleus (IPN) and (D) midline VTA regions. Each column represent a single cell; in each cell multiple probes were tested in parallel. Ct values were normalized to actin Ct controls (data not shown), where dark colors display no detectable expression, and warm colors display high expression levels. Note that parvalbumin (Pvalb) transcripts, which are present in VTA GABAergic neurons (Neuhoff et al., 2002), can be detected more than twice as often in cells lacking DAergic marker transcripts TH, DAT and VMAT2 (53.7%, n=22/41 cells) than in cell that co-express these markers (25%, n=7/28 cells).
Transcripts for calbindin (*Calb1*), calretinin (*Calb2*) and HCN current channels (*HCN2*) were detected more equally in both of these groups. *Calb1* and *HCN2*, however, were detected more frequently in cells that co-express TH, DAT and VMAT2.

**Figure S4. Neurochemical identity of mesohabenular neurons (is related to Figure 3).**

(A) Coronal brain section from a C57Bl6 mouse stained with nissl (red) showing injection site of fluorescently labeled beads (546 nm, green) in the lateral habenula (LHb) (arrow). (Scale bar 200 µm)

(B) Confocal image showing the anatomical position of retrogradely labeled neurons (= beads containing, 546 nm, white) in the posterior (left) and anterior (right) midbrain. Note, that cells projecting to lateral habenula are mainly localized in midline VTA regions which almost completely lack TH (488 nm, red) -immunopositive cells (IPN – interpeduncular nucleus, fr – fasciculus retroflexus). (Scale bars 200 µm)

(C) Bar graph shows the mean number of retrogradely labeled neurons for the areas depicted in Figure 1A and 1D (posterior midbrain: lateral VTA: 4.3±0.3 cells, midline VTA: 19±4.6 cells, IPN: 0 cells, anterior midbrain: lateral VTA: 2 cells, midline VTA: 41.3±12 cells; n=3 mice). (Data represent means ± SEM)

(D) Sample confocal image showing beads (546 nm, white) –containing TH (488 nm, red) –immunopositive and TH-immunonegative cells. Pie charts illustrate the percentage of retrogradely labeled cells that are TH-immunopositive (red) or TH-immunonegative (blue). Only 5 out of 200 retrogradely labeled neurons (2.5%) were TH-immunopositive (n=3 mice). (Scale bars 20 µm)

(E) Confocal images from TH (488 nm, green) stained midbrain sections (posterior-left, anterior-right) following rabies virus tdTomato (RVtdT, 546 nm, red) injection into the lateral habenula of a C57Bl6 mouse (Scale bars 200 µm). E’ and E’’ show magnifications of the regions indicated in E (Scale bars 40 µm). Rabies virus was used as an alternative tracing method in order to determine whether the limited spread of retrobeads (Figure S4A) caused us to underestimate the number of TH-immunopositive LHb-projecting VTA neurons in this experiment (see pie chart in Figure S4D).

(F) Bar graph shows the mean number of tdTomato expressing neurons for the areas depicted in Figure 1A and 1D (posterior midbrain: lateral VTA: 4±1.9 cells, midline VTA: 30.8±4.8 cells,
IPN: 0.8±0.8 cells; anterior midbrain: lateral VTA: 2.5±1.5 cells, midline VTA: 45.3±10 cells, n=4 mice. (Data represent means ± SEM)

(G) Pie charts illustrate the percentage of tdTomato expressing cells that are TH-immunopositive (red, 3/333 cells, 0.9%) or TH-immunonegative (blue) (n=4 mice).

(H) Injection-site of RV-tdT (546 nm, red) into the LHb (arrow) of a TH (488 nm, green)-stained coronal brain section. Note, that in rats DA terminals are confined to the medial and caudal parts of the LHb (Gruber et al., 2007; Skagerberg and Lindvall, 1984). However, neither the anatomical distribution (Figures S4F vs. S4C) nor the number of TH-immunopositive neurons (Figure S4G vs. S4D) changed if RV-tdT injections was used to cover large parts of the habenular complex, as compared to more restricted injections achieved with retrobead injections (Fig. S4A). (Scale bar 200 µm)

(I) In order to determine whether LHb-projecting VTA neurons were GABAergic, retrobeads were injected in the LHb of a GAD2-Cre x Ai14 reporter mouse. In an effort to remove subjective bias in determining which cells were GAD2+ (due to high background signal), for each cell the fluorescence intensity is reported as a ratio of the soma/local background signal. High ratios (>1) indicate GAD2+ cells. Upper left: Confocal image of retrogradely labeled VTA cells following injection of beads (488 nm, white) into the LHb of a GAD2-Cre (546 nm, green) Ai14 reporter line mouse with TH (647 nm, red)-immunostaining. For each bead-positive cell, GAD2 and TH fluorescence signals were evaluated. Arrows indicate examples of analyzed cells. Lower left: Dots indicate somatic (cell) to background (bg) ratios for GAD2 (green) and TH (red). Note, that the TH cell/bg ratio is ~1 (i.e. somatic and background immunosignals are similar) in almost all retrogradely labeled cells. In contrast, many of these cells have GAD2 cell/bg ratios > 1. Upper right: Confocal image from the same section and animal but in the lateral VTA where bead-positive cells are absent as a control comparison. Lower right: Dots indicate cell/bg ratios for TH (red) and GAD2 (green) from bead-negative cells (i.e., non-LHb-projecting) in the lateral VTA. In all cells examined TH cell/bg ratios were high indicating DAergic identity. In the same cells, GAD cell/bg ratios were low. (Scale bar 20 µm).

(J) Same as Figure S4I, but for glutamatergic VTA neurons. Retrobeads were injected in the LHb of a VGlut2 x Ai14 reporter mouse. Upper panel: Confocal image of retrogradely labeled VTA cells following injection of beads (488 nm, white) into the LHb of a VGlut2-Cre (546 nm, blue) Ai14 reporter line mouse with TH (647 nm, red)-immunostaining. Arrows indicate examples of
analyzed cells. Lower panel: Dots indicate cell/bg ratios for VGlut2 (blue) and TH (red) in bead-positive cells. Note, that the TH cell/bg ratio is ~1 or less in all but one retrogradely labeled cell. In contrast, many, but not all, retrogradely labeled cells have VGlut2 cell/bg ratios > 1. (Scale bar 20 µm).

DETAILED EXPERIMENTAL PROCEDURES

Subjects and Stereotactic Surgeries

The following mouse lines (male and female, 25-30 g, >10 weeks old) were used for the experiments: C57B16 mice (Charles River), TH::IRES-Cre (Jackson Laboratory, stock number: JAX:8601, strain name: B6.Cg-Tg(Th-cre)1Tmd/J) (Lindeberg et al., 2004), TH::IRES-Cre (European Mouse Mutant Archive, stock number: EM:00254, strain name: B6 129X1-Thtm1(cre)Te/Kieg) (Savitt, 2005), DAT::IRES-Cre (Jackson Laboratory, stock number: 006660, strain code: B6.SJL-Slc6a3tm1(cre)Hze/J) (Zhuang et al., 2005), TH-GFP (Sawamoto et al., 2001), GAD2::IRES-Cre (Jackson Laboratory, stock number: 010802, strain code: Gad2tm1(cre)Hze/J), VGlut2::IRES-Cre (Jackson Laboratory, stock number: 016963, strain code: S1c17a6tm2(cre)Lov/Hze/J), Ai14 Cre reporter mice (Jackson Laboratory, stock number: 007908, strain code: B6;129S6-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J). Ai14 Cre reporter mice were crossed to TH::IRES-Cre (JAX:8601), DAT::IRES-Cre, GAD2::IRES-Cre or VGlut2::IRES-Cre mice. Mice were maintained on a 12:12 light cycle (lights on at 07:00). All procedures complied with the animal care standards set forth by the National Institutes of Health and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

As previously described (Lammel et al., 2008) all stereotaxic injections were performed under general ketamine–medetomidine anaesthesia and using a stereotaxic instrument (Kopf Instruments). For retrobead labelling mice were injected unilaterally with fluorescent retrobeads (120 nl; LumaFluor Inc.) in the lateral habenula using a 1 µl Hamilton syringe (Hamilton) (bregma: -1.58 mm, lateral: 0.45 mm, ventral: 2.7 mm). The rabies virus tdTomato and AAVs (adeno associated virus) used in this study were generated as previously described (Lammel et al., 2012; Zhang et al., 2010) and were from either the Deisseroth laboratory (AAV5 EF1α.DIO hChR2(H134R)-eYFP; AAV5 EF1α.DIO eYFP; ~10^{12} infections units per ml, packaged and titered by the UNC Vector Core Facility) or the Stanford Neuroscience Gene Vector and Virus Core (AAV-DJ EF1α.DIO hChR2(H134R)-eYFP; AAV-DJ EF1α.DIO eYFP; ~10^{12} infections
units per ml). For viral infections 1 µl of concentrated AAV solution was injected unilaterally into the VTA (bregma, -3.5 mm; lateral, 0.3 mm; ventral, 4.2 mm) or 400 nl rabies virus tdTomato unilaterally into the lateral habenula (same coordinates as for retrobead injections) using a syringe pump (Harvard Apparatus) at 100 nl/min. (Lindeberg et al., 2004). The injection needle was withdrawn 5 min after the end of the infusion. For behavioral experiments, Channelrhodopsin-2 (ChR2)-injected mice received bilateral implantation of a chronically implantable optical fiber (NA = 0.22; Doric lenses) over the LHb (bregma, -1.58 mm, lateral, 0.5 mm, ventral, 2.4 mm). One layer of adhesive cement (C&B metabond, Parkell) followed by cranioplastc cement (Dental cement) was used to secure the fiber to the skull. The incision was closed with a suture and tissue adhesive (Vetbond; Fisher). The animal was kept on a heating pad until it recovered from anesthesia. Experiments were performed 4 weeks (for AAV-DJ), 8 weeks (for AAV5), 2 weeks (for retrobeads) or 1 week (for rabies virus) after stereotactic injection. Injection sites and optical fiber placements were confirmed in all animals by preparing coronal sections (100 µm) of injection sites and counterstaining with green or red Nissl (NeuroTrace 500/525 or 530/615, Molecular Probes). We routinely carried out complete serial analyses of the injection sites and animals with either misplaced injections or notable contaminations outside target areas were discarded (see Lammel et al., 2008) for serial analysis of injection-sites. Although optical fiber placements varied slightly from mouse to mouse, behavioral data from all mice were included in the study.

**Electrophysiological Recordings**

Mice were deeply anaesthetized with pentobarbital (200 mg/kg ip, Ovation Pharmaceuticals). Coronal midbrain slices (250 µm) were prepared after intracardial perfusion with ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 50 sucrose, 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 0.1 CaCl2, 4.9 MgCl2, and 2.5 glucose (oxygenated with 95% O2/5% CO2). After 90 min of recovery, slices were transferred to a recording chamber and perfused continuously at 2-4 ml/min with oxygenated ACSF (in mM): 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 11 glucose, 1.3 MgCl2 and 2.5 CaCl2 at ~30 °C. Cells were visualized with a 40x water-immersion objective on an upright fluorescent microscope (BX51WI, Olympus) equipped with infrared-differential interference contrast video microscopy and epifluorescence (Olympus).

For physiological characterization of eYFP-expressing neurons, picrotoxin (50 µM,
Sigma) was added to block inhibitory currents mediated by GABA-A receptors and excitatory transmission was inhibited by 20 µM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, Tocris). Patch pipettes (3.8-4.4 MΩ) were pulled from borosilicate glass (G150TF-4, Warner Instruments) and filled with internal solution containing (in mM): 135 K-gluconate, 5 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl2, 2 MgATP, 0.2 NaGTP, and 0.1% neurobiotin, pH 7.35 (290-300 mOsm). Electrophysiological recordings were made at 32 ºC using a MultiClamp700B amplifier and Pclamp software (Molecular Devices). Cells were held in current clamp mode at their respective resting membrane potential, and the following protocols were used (1) for analyzing the sag amplitude and rebound firing: hyperpolarizing current steps of -50 pA were injected for 2 s until the membrane potential reached -80 mV, (2) for examining maximal firing frequency: steady state current was injected in +20 pA increments until the cell reached depolarization block. No current injections were made for measuring the spontaneous firing frequency and single action potential analysis. Recordings were filtered at 10 kHz, and digitized at 100 kHz. Data were analyzed offline using Clampfit (Molecular Devices). To determine the neurochemical identity of eYFP-expressing neurons (i.e. TH-immunopositive or TH-immunonegative), neurons were filled with neurobiotin (Vector) during the patch clamp experiment, then fixed in 4% paraformaldehyde (PFA) and 24 h later immunostained for TH.

For recording of inhibitory postsynaptic currents (IPSCs) in the lateral habenula the internal solution contained (in mM): 130 CsCl, 1 EGTA, 10 HEPES, 2 MgATP and 0.2 NaGTP, pH 7.35 (270–285 mOsm). For excitatory postsynaptic currents (EPSCs) the internal solution contained (in mM): 117 CsCH3SO3, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA, 4 MgATP, 0.3 NaGTP, 5 QX314 and 0.1 spermine, pH 7.35 (270–285 mOsm). ChR2 was stimulated by flashing 473nm light (5-ms pulses; 0.1 Hz; 1–2mW) through the light path of the microscope using an ultrahigh-powered light-emitting diode (LED) powered by an LED driver (Prizmatix) under computer control. The light intensity of the LED was not changed during the experiments and the whole slice was illuminated. IPSCs and EPSCs were recorded in whole-cell voltage clamp at -70 mV, filtered at 2 kHz, digitized at 10 kHz (ITC-18 interface, HEKA) and collected online using custom IgorPro software (Wavemetrics). Series resistance (15–25 mV) and input resistance were monitored online with a 4-mV hyperpolarizing step (50 ms) given with each light stimulus. For IPSCs, we added a Na+-channel blocker (1 µM Tetrodotoxin citrate, Tocris) and a K+-channel blocker (1 mM 4-Aminopyridine, Sigma) to the bath as previously described.
(Cruikshank et al., 2010; Stamatakis et al., 2013). For EPSCs, the bath solution contained 50 μM picrotoxin (Sigma). IPSC and EPSC amplitudes were calculated by measuring the peak current from the average response from 10 sweeps before and after bath application of 50 μM picrotoxin or 10 μM CNQX, respectively.

**Behavioral Assays**

For real-time place preference/aversion experiments TH-Cre (JAX:8601), GAD2-Cre and VGlut2-Cre mice injected with 1 μl AAV5-DIO-ChR2-eYFP into the VTA and bilaterally implanted with optical fibers above the LHb were placed in a custom-made behavioral arena (as described previously in Lammel et al., 2012) for 15 min. One counterbalanced side of the chamber was assigned as the stimulation side. At the start of the session, the mouse was placed in the non-stimulated side of the chamber. For stimulation the bilateral optical fiber was connected to a 473 nm laser diode (OEM Laser Systems) through an FC/PC adaptor and laser output was controlled using a Master-8 pulse stimulator (A.M.P.I.). Light output through the optical fibers was adjusted to 30 mW (combined light intensity for both hemispheres) using a digital power meter console (Thorlabs) and was checked before and after each experimental animal. Every time the mouse crossed to the stimulation side of the chamber, 20 Hz (5 ms pulse width) constant laser stimulation was delivered until the mouse crossed back into the neutral and non-stimulation side. For reversal experiments the stimulation side was switched for additional 15 min. There was no interruption between the standard and the reversal real-time aversion experiment. The movement of the mice was recorded via a video tracking system (Biobserve) and the percentage of time the mice spent on each side of the chamber (stimulated, non-stimulated, neutral) was calculated.

**Single-cell Gene Expression Profiling**

Single cell profiling was performed essentially as previously described (Citri et al., 2011). In short, TH-Cre mice (JAX:8601) received intra-VTA injections of AAV-DJ-DIO-eYFP virus. Subsequently, Cre+ cells were identified in midline and lateral VTA regions as well as in the interpeduncular nucleus (IPN) based on eYFP expression. For comparison we also analyzed neurons in the substantia nigra pars compacta (SNc). Cytoplasm from single neurons were collected into 2x CellsDirect buffer (Invitrogen) by using glass patch pipettes. Samples were
snap frozen immediately on dry ice, and stored at \(-80^\circ\text{C}\) until further processing. Then, single cell mRNA samples were reverse transcribed, and subsequently PCR amplified by using target–specific probes. For internal controls, brain homogenates of the broader VTA region were also collected and purified using Trizol (Invitrogen). Control samples were reverse transcribed and PCR amplified together with the single cell samples. Critical threshold cycles (Ct) values were then determined by using Taqman assays (IDTDNA) and BioMark 48x48 Dynamic Array integrated microfluidic assays (Fluidigm Corporation). The resulting data were analyzed by custom made scripts in Mathematica 9 (Wolfram Research), and plotted as normalized expression relative to actin levels measured in the same cells. All presented probes had Ct values less than 27 in control tissue, and cells in which the normalized mRNA values were less than 0.1 (i.e. less than 5\% of actin levels) were classified as non–expressing. The probes were designed to have similar amplicon lengths (100–120 bp) to minimize amplification bias during PCR amplification and include transcripts for DAergic markers: TH (tyrosine hydroxylase), DAT (dopamine transporter), VMAT2 (vesicular monoamine transporter 2), GABAergic markers: GAD1 (glutamate decarboxylase 1, 67kDa), GAD2 (glutamate decarboxylase 2, 65kDa); calcium-binding proteins: Pvalb (parvalbumin), Calb1 (calbindin 1, 28kDa), Calb2 (calretinin); and an ion-channel: HCN2 (hyperpolarization-activated cyclic nucleotide-gated ion channel 2).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Forward primer</th>
<th>Probe sequence</th>
<th>Reverse primer</th>
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Histology, Immunohistochemistry, and Confocal Microscopy

Immunohistochemistry and confocal microscopy were performed as described previously (Lammel et al., 2008, 2012). Briefly, after intracardial perfusion with 4% paraformaldehyde in PBS, pH 7.4, the brains were post-fixed overnight and coronal midbrain slices (50 or 100 µm) were prepared. The primary antibodies used were rabbit anti-tyrosine hydroxylase (TH) (1:1000, Calbiochem), mouse anti-TH (1:1000, Millipore), rabbit anti-TH (1:1000, Pel-Freez) and rabbit anti-dopa decarboxylase (DDC) (1:1000, Millipore). The secondary antibodies used were AlexaFluor488 goat anti-rabbit, AlexaFluor488 goat anti-mouse, AlexaFluor546 goat anti-rabbit, AlexaFluor546 goat anti-mouse, Alexa Fluor Alexa Fluor647 goat anti-rabbit, Alexa Fluor Alexa Fluor647 goat anti-mouse (all 1:750, Molecular Probes), Rhodamine Avidin D (1:1000, Vector). Image acquisition was performed with a Zeiss LSM510 confocal microscope using 10x and 40x objectives, with an Olympus Fluoview FV1200 laser scanning confocal microscope using a 10x objective, and on a Zeiss AxioImager M1 upright wide-field fluorescence/differential interference contrast microscope with charged-coupled device camera using 2.5x and 10x objectives. Confocal images were analyzed using the Zeiss LSM Image Browser software and ImageJ software.

For anatomical characterization and quantification of eYFP-expressing and GFP-expressing neurons in transgenic mouse lines, 4 coronal brain sections (each 50 µm) in the posterior (approximately at bregma −3.4 mm, −3.45 mm, −3.5 mm, −3.55 mm) and 4 sections (each 50 µm) in anterior (approximately at bregma −2.8 mm, −2.85 mm, −2.9 mm, −2.95 mm) ventral midbrain were analyzed. For posterior midbrain sections, confocal images were taken in 3 different areas: midline VTA, lateral VTA and IPN (interpeduncular nucleus). The size of each area was ~300 x 300 µm. 4 non-overlapping confocal images, each covering ~150 x 150 µm, were acquired within this area using a 40x objective. The midline VTA contained parts of the interfascicular nucleus (IF) and the rostral linear nucleus (RLi) (blue area in Figures 1A, 1B). The lateral VTA was defined as the region comprising the paragigral nucleus (PN) and parabrachial pigmented nucleus (PBP) (green area in Figures 1A, 1B). The PBP contains mainly mesolimbic lateral shell DA neurons, while the PN contains mesocortical, mesolimbic medial shell and core as well as mesoamygdaloïd DA neurons (Lammel et al., 2008). Thus, in this study the analysis of the lateral VTA takes all DA subpopulations that were described previously (Lammel et al., 2008) into consideration. The IPN area comprised rostral (IPR), caudal (IPC),
intermediate (IPI), lateral (IPL), dorsolateral (IPDL) and dorsomedial (IPDM) interpeduncular subnuclei (red area in Figures 1A, 1B). For anterior midbrain sections, confocal images were taken in 2 different areas: midline VTA and lateral VTA with the same physical dimension as for the posterior midbrain sections. Anatomically, the midline VTA comprised the area between the fasciculus retroflexus (fr) and supramamillary decussation (sumx) (orange area in Figures 1D, 1E). The lateral VTA of the anterior midbrain contained the parabrachial pigmented nucleus (PBP) (brown area in Figures 1D, 1E). Sections were labelled relative to bregma using landmarks and neuroanatomical nomenclature as described in “The Mouse Brain in Stereotaxic Coordinates” (Franklin and Paxinos, 2001). Confocal images were acquired using identical pinhole, gain and laser settings. Then the total number of eYFP-expressing TH-immunopositive and TH-immunonegative neurons was calculated for each of the 5 areas. A similar methodological approach was used for quantification of retrogradely labeled (i.e. bead-containing or tdTomato-expressing) neurons projecting to lateral habenula in C57Bl6 mice.

For quantification of GAD2, VGlut2 and TH fluorescence intensities, confocal images from retrogradely labeled (i.e. beads-containing) VTA neurons projecting to lateral habenula as well as DA neurons in the lateral VTA (i.e. TH-immunopositive cells) from the same tissue sections were acquired at the same focus level. No additional post-processing was performed on any of the collected images. In these images, regions of interest (ROI) were marked around the somata (cell). For each ROI the frequency distribution of GAD2, VGlut2 and TH signal intensities was then quantified using a scale from 0 to 255 in ImageJ. Frequency distributions of GAD2, VGlut2 and TH immunosignal intensities were described by Gaussian functions to determine their mean intensity. GAD2, VGlut2 and TH background (bg) levels were determined in each image and the somatic signal intensity was normalized to the background signal to account for small variances in the background between different slices and animals.

**Statistics**

Student's t tests or one-way ANOVA tests were used to determine statistical differences for anatomical and electrophysiological data using GraphPad prism 6 (Graphpad Software). Bonferroni post hoc analysis was applied, when necessary, to correct for multiple comparisons. One- or two-way repeated measures ANOVAs were used to analyze behavioral data, with genotype and/or compartment as factors, followed by Student-Newman-Keuls posthoc tests.
SigmaStat software was used for these comparisons. Statistical significance was *p < 0.05, **p < 0.01, ***p < 0.001. All data values are presented as means ± SEM.

SUPPLEMENTAL REFERENCES


