



Published in final edited form as:

*Mol Psychiatry*. 2022 March ; 27(3): 1515–1526. doi:10.1038/s41380-021-01424-3.

## Developmental impact of glutamate transporter overexpression on dopaminergic neuron activity and stereotypic behavior

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### Abstract

Obsessive-compulsive disorder (OCD) is a disabling condition that often begins in childhood. Genetic studies in OCD have pointed to *SLC1A1*, which encodes the neuronal glutamate transporter EAAT3, with evidence suggesting that increased expression contributes to risk. In mice, midbrain *Slc1a1* expression supports repetitive behavior in response to dopaminergic agonists, aligning with neuroimaging and pharmacologic challenge studies that have implicated the dopaminergic system in OCD. These findings suggest that *Slc1a1* may contribute to compulsive behavior through altered dopaminergic transmission; however, this theory has not been mechanistically tested. To examine the developmental impact of *Slc1a1* overexpression on compulsive-like behaviors, we therefore generated a novel mouse model to perform targeted, reversible overexpression of *Slc1a1* in dopaminergic neurons. Mice with life-long overexpression of *Slc1a1* showed a significant increase in amphetamine (AMPH)-induced stereotypy and hyperlocomotion. Single-unit recordings demonstrated that *Slc1a1* overexpression was associated with increased firing of dopaminergic neurons. Furthermore, dLight1.1 fiber photometry showed

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#### Author Contributions

MOC, JMK, JV and SEA designed the study and analyzed the data. MOC, JMK, HY, ZF, and NG performed experiments. JV and SEA acquired funding for the project and supervised experiments. AZH, PDB, EDL, and ERK provided key resources, assisted in methods implementation, and interpreted the resulting data. MOC and JV drafted the manuscript. MOC, JMK, HY, ZF, NG, AZH, PDB, EDL, ERK, JV and SEA revised and edited the manuscript for critical content.

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that these behavioral abnormalities were associated with increased dorsal striatum dopamine release. In contrast, no impact of overexpression was observed on anxiety-like behaviors or SKF-38393-induced grooming. Importantly, overexpression solely in adulthood failed to recapitulate these behavioral phenotypes, suggesting that overexpression during development is necessary to generate AMPH-induced phenotypes. However, doxycycline-induced reversal of *Slc1a1*/EAAT3 overexpression in adulthood normalized both the increased dopaminergic firing and AMPH-induced responses. These data indicate that the pathologic effects of *Slc1a1*/EAAT3 overexpression on dopaminergic neurotransmission and AMPH-induced stereotyped behavior are developmentally mediated, and support normalization of EAAT3 activity as a potential treatment target for basal ganglia-mediated repetitive behaviors.

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## Introduction

Obsessive-compulsive disorder (OCD) is a debilitating neuropsychiatric condition characterized by persistent intrusive thoughts (obsessions) and/or repetitive behaviors (compulsions) (1). OCD affects 2–3% of the general population (2) and is a leading cause of disability (3). Available first-line treatments include exposure therapy with response prevention (E/RP) and serotonin-reuptake inhibitors (SRIs) (4–6); however, full remission is achieved in only 10–20% of patients (7). Several other neuropsychiatric disorders, including Tourette Syndrome (TS) and Autism Spectrum Disorders (ASD), also include repetitive behavior and show elevated co-occurrence rates with OCD (8–10). Approximately half of individuals with OCD have onset in childhood (11), suggesting that it has developmental origins. A greater understanding of the neurobiological underpinnings of OCD, and related disorders, is needed to develop novel treatments.

Converging evidence indicates that dysfunction of basal ganglia circuits contributes to abnormal repetitive behaviors characteristic of OCD and related disorders, such as TS (12–14). Structural neuroimaging finds abnormal caudate volume in OCD and TS (15–19), and functional imaging demonstrates abnormal activity in basal ganglia circuits in these disorders (20–25). Neuronal recordings also demonstrate abnormal activity in the caudate, globus pallidus, thalamus and subthalamus (26–30), supporting the hypothesis that dysregulated basal ganglia signaling is linked to obsessive-compulsive and tic symptoms.

Evidence also indicates involvement of the dopaminergic system in OCD and TS. For example, in humans, dopaminergic agents such as amphetamine (AMPH) and L-DOPA can trigger repetitive behaviors and can exacerbate or worsen tics in patients with TS (31–36). Molecular imaging studies in OCD and TS also find altered dopamine transporter or D<sub>2</sub> receptor binding in the striatum (37–39). Notably, increased neuromelanin signal, a marker of dopaminergic neurons, has been reported in pediatric OCD (40). Increased striatal intrasynaptic dopamine release is also implicated in TS from PET imaging findings (41, 42). Consistent with these findings, augmentation with D<sub>2</sub> antagonists is a common pharmacological strategy for SRI-resistant OCD and TS (43–46). Finally, data from a wide range of animal models of repetitive behavior suggests that alterations in dopaminergic transmission contribute to abnormal repetitive behavior (47, 48).

Family and twin studies demonstrate substantial heritability of OC symptoms, with greater support for heritability in early-onset OCD (49). Genetic linkage (50, 51) and association (52–54) studies point to the *SLC1A1* gene located on chromosome 9p24, which encodes the neuronal glutamate transporter Excitatory Amino Acid Transporter 3 (EAAT3/EAAC1), with strongest association in males and in early-onset OCD. Notably, the OCD families contributing to linkage at the chromosome 9p24 region and association to *SLC1A1* also included individuals with Tic Disorder or TS (50, 52, 53), highlighting the co-occurrence and shared genetic risk of these repetitive behavior disorders. Most of the findings have clustered at the 3' gene region with the greatest evidence for association with the rs301430C allele, which is linked to increased expression in brain tissue and in cell models (54, 55). Of note, however, a meta-analysis found only a modest association of *SLC1A1* variants with OCD (56), and modestly sized genome-wide association studies have reported no genes reaching genome-wide significance in OCD (57, 58). *SLC1A1/EAAT3* is expressed broadly throughout the brain, including in spiny projection neurons in the striatum (59, 60) and midbrain dopaminergic neurons (61, 62). Within neurons, EAAT3 is localized to peri-synaptic regions where it modulates glutamate signaling (63, 64). This is consistent with evidence of altered glutamate signaling in OCD and TS from cerebrospinal fluid, magnetic resonance spectroscopy, and gene expression studies (65–71).

Recently, Delgado-Acevedo and colleagues (72) used CaMKII $\alpha$ -Cre to activate transgenic overexpression of EAAT3 in glutamatergic neurons in the cortex, striatum, and hippocampus, finding increased anxiety-like and grooming behavior. In a follow-up study (73), the authors found decreased baseline dopamine levels and increased AMPH-induced dopamine release in the dorsal striatum, suggesting that CaMKII $\alpha$ -Cre-based EAAT3 overexpression impacts dopaminergic signaling in the striatum; although AMPH-induced locomotor activity was not affected. Here, we investigated the functional impact of EAAT3 manipulation on circuits implicated in OCD. We focused on EAAT3 activity in dopaminergic neurons because of the well-established importance of dopamine in repetitive behaviors (37, 43, 47, 48, 74), and our previous work in EAAT3 knockout mice (75) that suggested involvement of midbrain EAAT3 expression in pharmacologically induced-repetitive behaviors. Here, we therefore directly tested the hypothesis that increased EAAT3 expression in dopaminergic neurons leads to increased compulsive-like behavior. We used a flexible *Slc1a1* knock-in cassette (76) to generate mice with selective and reversible overexpression of EAAT3 in tyrosine hydroxylase (TH)-expressing neurons in both development and adulthood to determine the impact on dopamine signaling and basal ganglia-mediated repetitive behavior.

## Materials and methods

A summary is provided here. Additional details are available in Supplementary Information.

### Mice

All experimental studies were approved by the New York State Psychiatric Institute or the University of Pittsburgh Institutional Animal Care and Use Committees. *Slc1a1*-STOP-tetO mice (75) were crossed to *Pgk1*-flpo mice (77) to obtain *Slc1a1*-tetO mice that were

then crossed to the *Th-tTA* line (78) to obtain *Slc1a1-Th*-OE and control mice. EAAT3 expression was regulated by doxycycline-supplemented chow (Envigo, Madison, Wisconsin, USA) and was quantified using real-time qRT-PCR and immunoblots. Adult male and female mice and littermate controls were used for all experiments.

### Behavioral assays

Behavioral procedures were adapted from (75). Two separate cohorts of naïve, *Slc1a1-Th*-OE mice and littermate controls were used to test baseline anxiety-like and repetitive (elevated plus maze, light-dark emergence, and marble-burying), and drug-induced (AMPH-induced locomotion, AMPH-induced stereotypy, and SKF-38393-induced grooming) behaviors. Developmental effects were tested in separate, naïve cohorts that were administered doxycycline/regular chow. Rescue experiments were tested separately, in naïve cohorts, using a longitudinal design and in drug-naïve mice. Timelines for each experiment, including the ages of mice, are provided in the figures.

### *In vivo* single-unit recordings

Naïve, adult chloral hydrate anesthetized mice were implanted with a glass electrode filled with 2M NaCl into the VTA (AP  $-3.2$ , ML  $\pm 0.6$ , DV  $-3.5$ mm). Neurons were sampled from 6 equally spaced tracks. Traces were processed using NeuroScope software. Dopamine neurons were identified by the broad action potential width ( $>3$  msec) and tri-phasic waveform (79–81). Bursts were defined as per the classical criteria of Grace and Bunney (79, 80). ISI distributions, burst properties, and firing rates for each dopamine neuron were quantified. Recording locations were histologically confirmed at the end of the experiment.

### *In vivo* fiber photometry

Naïve, adult isoflurane anesthetized mice were injected with 0.3  $\mu$ l of AAV5-CAG-dLight1.1 (82) into the dorsal striatum (AP  $+1.0$ , ML  $\pm 1.5$ , DV  $-3.2$  mm) followed by implantation of a fiber optic cannula (200  $\mu$ m core, 0.48 NA, Doric, Quebec, Canada) above the site of virus injection. 3 weeks after virus expression dopamine imaging was conducted using a fiber photometry system (Neurophotometrics) with capabilities for 470 nm and 410 nm excitation wavelengths. Raw 470 nm and 410 nm signals were extracted using MATLAB (Mathworks, R2015a); extracted signals were baseline-corrected to remove the slope (Origin(Pro), Version 2021) and then standardized using the median and standard deviation of the 20 min baseline period (83). The standardized 410 nm signal was then regressed onto the standardized 470 nm signal. The regressed 410 nm signal was then subtracted from the standardized 470 nm signal to obtain  $dF/F$  values.

### Statistical analysis

Data analysis was performed with GraphPad Prism (version 9.0.0, GraphPad Software, San Diego, CA, USA). Two-tailed, unpaired *t* test, 2-way RM ANOVA, or 3-way RM ANOVA was used to analyze the primary data. Locomotion data was analyzed using non-linear curve fit and area under curve (AUC) analysis. Table S1 in Supplementary Information shows a summary of all statistics. Secondary analysis using sex as an independent variable

is presented in **Sex as a biological variable** in Supplementary Information. All data are reported as the mean  $\pm$  SEM (standard error of mean).

## Results

### EAAT3 is selectively and reversibly overexpressed in *Slc1a1-Th-OE* mice

We took advantage of the Flexible Accelerated STOP TetO-knockin (FAST) system (76) to selectively overexpress EAAT3 in TH-positive neurons via the tetracycline controlled transcriptional activator (tTA) (Figure 1, Supplementary Figures S1–2). We first created *Slc1a1-tetO* mice by crossing to a *Pgk1-flpo* line (77) to delete the STOP cassette from *Slc1a1-STOP-tetO* mice (75). To obtain TH-positive neuron-restricted overexpression, *Slc1a1-tetO* mice were crossed to mice expressing tTA under control of the *Th* promoter (*Th-tTA*) (78), yielding mice that were homozygous for the *Slc1a1-tetO* allele and possessed one copy of the *Th-tTA* transgene (*Slc1a1-tetO<sup>homo</sup>/Th-tTA<sup>+</sup>*, termed *Slc1a1-Th-OE* or *Th-OE* mice) (see Supplementary Information for detailed breeding scheme). In the absence of doxycycline (tet-OFF), tTA binds and activates the tetO promoter, leading to increased expression of the tetO-associated gene (Figure 1A). As expected, western blot analysis of midbrain whole cell-lysis extracts (Figure 1B) showed increased EAAT3 protein expression in life-time overexpressing *Slc1a1-Th-OE* mice in comparison with littermate controls (Figure 1C, unpaired  $t(22) = 6.435$ ,  $P < 0.0001$ ). Administration of doxycycline-supplemented chow in adulthood resulted in restoration of expression in *Slc1a1-Th-OE* mice to littermate control group levels (Figure 1D, unpaired  $t(10) = 0.01105$ ,  $P = 0.9142$ ). The increased EAAT3 protein expression in *Slc1a1-Th-OE* mice was restricted to the midbrain; no genotype differences in EAAT3 expression were observed in regions outside the midbrain, including cortex, hippocampus, and striatum (Supplementary Figure S2). Together, these results indicate efficient and selective induction and reversal of midbrain EAAT3 overexpression in *Slc1a1-Th-OE* mice (76, 84, 85).

### *Slc1a1-Th-OE* mice show no baseline changes in anxiety-like or repetitive behavior

We next evaluated life-time overexpressing *Slc1a1-Th-OE* mice and littermate controls for baseline anxiety-like and repetitive behavioral phenotypes (Supplementary Figures S3, S4). In the open field (a novel environment), *Slc1a1-Th-OE* mice showed a slight but significant increase in novelty-induced locomotion in comparison with littermate controls over 30 minutes of exploration (Supplementary Figure 3A, third order polynomial least squares fit,  $F(4, 106) = 2.483$ ,  $P = 0.0481$ ). Despite this small increase in locomotion in *Slc1a1-Th-OE* mice during the first open field exposure, no significant time by genotype interaction was found across subsequent days of exposure (Supplementary Figure 3A), indicating preserved habituation in *Slc1a1-Th-OE* mice. We found no differences in indices of anxiety-like behavior in the open field (Supplementary Figure S4). No significant genotype differences were found in time spent in the open sections of the elevated zero maze (considered a more anxiety-provoking environment) (Supplementary Figure S3B) or in the time spent or distance traveled in the lit chamber in the light-dark test (Supplementary Figure S3C). Finally, we found no significant genotype differences in the number of marbles buried in the marble burying test (Supplementary Figure S3D), or in baseline grooming behavior in a novel cage (Figure 2E).

## Amphetamine reveals increased basal ganglia-dependent repetitive behavior in *Slc1a1-Th-OE* mice

To examine whether life-time overexpression of *Slc1a1* would lead to an increase in basal ganglia-mediated repetitive behavior mirroring the decrease in AMPH-induced repetitive behaviors in *Slc1a1*-STOP-tetO mice (75), *Slc1a1-Th-OE* mice and littermate controls were evaluated for AMPH-induced locomotion and stereotypic behavior (Figure 2). Injection of low dose AMPH (1.8 mg/kg) resulted in significantly increased locomotor activity in *Slc1a1-Th-OE* mice relative to littermate controls (Figure 2B, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 334) = 13.47$ ,  $P < 0.0001$ ). At a moderate dose (3.0 mg/kg), this difference became even more pronounced (Figure 2C, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 334) = 16.12$ ,  $P < 0.0001$ ). At the highest dose tested (8.0 mg/kg), video-scoring of stereotypic behavior at 50- and 80-mins post-injection revealed a significant drug by genotype interaction and a significant main effect of genotype (Figure 2D, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 34) = 26.73$ ,  $P < 0.0001$ ; drug,  $F(1, 34) = 139.8$ ,  $P < 0.0001$ , genotype,  $F(1, 34) = 25.84$ ,  $P < 0.0001$ ), indicating increased stereotypic activity in *Slc1a1-Th-OE* mice. To examine stereotypic behavior that is independent of pre-synaptic dopamine release, we acutely challenged *Slc1a1-Th-OE* mice and littermate controls with the D<sub>1</sub> agonist SKF-38393. While there was a significant main effect of drug, there was no significant drug by genotype interaction or main effect of genotype (Figure 2E, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 17) = 1.219$ ,  $P = 0.2849$ ; drug,  $F(1, 17) = 38.53$ ,  $P < 0.0001$ , genotype,  $F(1, 17) = 0.9749$ ,  $P = 0.3373$ ). These results indicate that EAAT3 overexpression impacts dopamine-induced repetitive behavior via a pre-, not post-synaptic, mechanism.

Because gene knock-ins and transgene insertions can cause unintended changes in native gene expression and function (86–91), we evaluated the impact of 1) the *Slc1a1*-tetO operator, and 2) the *Th*-tTA transgene independent of the *Slc1a1*-tetO operator in mice that were administered regular chow. While mice that were homozygous for the *Slc1a1*-tetO allele (*Slc1a1*-tetO<sup>homo</sup>/*Th*-tTA<sup>-</sup>) displayed ~50% reduction in EAAT3 expression compared to WT mice (Supplementary Figure S5B), they did not show significant differences in AMPH-induced responses or basal dopaminergic transmission (Supplementary Figure S5C–M). We similarly observed no significant effect of the *Th*-tTA transgene alone on AMPH-induced responses (Supplementary Figure S6).

## Anesthetized *Slc1a1-Th-OE* mice show increased dopamine neuron burst firing

To determine whether life-time overexpression of EAAT3 in dopaminergic neurons alters neuron activity patterns *in vivo*, we recorded spontaneous action potential firing in anesthetized *Slc1a1-Th-OE* mice and littermate controls (Figure 3A–I). Putative dopamine neurons were identified by the established long duration (>3 msec), triphasic waveform and by their tonic irregular firing and intermittent bursts (79–81, 92) (Figure 3B), defined per the standard 80/160 ms criteria (79). We found an increased proportion of short duration interspike intervals (ISIs) in life-time overexpressing *Slc1a1-Th-OE* mice relative to littermate controls (Figure 3C, 2-way RM ANOVA: ISI  $\times$  genotype,  $F(50, 500) = 5.254$ ,  $P < 0.0001$ ; ISI,  $F(50, 500) = 103.5$ ,  $P < 0.0001$ , genotype,  $F(1, 10) = 6.269$ ,  $P = 0.0312$ ; Sidak's multiple comparisons:  $P < 0.0001$  for ISIs 30–90 msec). Evaluation of burst parameters



revealed that *Slc1a1-Th*-OE mice displayed an increased percentage of total spikes fired in bursts (Figure 3D, unpaired  $t(10) = 4.684$ ,  $P = 0.0009$ ), spikes per burst (Figure 3E, unpaired  $t(10) = 4.905$ ,  $P = 0.0006$ ), and increased burst set rate (Figure 3F, unpaired  $t(10) = 2.825$ ,  $P = 0.0180$ ). We did not find differences in average burst ISI (Figure 3G, unpaired  $t(10) = 1.191$ ,  $P = 0.2612$ ); however, average before-burst ISI trended toward a decrease in *Slc1a1-Th*-OE mice (Figure 3H, unpaired  $t(10) = 2.030$ ,  $P = 0.0699$ ). Overall, we found increased firing rate in *Slc1a1-Th*-OE mice (Figure 3I, unpaired  $t(10) = 3.808$ ,  $P = 0.0034$ ).

### **Awake, freely moving *Slc1a1-Th*-OE mice show increased baseline and AMPH-induced dopamine release in dorsal striatum**

To determine whether life-time overexpression of EAAT3 in TH-positive neurons alters dopamine release, we measured dopamine transients in freely moving mice with fiber photometry (Figure 3J–P). To visualize dopamine release, we infused an AAV vector encoding dLight1.1 (AAV5-CAG-dLight1.1), a highly-specific, intensity-based dopamine sensor (82), into the dorsal striatum of *Slc1a1-Th*-OE and littermate control mice. Optic fibers were simultaneously implanted (Figure 3J). After 3 weeks, we reliably detected dopamine transients during a 20-minute baseline evaluation period in an empty cage (Figure 3K). We used a stringent threshold of one interquartile range (IQR) above median and a minimum of 100 msec duration (to account for dLight1.1 decay time (82)) to detect dopamine transients in each group. Analysis of these dopamine transients revealed significantly increased median fluorescence (Figure 3L, unpaired  $t(9) = 3.379$ ,  $P = 0.0081$ ) but unaltered transient frequency (Figure 3M, unpaired  $t(9) = 0.7751$ ,  $P = 0.4582$ ) in *Slc1a1-Th*-OE mice in comparison with littermate controls. We next challenged mice with a 3.0 mg/kg dose of AMPH and monitored fluorescence for 60 mins. As expected, AMPH induced a significant elevation of dLight1.1 signal compared to baseline in both groups. AUC analysis of the 60-minute post-AMPH injection period revealed significantly increased fluorescence in *Slc1a1-Th*-OE mice compared with littermate controls (Figure 3N, unpaired  $t(8) = 2.740$ ,  $P = 0.0254$ ). Like pre-AMPH, we found increased median fluorescence of dopamine transients (Figure 3O, unpaired  $t(8) = 5.065$ ,  $P = 0.0010$ ) but unaltered transient frequency (Figure 3P, unpaired  $t(8) = 1.788$ ,  $P = 0.1115$ ) following AMPH in *Slc1a1-Th*-OE mice compared to littermate controls.

### **Overexpression of EAAT3 during development is necessary for increased AMPH-induced locomotion and stereotypic behavior in *Slc1a1-Th*-OE mice**

To determine whether the effects of EAAT3 overexpression are developmental in origin, we administered doxycycline-supplemented chow to *Slc1a1-Th*-OE mice and littermate controls for 2 months, and then switched them to regular chow for 1 month to induce adult-specific tTA activation (termed ‘adult-specific overexpressing mice’, Figure 4M–R). *Slc1a1-Th*-OE mice and littermate controls that were administered doxycycline-supplemented chow for whole-life (termed ‘life-time rescue mice’, Figure 4A–F) or regular chow for whole-life (termed ‘life-time overexpressing mice’, Figure 4G–L) served as control groups and were run in parallel to the adult-specific overexpressing mice.

As expected, whole-life tTA suppression by doxycycline resulted in rescue of *Slc1a1* expression via qRT-PCR of the midbrain (Figure 4B, unpaired  $t(6) = 0.7816$ ,  $P = 0.4642$ ).

We found no genotype differences in novelty-induced locomotion (Figure 4C, third order polynomial least squares fit,  $t = 0-30$ ;  $F(4, 94) = 0.3311$ ,  $P = 0.8564$ ) or anxiety-like behavior measures (Supplementary Figure S7A–C) in life-time rescue mice. We found a slight increase in AMPH-induced locomotor activity in *Slc1a1-Th*-OE mice at 1.8 mg/kg via curve fit analysis (Figure 4D, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 298) = 2.774$ ,  $P = 0.0273$ ); however, no significant drug by genotype interaction or a main effect of genotype was found in AUC analysis at this dose (Figure 4D, **inset**, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 15) = 1.341$ ,  $P = 0.2650$ ; drug,  $F(1, 15) = 21.85$ ,  $P = 0.0003$ , genotype,  $F(1, 15) = 0.2310$ ,  $P = 0.6377$ ). Locomotor activity at 3.0 mg/kg (Figure 4E, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 298) = 2.144$ ,  $P = 0.0754$ ) and stereotypic behavior at 8.0 mg/kg (Figure 4F, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 30) = 1.581$ ,  $P = 0.2183$ ; drug,  $F(1, 30) = 341.4$ ,  $P < 0.0001$ , genotype,  $F(1, 30) = 2.017$ ,  $P = 0.1659$ ) in life-time rescue *Slc1a1-Th*-OE mice was comparable to controls.

As above, administration of regular chow to life-time overexpressing mice resulted in increased *Slc1a1* expression in *Slc1a1-Th*-OE mice (Figure 4H, unpaired  $t(6) = 4.168$ ,  $P = 0.0059$ ), and life-time overexpressing *Slc1a1-Th*-OE mice displayed robust increases in novelty-induced locomotion (Figure 4I, third order polynomial least squares fit,  $t = 0-30$ ;  $F(4, 124) = 9.196$ ,  $P < 0.0001$ ). No genotype differences were observed in open-field anxiety-like measures (Supplementary Figure S7D–F). AMPH-induced locomotor activation in response to 1.8 mg/kg (Figure 4J, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 388) = 20.86$ ,  $P < 0.0001$ ) and 3.0 mg/kg (Figure 4K, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 388) = 22.41$ ,  $P < 0.0001$ ) was significantly increased in life-time overexpressing *Slc1a1-Th*-OE mice; AMPH-induced stereotypic behavior at 8.0 mg/kg (Figure 4L, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 40) = 12.34$ ,  $P = 0.0011$ ; drug,  $F(1, 40) = 193.7$ ,  $P < 0.0001$ , genotype,  $F(1, 40) = 11.88$ ,  $P = 0.0013$ ) was also significantly elevated.

Remarkably, adult-specific overexpression of *Slc1a1* (Figure 4N, unpaired  $t(6) = 4.662$ ,  $P = 0.0035$ ) failed to increase novelty-induced locomotion in *Slc1a1-Th*-OE mice (Figure 4O, third order polynomial least squares fit,  $t = 0-30$ ;  $F(4, 142) = 0.6595$ ,  $P = 0.6212$ ). Furthermore, no genotype differences were found in AMPH-induced locomotor response at 1.8 mg/kg (Figure 4P, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 442) = 2.341$ ,  $P = 0.0544$ ) or 3.0 mg/kg (Figure 4Q, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 442) = 0.04487$ ,  $P = 0.9962$ ) in adult-specific overexpressing mice. Stereotypic behavior at the 8.0 mg/kg AMPH dose (Figure 4R, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 46) = 2.605$ ,  $P = 0.1134$ ; drug,  $F(1, 46) = 199.5$ ,  $P < 0.0001$ , genotype,  $F(1, 46) = 2.407$ ,  $P = 0.1277$ ) in *Slc1a1-Th*-OE mice was also comparable to controls. These data suggest that increased EAAT3 activity during early development may increase susceptibility to pharmacologically-induced compulsive-like behavior.



## Reversal of EAAT3 overexpression in adulthood rescues AMPH-induced locomotor and stereotypic behavior and dopamine neuron activity

To evaluate whether the heightened AMPH-induced repetitive behavior in life-time *Slc1a1-Th*-OE mice could be rescued by normalizing EAAT3 overexpression in adulthood, we performed a series of longitudinal experiments. We first assessed AMPH-induced behavior prior to 4 weeks of tTA suppression by doxycycline-supplemented chow (see Fig 1D for rescue of EAAT3 expression). As above, we found robust increases in AMPH-induced locomotion at the low (Figure 5B, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 370) = 9.326$ ,  $P < 0.0001$ ) and moderate doses (Figure 5D, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 370) = 16.33$ ,  $P < 0.0001$ ), and increased AMPH-induced stereotypic behavior at 8.0 mg/kg (Figure 5F, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 38) = 13.89$ ,  $P = 0.0006$ ; drug,  $F(1, 38) = 126.9$ ,  $P < 0.0001$ , genotype,  $F(1, 38) = 9.839$ ,  $P = 0.0033$ ) in *Slc1a1-Th*-OE mice. After 4 weeks of doxycycline-supplemented chow, we retested AMPH-induced behavior (Figure 5A) and found significantly reduced AMPH responses in *Slc1a1-Th*-OE mice compared to controls—after doxycycline treatment, *Slc1a1-Th*-OE mice actually displayed less locomotor activity at the low dose than controls (Figure 5C, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 370) = 5.153$ ,  $P = 0.0005$ ). Locomotor activation at the moderate AMPH dose (Figure 5E, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 370) = 2.105$ ,  $P = 0.0796$ ), and stereotypic behavior at the high AMPH dose (Figure 5G, 3-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 38) = 0.05213$ ,  $P = 0.8206$ ; drug,  $F(1, 38) = 96.66$ ,  $P < 0.0001$ , genotype,  $F(1, 38) = 0.040704$ ,  $P = 0.8411$ ) was comparable to controls.

To eliminate the potential confound of repeated exposure to high-dose AMPH, a separate cohort of drug naïve mice were switched to doxycycline-supplemented chow at 2 months of age and then evaluated for pharmacological-induced behaviors starting at 4 months (Supplementary Figure S8). We found a slightly increased locomotor response at the low dose in *Slc1a1-Th*-OE mice compared to littermate controls in this cohort (Supplementary Figure S8C, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 298) = 6.411$ ,  $P < 0.0001$ ) and no differences at the moderate (Supplementary Figure S8D) or high AMPH doses (Supplementary Figure S8E), or in SKF-38393-induced grooming behavior (Supplementary Figure S8F).

To determine whether the increased dopaminergic neuron spike firing in life-time overexpressing *Slc1a1-Th*-OE mice could be rescued by normalizing EAAT3 expression, both adult naïve *Slc1a1-Th*-OE and littermate control mice were placed on doxycycline-supplemented chow (Figure 5H). One month after initiating doxycycline, no significant genotype differences were observed in the proportion of short duration ISIs in adult-specific rescue *Slc1a1-Th*-OE mice compared to controls (Figure 5J, 2-way RM ANOVA: ISI  $\times$  genotype,  $F(50, 500) = 0.1851$ ,  $P > 0.9999$ ; ISI,  $F(50, 500) = 60.06$ ,  $P < 0.0001$ , genotype,  $F(1, 10) = 3.171$ ,  $P = 0.1053$ ). Furthermore, no significant genotype differences were observed in percentage of total spikes fired in bursts (Figure 5K, unpaired  $t(10) = 0.2226$ ,  $P = 0.8283$ ), spikes per burst (Figure 5L, unpaired  $t(10) = 0.3098$ ,  $P = 0.7631$ ), burst set rate (Figure 5M, unpaired  $t(10) = 0.1082$ ,  $P = 0.9160$ ), average burst ISI (Figure 5N, unpaired  $t(10) = 0.4365$ ,  $P = 0.6717$ ), and average before-burst ISI (Figure 5O, unpaired  $t(10) =$

0.5456,  $P = 0.5973$ ). Finally, no genotype differences were observed in firing rate (Figure 5P, unpaired  $t(10) = 0.2049$ ,  $P = 0.8417$ ) after adult-specific administration of doxycycline. Together, these results indicate that normalizing EAAT3 expression in adulthood leads to both normalization of AMPH-induced behaviors and of dopamine neuron firing.

## Discussion

Here, using a novel transgenic strategy, we found that overexpression of *Slc1a1*/EAAT3 in TH-positive neurons can increase AMPH-induced hyperlocomotion and repetitive behavior. This was accompanied by increased baseline burst firing of dopaminergic neurons in anesthetized mice and increased dopamine release in striatum in awake, freely moving animals. Surprisingly, we also observed a developmental effect of *Slc1a1*/EAAT3 expression on AMPH-induced repetitive behavior. Specifically, we found that both developmental and adult overexpression of *Slc1a1* are necessary to induce increases in AMPH-induced hyperlocomotion and stereotypy, whereas neither by itself is sufficient. Our findings identify dopaminergic *Slc1a1*/EAAT3 activity over the life course as a mechanism that shapes susceptibility to AMPH-induced compulsive-like behavior, and provide a causal link between elevated dopaminergic EAAT3 activity that begins in early development and increased stereotypic activity induced by AMPH. Lastly, we found that rescue of *Slc1a1* expression in adulthood is sufficient to restore AMPH-induced responses, suggesting normalization of EAAT3 as a potential treatment strategy in basal ganglia-mediated repetitive behaviors.

Our data indicate that altered EAAT3 expression in dopaminergic neurons impacts baseline dopamine release, as well as AMPH-induced release and downstream repetitive behavior, as is seen in some people with OCD or TS (31–34). We did not, however, observe increased grooming, a behavior that is also dependent upon the basal ganglia and has face validity for OCD (48, 93, 94). We also did not find increased anxiety-like behavior, despite the prominence of anxiety in OCD. The increased grooming and anxiety-like behavior previously reported with a CamKII-Cre-mediated transgenic approach (72, 73) may indicate that EAAT3 overexpression in other brain regions or circuits is responsible for other OCD-relevant phenotypes. Future work will be necessary to fully disentangle the cell type-specific and developmental impact of EAAT3 on OCD-relevant circuits and behavior.

We observed decreased expression of EAAT3 in *Slc1a1*-tetO mice, our controls for the *Slc1a1*-Th-OE mice, in comparison to wildtype mice. Importantly, there was no difference in baseline dopaminergic neuron activity or AMPH-induced behaviors in these animals, supporting their use as the appropriate controls. Previous work in EAAT3 heterozygous mice similarly found intact baseline anxiety-like and compulsive-like behaviors, AMPH-induced response, and glutamate and dopamine levels (95), suggesting that a larger change in EAAT3 expression is necessary to induce a functional change.

We previously reported that constitutive ablation of *Slc1a1*/EAAT3 caused decreased AMPH-induced locomotion and stereotypy, as well as decreased SKF-38393-induced grooming behavior (75). Rescue of midbrain EAAT3 expression in this previous study using a constitutive viral Cre promoter partially rescued the AMPH response in these

animals but was not specific to a neuronal subtype (75). Based on previous studies, midbrain GABAergic and dopaminergic neurons were candidates for this rescue effect (62, 96, 97). Unfortunately, transgenic lines that drive Cre expression in GABAergic neurons yield broad expression that extends beyond the midbrain (98), and it has been difficult to specifically drive Cre expression in GABAergic neurons using viral vectors (99, 100). Furthermore, transgenic animals that drive dopaminergic Cre expression (101, 102) could not be used to further localize this effect because we found that they have baseline changes in AMPH response (87). Our data in *Slc1a1-Th*-OE mice support and refine previous imaging, pharmacologic, and transgenic work that implicates altered dopaminergic transmission in compulsive behavior and provide *Slc1a1*-driven increases in dopaminergic transmission during development as a pathophysiological mechanism that contributes to susceptibility to pharmacologically-induced compulsive-like behavior.

The impact of EAAT3 overexpression on dopaminergic transmission and AMPH-induced repetitive behavior could result from multiple potential mechanisms. Notably, AMPH triggers endocytosis of EAAT3 upon entry into dopamine neurons and leads to potentiation of glutamatergic transmission via activation of extra-synaptic NMDA receptors (59, 61, 62). Although we did not directly evaluate glutamate transmission in dopamine neurons, it is conceivable that long-term decreases in exposure to glutamate due to overexpression of EAAT3 leads to compensatory increases in NMDA receptor expression and/or sensitivity. Indirect evidence for altered NMDA receptor function comes from our *in vivo* recording data where, compared with control animals, an increase in burst firing is observed in EAAT3 overexpressing mice. Notably, EAAT3 also plays roles in supplying glutamate for GABA synthesis (96, 97) and cysteine for glutathione synthesis (103, 104), so alternative mechanisms could also account for the changes in dopaminergic neuron burst firing, striatal dopamine release, and AMPH response in overexpressing mice. Future studies examining glutamate release (105), receptor subunit composition of glutamate and GABA receptors, and the impact on other glutamate transporters' function, will be important for revealing molecular mechanisms underlying increased dopaminergic transmission in *Slc1a1-Th*-OE mice.

We observed a slight increase in response to 1.8 mg/kg AMPH in *Slc1a1-Th*-OE mice (Fig 4D, S8C) despite life-time suppression of overexpression by doxycycline. This increase was significant in the curve fit analysis but not the AUC analysis, and no change in response was seen to higher dose AMPH, suggesting a small effect size. It seems possible that this change reflects a small amount of residual EAAT3 overexpression, which could be assessed in future experiments using higher doses of doxycycline. Remarkably, inducing *Slc1a1*/EAAT3 overexpression in adulthood alone did not lead to increases in AMPH-induced locomotion or stereotypy. Failure to induce increases in AMPH response in adult mice is especially intriguing as the effects of both EAAT3 overexpression and knockdown (Figure 5 and (75)) can still be rescued in adulthood. Our data showing the necessity of both developmental and adult EAAT3 overexpression in the induction of increases in AMPH-induced repetitive behavior also parallel genetic data that show a stronger association between *SLC1A1* and early-onset OCD (50–53, 55). These temporal-specific aspects of EAAT3 function may reflect the protracted course of dopaminergic system development that continues into adolescence (106, 107). Our data highlight the need to study the role of genetic influences

during early developmental epochs when cognitive circuits and processes are maturing. Future work will be needed to evaluate potential effects of altered dopaminergic signaling on OCD-relevant cognitive tasks, such as reversal learning or set shifting (108–111).

Finally, we found that the effects of EAAT3 overexpression in TH-positive neurons are reversible. Specifically, restoration of normal levels of expression in adult animals reverses the increases in dopaminergic neuron spiking activity and the novelty- and AMPH-induced increases in locomotion and AMPH-induced stereotypic behavior, indicating that maintenance of these phenotypes requires continuing EAAT3 overexpression in TH-positive neurons. This could suggest therapeutic potential of EAAT3 inhibition for abnormal repetitive behaviors, including those that have an early onset. The apparent over-rescue of the 1.8 mg/kg AMPH response in *Slc1a1-Th-OE* mice following doxycycline-mediated suppression in the longitudinal study (Fig 5C) is intriguing and could suggest opposite impacts of developmental and adult expression of EAAT3. However, the lack of any behavioral differences in response to 3.0 mg/kg and 8.0 mg/kg of AMPH suggest that any such effect is small.

In contrast to the robust and reversible effects on AMPH-induced repetitive behaviors, we observed minimal or no change in several other behaviors. Increased dopaminergic burst firing in *Slc1a1-Th-OE* mice did align with a slight increase in locomotion in a novel environment, which is known to induce a transition from tonic to burst firing of dopaminergic neurons (112–116). However, we did not observe changes in baseline anxiety-like or repetitive behavior. This is not altogether surprising, as the directionality of the relationship between anxiety and dopaminergic activity may vary by brain region based on the literature. For example, although genetic suppression of dopaminergic neuron firing promotes anxiety-like behavior (117), infusions of dopaminergic agonists in limbic forebrain regions can also increase behavioral measures of anxiety (118). Indeed, evidence from pharmacological (119) and imaging studies (120) lend support for both hypodopaminergic and hyperdopaminergic states leading to anxiety in humans, highlighting the need to study the cellular targets of dopaminergic neurons that mediate anxiety phenotypes. In contrast with our specific overexpression in TH-positive neurons, a recent study evaluated the impact of forebrain-specific overexpression of EAAT3 and found increased anxiety-like and grooming behavior (72), suggesting a possible separate role of forebrain *Slc1a1/EAAT3* expression in these phenotypes. Intriguingly, in a prior study, mice with *CaMKII $\alpha$ -Cre*-dependent *Slc1a1* overexpression exhibited reduced basal dopamine levels in the striatum (73). Decreased basal dopaminergic tone may reflect changes in dopamine D2-mediated inhibition, which was found to be increased in this line (73). Because *CaMKII $\alpha$*  is broadly expressed in the forebrain (121, 122), and is expressed in heterogenous cell types, the increased anxiety-like and compulsive behaviors at baseline could potentially result from changes in multiple brain regions.

As noted above, there is substantial evidence suggesting that OCD and TS are etiologically linked. Tic disorders were present in the pediatric OCD populations in which linkage and association with *SLC1A1* was initially studied (50, 52, 53). Multiple carriers of a loss of function variant in *HDC*, which segregated perfectly with TS (123), had co-occurring OCD (33). Of relevance, AMPH can trigger compulsive-like behavior in humans and exacerbate

tics in individuals with TS (31, 34), as well as increase stereotypies in *Hdc*-deficient mice (33). Interestingly, an early case-control study of *SLC1A1* in schizophrenia identified a single-nucleotide polymorphism (rs7022369) that was associated with increased *SLC1A1* expression in postmortem tissue (124); however, a recent well-powered genome-wide association study of schizophrenia did not find genome wide significance for *SLC1A1*, making its role in schizophrenia unclear (125). Our findings of increased AMPH sensitivity in *Slc1a1-Th*-OE mice are reminiscent of the increased AMPH sensitivity that is observed in rodent models of schizophrenia and in human patients (126), potentially warranting examination of schizophrenia-relevant behavior in these animals.

One caveat of these studies is that we cannot exclude the contribution of perturbed norepinephrine signaling in the observed phenotypes. AMPH can induce endocytosis of EAAT3 in cultured locus coeruleus (LC) norepinephrine neurons, indicating that AMPH also potentiates glutamatergic signaling in this cell population (127). Notably, norepinephrine-dopamine co-release from the LC is extensive and has been shown to be involved in novelty-associated memory formation (112, 128, 129). Dopamine release from norepinephrine neurons could thus be an additional mechanism by which EAAT3 modulates dopamine-dependent behaviors. However, we do not expect this is a significant contributor to these results because our previous experiments with midbrain viral injections would not have rescued EAAT3 expression in the LC (75).

In summary, we used a circuit- and cell-type specific approach to begin to dissect the impact of overexpression of the OCD candidate gene *Slc1a1*/EAAT3. We found that dopamine neuron overexpression of EAAT3 that begins in early life leads both to increased AMPH-induced stereotypic behavior and increased dopaminergic transmission in the striatum. Importantly, overexpression of *Slc1a1*/EAAT3 in dopaminergic neurons did not affect anxiety-like or self-grooming behavior, suggesting that direct effects of EAAT3 manipulation in dopaminergic neurons affect some but not all OCD-relevant behaviors. Surprisingly, adult-specific overexpression of *Slc1a1*/EAAT3 failed to recapitulate the phenotypes that emerged after induction of overexpression in early development, suggesting that the effects of EAAT3 overexpression are developmentally mediated. Our electrophysiological data suggest that alterations in dopaminergic transmission during developmentally sensitive periods may be a mechanism underlying the effects of *Slc1a1*/EAAT3 overexpression, warranting additional work to understand the dynamics of glutamatergic and dopaminergic signaling in basal ganglia circuits during early developmental periods. Finally, our electrophysiological and behavioral data following reversal of EAAT3 overexpression suggest that EAAT3 inhibitors should be evaluated as a potential treatment for basal ganglia-mediated repetitive behavior.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We would like to thank Ziwen Wang and Hanxiao Liu for assistance with general laboratory support. This work was funded by NIH Grant MH114296 (to J.V. and S.E.A.).

### Conflict of Interest

Dr. Veenstra-VanderWeele has received research support from the National Institutes of Health, the Simons Foundation, Roche, and Janssen. He has served on advisory boards for Roche, Autism Speaks, the Brain and Behavior Research Foundation, and the Autism Biomarkers Consortium for Clinical Trials. He has served as associate editor of *Autism Research*, as a special issue editor for *Pediatrics*, and on the editorial boards of *JAMA Psychiatry*, the *Journal of Autism and Developmental Disorders*, *Autism*, and the *Journal of Neurodevelopmental Disorders*. Dr. Ahmari has received research support from the National Institutes of Health, the International OCD Foundation, the One Mind Foundation, the Brain Research Foundation, the Klingenstein-Simons Foundation, the McKnight Foundation, and the Foundation for OCD Research. She serves on advisory boards for the International OCD Foundation, ORCHARD, and the Brain and Behavior Research Foundation. She serves as an associate editor of *Neuropsychopharmacology*. The other authors disclose no biomedical financial interests or potential conflicts of interest.

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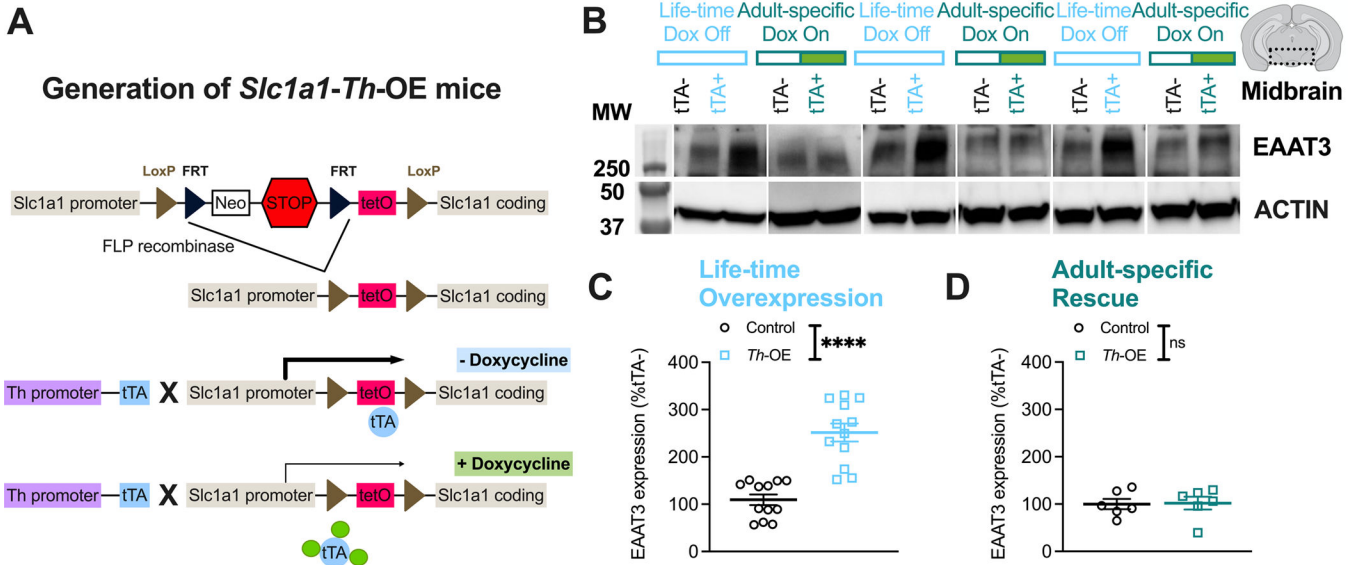
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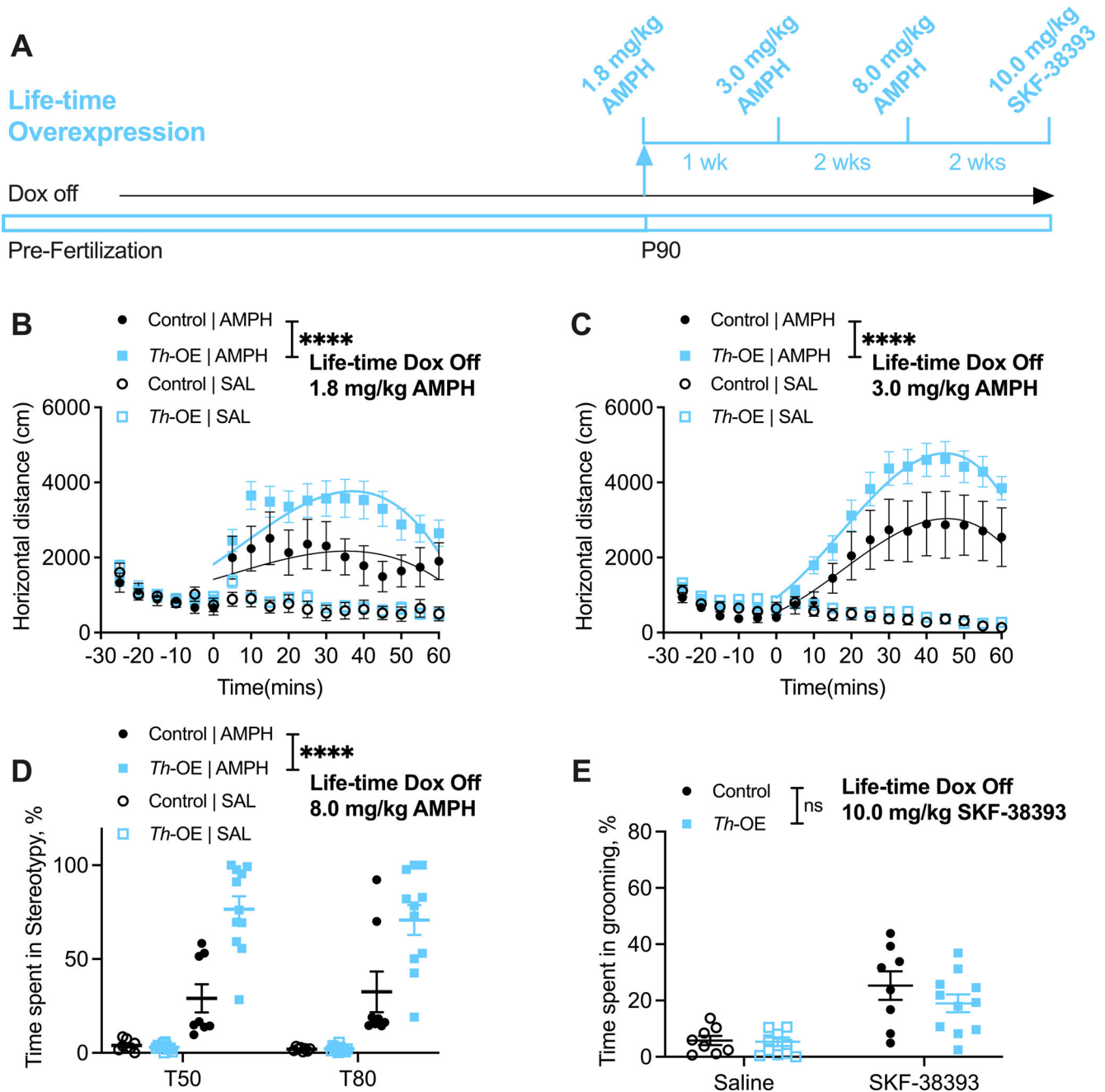
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**Figure 1. EAAT3 is selectively and reversibly overexpressed in *Slc1a1-Th-OE* mice.** (A) Generation of *Slc1a1-Th-OE* mice. *Slc1a1-STOP-tetO* mice were crossed with *Pgk1*-flpo deleter line to generate *Slc1a1-tetO* mice that were then crossed with *Th-tTA* line to generate tTA-mediated overexpression of EAAT3 selectively in TH-expressing neurons. (Neo, PGK-EM7-NEO minigene; STOP, Stop signal; tetO, tetracycline operon; tTA, tetracycline transactivator). (B) Representative western blot images showing increased midbrain EAAT3 expression in *Slc1a1-Th-OE* (*Th-OE*) mice following administration of regular chow and restored expression following administration of doxycycline-supplemented chow. Molecular weight (MW) in kDa is indicated on the left. (C) EAAT3 overexpression (\*\*\*\**P* < 0.0001, n = 12 per genotype), and (D) rescue of expression following treatment with doxycycline-supplemented chow (ns*P*, not significant, n = 6 per genotype). Also see Figure 4H for qRT-PCR, Supplementary Figure S1 for full blot image and Supplementary Figure S2 for regional protein expression.



**Figure 2. Amphetamine reveals increased basal ganglia-dependent repetitive behavior in *Slc1a1-*Th*-OE* mice.**

(A) Experimental timeline. For all panels,  $n = 8$  control, 11 *Th*-OE mice. No genotype differences were found in basal locomotion in saline-injected *Th*-OE and control mice in a familiar environment (B, third order polynomial least squares fit,  $t = 0-60$ ;  $F(4, 334) = 1.349$ ,  $P = 0.2515$ ). *Th*-OE mice show significantly increased locomotor response to (B) low and (C) moderate doses of AMPH. (D) *Th*-OE mice display increased stereotypic behavior following 8.0 mg/kg AMPH dose. (E) SKF-38393-induced grooming response is not altered in *Th*-OE mice. \*\*\*\* $P < 0.0001$ ; <sup>ns</sup> $P$ , not significant. Also see Supplementary Figures S3,

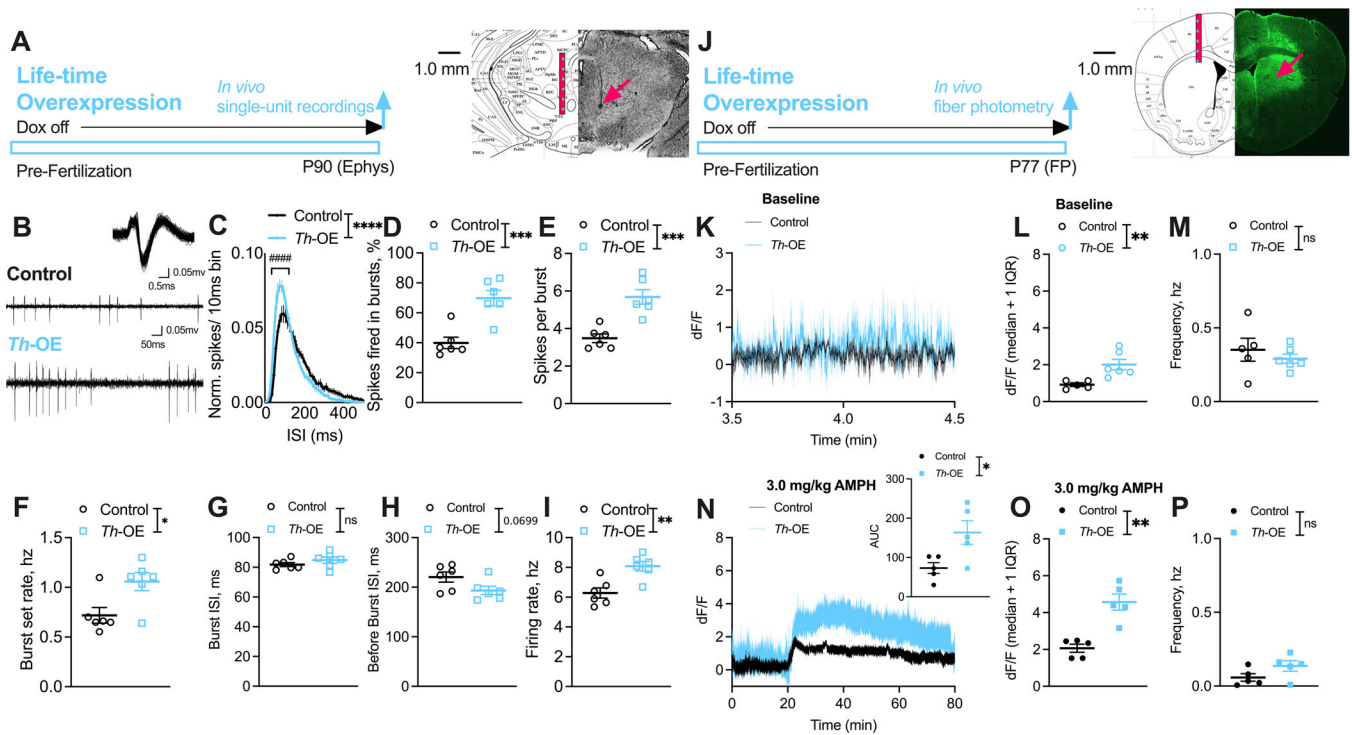
S4 for baseline anxiety-like and repetitive behaviors and Supplementary Figures S5, S6 for tet-operator and tTA transgene control experiments.

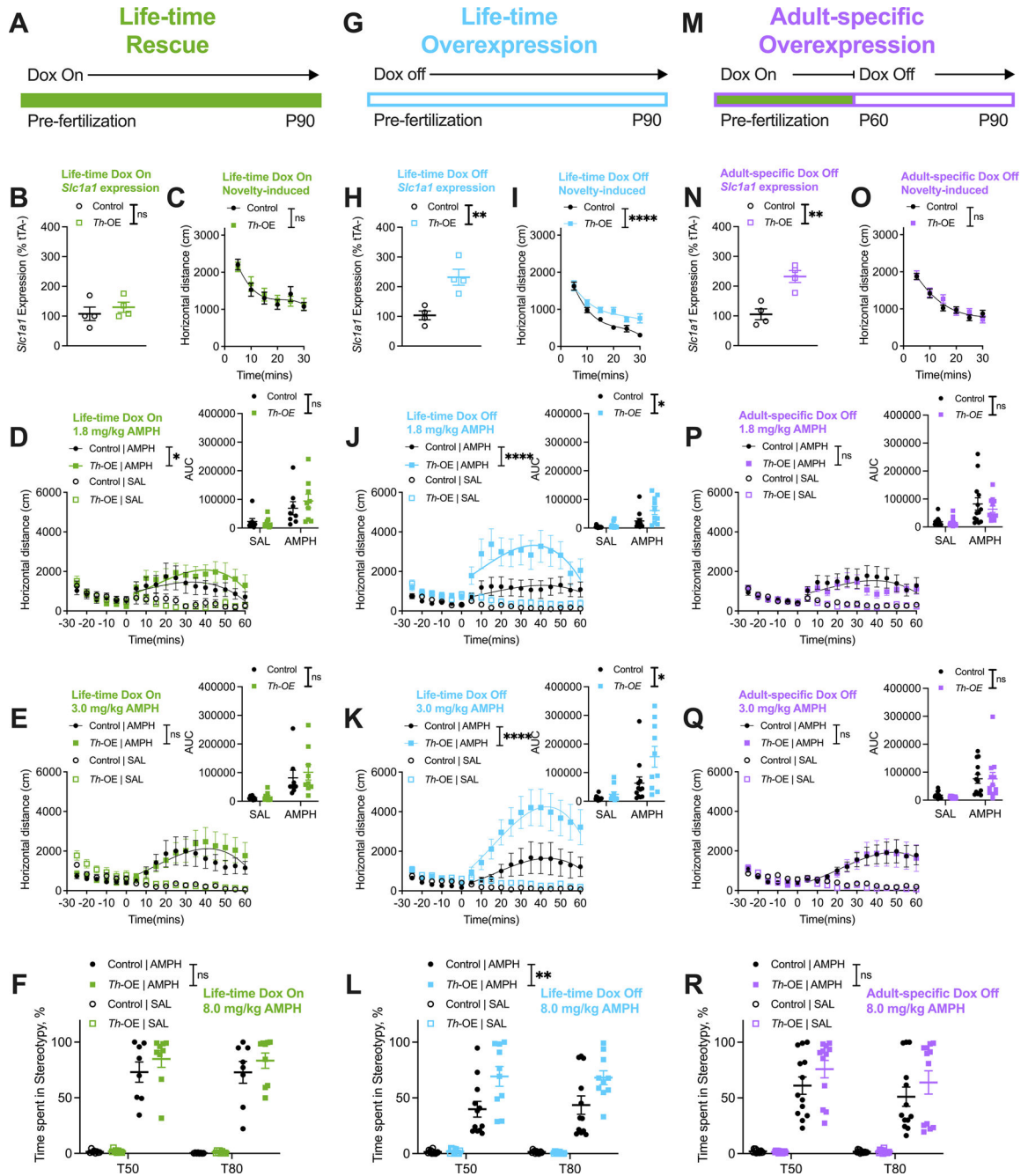
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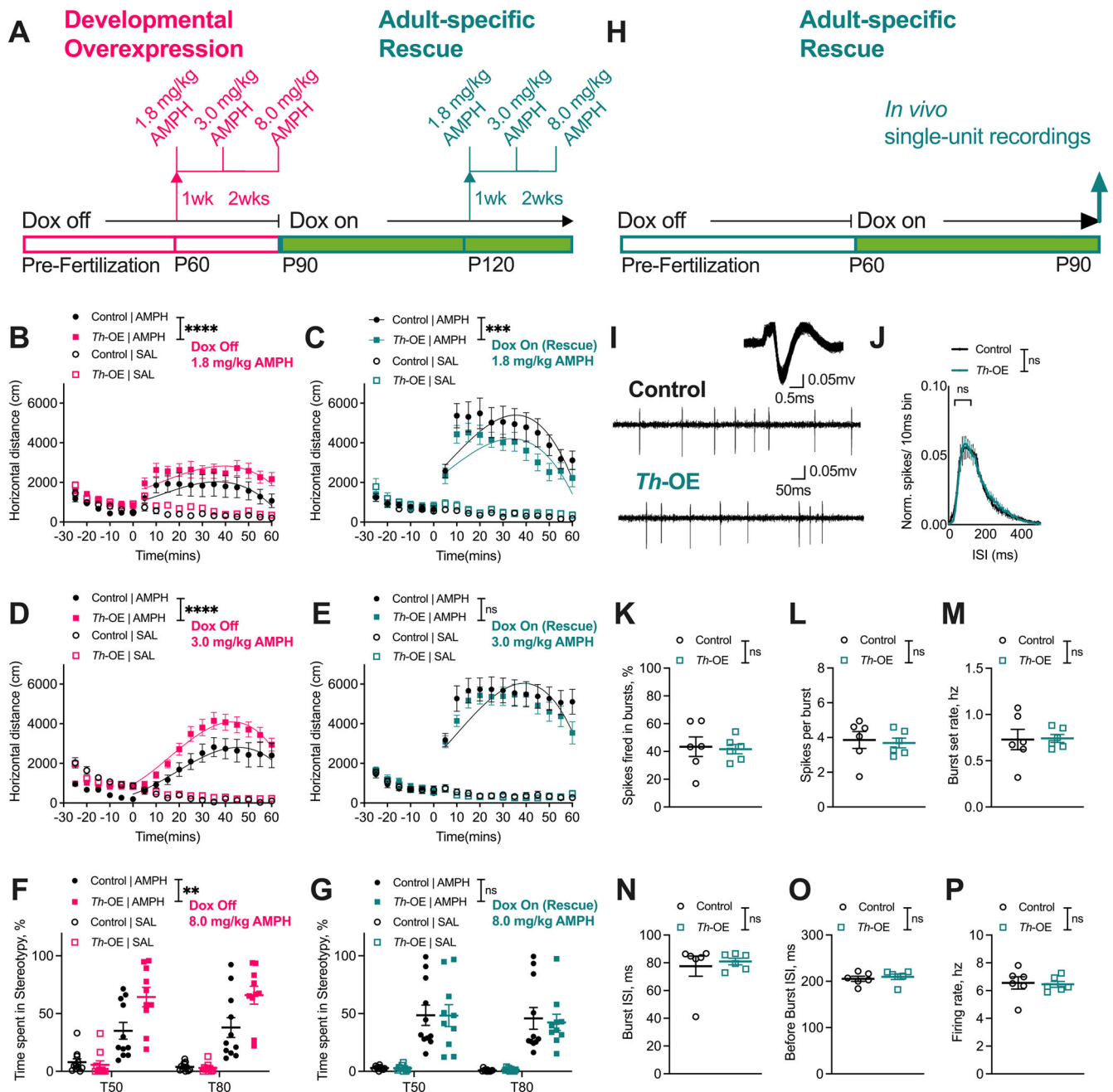


**Figure 4. Overexpression of EAAT3 during development is necessary for increased AMPH-induced locomotion and stereotypic behavior in *Slc1a1*-*Th*-OE mice.**

(A-F) Life-time rescue experiments. (B) qRT-PCR showing complete rescue of *Slc1a1* mRNA expression in *Th*-OE mice ( $n = 4$  per genotype). For all life-time rescue behavior panels,  $n = 8$  controls, 9 *Th*-OEs. (C) Life-time rescue *Th*-OE mice display novelty-induced locomotion that is indistinguishable from littermate controls. (D) Life-time rescue *Th*-OE mice show a slightly increased locomotor response to a low dose of AMPH via curve-fit analysis; however, no genotype differences are observed in AUC analysis (D, inset). (E) Locomotor response of life-time rescue *Th*-OE mice to a moderate AMPH dose (AUC,



2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 15) = 0.2081$ ,  $P = 0.6548$ ; drug,  $F(1, 15) = 18.74$ ,  $P = 0.0006$ , genotype,  $F(1, 15) = 0.3003$ ,  $P = 0.5917$ ) is indistinguishable from littermate controls. **(F)** Stereotypic activity at the high AMPH dose in life-time rescue *Th*-OE mice does not differ from controls. **(G-L)** Life-time overexpression experiments. **(H)** qRT-PCR showing increased *Slc1a1* mRNA expression in *Th*-OE mice ( $n = 4$  per genotype). For all life-time overexpression behavior panels,  $n = 12$  controls, 10 *Th*-OEs. **(I)** Life-time overexpressing *Th*-OE mice show increased novelty-induced locomotion. **(J-K)** Life-time overexpressing *Th*-OE mice show increased locomotor activation at **(J)** low (AUC, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 20) = 4.446$ ,  $P = 0.0478$ ; drug,  $F(1, 20) = 23.90$ ,  $P < 0.0001$ , genotype,  $F(1, 20) = 5.347$ ,  $P = 0.0315$ ), and **(K)** moderate (AUC, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 20) = 4.915$ ,  $P = 0.0384$ ; drug,  $F(1, 20) = 24.34$ ,  $P < 0.0001$ , genotype,  $F(1, 20) = 5.997$ ,  $P = 0.0237$ ) doses of AMPH. **(L)** Stereotypic behavior at 8.0 mg/kg AMPH dose is elevated in *Th*-OE mice. **(M-R)** Adult-specific overexpression experiments. **(N)** qRT-PCR showing increased *Slc1a1* expression in *Th*-OE mice ( $n = 4$  per genotype). For all adult-specific overexpression behavior panels,  $n = 13$  controls, 12 *Th*-OEs. **(O)** Adult-specific overexpressing *Th*-OE mice show novelty-induced locomotor behavior that is indistinguishable from littermate control mice. **(P-Q)** Locomotor responses in *Th*-OE mice to **(P)** low (AUC, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 23) = 0.3956$ ,  $P = 0.5356$ ; drug,  $F(1, 23) = 18.19$ ,  $P = 0.0003$ , genotype,  $F(1, 23) = 0.6658$ ,  $P = 0.4229$ ), and **(Q)** moderate (AUC, 2-way RM ANOVA; drug  $\times$  genotype interaction,  $F(1, 23) = 0.06689$ ,  $P = 0.7982$ ; drug,  $F(1, 23) = 19.62$ ,  $P = 0.0002$ , genotype,  $F(1, 23) = 0.07915$ ,  $P = 0.7810$ ) doses of AMPH are comparable to control group responses. **(R)** No genotype differences are observed in AMPH-induced stereotypic behavior in adult-specific overexpressing mice. \*\*\*\* $P < 0.0001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; <sup>ns</sup> $P$ , not significant. Also see Supplementary Figure S7 for anxiety-like measures.



**Figure 5. Reversal of EAAT3 overexpression in adulthood rescues AMPH-induced locomotion and stereotypic behavior and dopaminergic neuron activity.**

(A) Behavior timeline. For all behavioral panels,  $n = 11$  controls, 10 *Th*-OEs.

Developmentally overexpressing *Th*-OE mice showed increased locomotor activation at low (B) and moderate (D) doses of AMPH and increased stereotypic behavior at 8.0 mg/kg AMPH dose (F). Following doxycycline administration to these same mice, *Th*-OE mice showed (C) reduced locomotor activation in response to low AMPH dose in comparison with control mice. (E) Locomotor activation of rescue *Th*-OE mice was indistinguishable from control mice at the moderate AMPH dose. (G) Further, adult-specific rescue *Th*-OE mice showed AMPH-induced stereotypic behavior that was comparable to the control group.

**(H)** Single-unit recordings timeline. **(I)** Representative dopaminergic neuron waveform and spiking patterns from naïve adult-specific rescue *Th*-OE and control animals. For all ephys panels,  $n = 55$  cells/ 6 control mice, 46 cells/ 6 *Th*-OE mice. **(J)** Normalized ISI histograms showing no changes in proportion of short duration ISIs in adult-specific rescue *Th*-OE mice. **(K)** % spikes fired in bursts, **(L)** spikes per burst, **(M)** burst set rate, **(N)** average burst ISI, **(O)** average before-burst ISI, and **(P)** firing rate are not altered in adult-specific rescue *Th*-OE mice in comparison to control mice. \*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; <sup>ns</sup> $P$ , not significant. Also see Supplementary Figures S8, S9 for naïve adult-specific rescue behavioral experiments.