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Genetic Approaches for the Cell-Specific Deletion of the Neuronal Glutamate Transporter EAAC1

Danielle Saint Hilaire
University at Albany, State University of New York, dsainthilaire@albany.edu

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Danielle Saint Hilaire

Research Mentor: Annalisa Scimemi, Ph.D.

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Abstract

With mice identified as an adaptable human disease model, researchers have been able to use genome editing to study a myriad of human disorders and diseases. One of these disorders, Obsessive Compulsive Disorder (OCD), has a prevalence of 2% in humans. At present, its underlying molecular and circuit mechanism remains incompletely understood. Genetic studies identified the gene encoding EAAC1, a neuronal glutamate transporter, as a candidate gene associated with OCD. Therefore, by understanding how EAAC1 shapes synaptic transmission and behavior, we can shed light on how it may ultimately contribute to OCD in humans. Here we show how bacterial artificial chromosome (BAC) transgenic mice can be used to remove EAAC1 either constitutively, or from identified populations of neurons. We show that mice that do not express EAAC1 (EAAC1$^{-/-}$) display altered timing of reward-based behaviors. These findings confirm a potential implication of EAAC1 in behaviors relying on the activity of brain structure that show hyperactivity in humans with OCD. Our findings open many new areas of study for understanding how OCD and OCD-like behaviors manifest.

Keywords: *Glutamate Transporters, EAAC1, Obsessive Compulsive Disorder*

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Introduction

Obsessive Compulsive Disorder (OCD) is characterized by an array of varying obsessions and compulsions; most patients experience both. An obsession is defined as an intrusive, frequent, stereotyped thought, while compulsions are defined as repetitive, ritualized behavior. OCD is a common neuropsychiatric disorder with an estimated prevalence of 2% in humans (Nestadt et al., 2010). OCD has a high familial prevalence, suggesting its potential genetic basis, and 80 candidate genes have been identified as potential contributing factors (Pauls, 2010). Genetic studies show a consistent association between non-functional mutations in the gene encoding the neuronal glutamate transporter EAAC1 (called *Slc1a1*) and OCD-like behaviors (Porton et al., 2013). At present, however, the mechanisms by which EAAC1 contributes to OCD remain unknown.

Glutamate transporter bind and remove glutamate from the extracellular space (Holmseth et al., 2012). Glutamate is an excitatory neurotransmitter, capable of binding to ionotropic and metabotropic receptors. The ionotropic receptors include ion channels permeable to cations (the AMPA receptors) and others that are also permeable to calcium (the NMDA receptors). AMPA and NMDA receptors are abundantly expressed in neurons throughout the brain (Vekhratsky & Kirchhoff, 2007). At rest, NMDA receptors are blocked by magnesium. Therefore, their activation requires not only glutamate release but also membrane depolarization to relieve the voltage-dependent magnesium block. NMDA receptors assemble as tetramers, composed of two NR1 and two NR2 subunit. The identity of the NR2 subunit alters the receptor activation kinetics and calcium permeability. Because of their high affinity for glutamate and their expression in extrasynaptic regions, NMDA receptors are perfectly tailored to bind glutamate diffusing out of the synaptic cleft. Varying their activation can have profound effects on synaptic plasticity, learning and memory (Rao & Finkbeiner, 2007). EAAC1 is expressed in perisynaptic regions. Therefore, EAAC1 prevents extrasynaptic NMDA receptor activation by binding glutamate on its way out of the synaptic cleft (Scimemi et al., 2009).

Glutamate also serves as a substrate for GABA synthesis, which requires the decarboxylation of glutamate by the enzyme glutamate decarboxylase. In the absence of EAAC1, there is a reduction in GABA synthesis and release (Mathews & Diamond, 2003). EAAC1 is expressed at low levels throughout the brain, in both glutamatergic and GABAergic neurons (Holmseth et al., 2012). More specifically, EAAC1 is expressed in various subcortical regions,

especially the striatum (Bellini et al., 2018). In the striatum, EAAC1 promotes D1 dopamine receptor expression, and in doing so, EAAC1 controls not only glutamatergic and GABAergic transmission, but also dopaminergic transmission. Loss of EAAC1 in mice is associated with behaviors seen in dopamine circuit dysregulations, such as increased repetitive and compulsive behaviors (Bellini et al., 2018). The D1 receptor expressing neurons of the striatum project onto the *substantia nigra* and are called D1 medium spiny neurons (D1-MSNs). D1-MSNs control movement initiation and reward. Another subset of neurons that project inhibitory effects from the striatum are D2-MSNs, which project onto the *globus pallidus* and, in contrast to the D1-MSNs, when activated, are involved in movement termination (Lee et al., 2016). D1-MSNs, also known as the direct pathway, are localized in the dorsolateral striatum, while the D2-MSNs, or, the indirect pathway, are localized along the ventral medial striatum (Gagnon et al., 2017). The activity of D1- and D2-MSNs may be a critical point of study when examining the effects of differential EAAC1 expression in the striatum.

The striatum is implicated in repetitive and compulsive behaviors, similar to those seen in OCD, which is why it was our main area of focus. In patients with OCD, abnormal firing rates of MSNs in the striatum have been implicated in the presence of compulsions and repetitive behaviors (Guehl et al., 2008). In mice, dysregulation of various striatal circuits, including the cortico-striato-thalamo-cortical (CSTC) circuit, have been correlated with the prevalence of repetitive behaviors (Ahmari et al., 2013). Though the mechanism by which this circuit acts remains unknown. We wanted to know how exactly EAAC1 contributes to the striatum's function in controlling repetitive behaviors and whether the loss of EAAC1 could alter the presentation of repetitive behaviors seen in our mice models. To study the activity of the striatum, we used lever press experiments specifically to study behavior since they are a valuable measure of compulsive behaviors, and thus are controlled by the striatum.

To determine how these two different cell populations contribute to the repetitive behaviors, we generated conditional mice lacking EAAC1 only in D1- or D2-MSNs, and studied their phenotypes, in the context of movement execution and reward-based behaviors. These mice are called $D1^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice, and $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice, respectively. We confirmed their genotype with polymerase chain reaction (PCR) and confocal microcopy imaging, and then studied their phenotypic effects through a series of lever press experiments.

Materials and Methods

1. Ethics statement

All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the State University of New York (SUNY)–Albany and guidelines described in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2. Mice

All mice were group housed and kept under a 12 h light cycle (7:00 A.M. on, 7:00 P.M. off) with food and water available *ad libitum*. Constitutive EAAC1 knockout mice ($EAAC1^{-/-}$) were obtained by targeted disruption of the *Slc1a1* gene via insertion of a pgk neomycin resistance cassette in exon 1 of the *Slc1a1* gene (Peghini et al., 1997). $EAAC1^{-/-}$ breeders were generated after back-crossing $EAAC1^{+/-}$ mice with C57BL/6 mice for >10 generations (Scimemi et al., 2009). One week prior to the lever press experiments, we added 2% citric acid to the home cage water of 2-month-old mice (Reinagel, 2018).

3. Genotyping Protocol

In order to genotype these mice and confirm the genes they did or did not have, we had to collect tissue samples from them. For mice of ages 6-12 days old (P6-P12), we collected two samples and determined the sex of the mice by visual inspection. We were then able to identify the mice pups by annotating them as the following, M10, for example. The letter M stands for Male (or F for female), and the number 1 stands for the fact that we clipped toe #1 from the hind paws and toe #0 from the fore paws (see **Figure 1**). We refrained from taking any tissue from toes #5 or #6 because these toes do not contain enough tissue for DNA extraction. The mice can be uniquely identified via their date of birth, and their sex followed by the toes they had taken. For breeders (any mice greater than 12 days old, or > P12), a different method was used for tissue collection: tail snip. In either case, the samples were placed in a labeled Eppendorf tube with either the breeder number or the unique mouse identification number. Once the tissue had been collected, the DNA was extracted from the samples, which was done using a series of steps. First, we created

a master mix for DNA extraction, which consisted of a 200:1 ratio of DNA Extraction Buffer and Proteinase K, respectively. The DNA Extraction Buffer consists of 100 mM Tris pH 8, 5 mM EDTA, 200 mM NaCl, and 0.2% SDS. 40 µl of this master mix was added to each Eppendorf tube that contained tissue, and we ensured that the tissue was fully submerged so that it would be fully digested. We then placed these tubes in the VWR Incubating Orbital Shaker at 330 rpm at 55°C, and typically letting them shake overnight. The following day we would collect the samples and prepare them for PCR following an extensive series of steps. The first major step was to inactivate the Proteinase K in the samples; we did this by incubating the samples at 97°C for 10 min, and then centrifuging the samples for 5 min at 13,000 rpm at 4°C. Then next step to prepare the samples for PCR was to dilute the DNA, and this was done using a series of steps. For every Eppendorf tube with a tissue sample in it, we labeled a 0.6 ml tube and added 20 µl of PCR water to each tube and followed this by adding 20 µl of the sample supernatant. Each dilution was then vortexed and centrifuged, and kept on ice. The third major step was setting up the PCR reactions, and this was done through the use of primers (see **Table 1)** (Delidow, 1993). These primers were created and were tested using BLAST for target detection (Ye et al., 2012). Each mouse needed to be typed for a varying number of genes, depending on the parental genotypes (typically, the mice were genotyped for every parental gene, unless both parents were homozygous for that gene). To create the Mastermix for each gene, we counted the number of samples that needed to be types for each gene, and added one for the control sample, or in the case of EAAC1, added three extra for the three controls: wild type (WT), heterozygous (het), and EAAC1$^{-/-}$. Then we multiplied this number by 12.5 µl to obtain the amount of KAPA needed, and 11.5 µl, to obtain the amount of Primer to add to each Mastermix. Each Mastermix was then vortexed, and 24 µl was added to each uniquely labeled PCR tube. Then, 1 µl of the diluted DNA sample (or control) was added to the corresponding PCR tube. These PCR tubes were vortexed and centrifuged and then finally were placed in the thermocycler and underwent PCR. After the PCR was complete (the KAPA program takes roughly 40 min), the samples were loaded into an agarose gel to undergo gel electrophoresis. The gel was prepared using 2% agarose in a 1X TAE buffer. We microwaved this for 2 min and added 4-5 µl of SmartGlow dye in order for the bands to show up clearly. We cast the gel and let it solidify (in darkness) for roughly 15 min. When the gel was ready, we submerged it in a 1X TAE buffer and loaded the wells. The leftmost lane always contained the DNA Ladder for a baseline of band lengths and then we added the samples to the gel in a particular order which was always

documented through photos. Once all the samples had been loaded, the gel was run at 220 V for 10-15 min (depending on the gel size).

The very last step of our genotyping protocols was imaging the gel. We imaged the gel using cSeries Capture Software, and captured the gel using Auto Exposure. Each image took 30-60 s to collect. Once the image was ready, we aligned the gel with our gel loading order and analyzed the results and determined which mice carried which genes. We compared the band lengths of the samples to the band length of the controls (see **Table 2**). Once we had determined the correct genotype of the pups and/or breeders being typed, we were able to do more extensive research into the effects of these genes on the mice.

4. Trans-Cardial Perfusions

In order to fix the brain tissue and obtain cellular patch-clamp recordings of the D1- and D2-MSNs, we performed trans-cardial perfusions on P25-P28 mice. These mice were anesthetized with halothane and underwent a trans-cardial perfusion; we checked alertness by testing tail-pinch reflexes. Once unconscious, the mice were dissected and 1X phosphate buffered saline was injected into their left ventricle, and this was circulated through the blood vessels. Once the blood vessels were efficiently washed out and drained, 30 ml of 4% paraformaldehyde was circulated through the vessels, in doing this, we ensured the proper fixation of the brain. At this point, the mice were decapitated in accordance with SUNY Albany Care and Use Committee guidelines. The brain was removed and placed into cold slicing solution which was bubbled with 95% oxygen and 5% carbon dioxide, which also contained 2.5 mM KCl, 0.5 mM $CaCl_2$, 4.0 mM $MgCl_2$, 26.2 mM $NaHCO_2$, 1.0 mM NaH_2PO_4, 119 mM NaCl, 1.3 $MgSO_4 \cdot H_2O$, 300 mOsm, and 22 mM glucose at pH 7.4. The brain was then removed, and we obtained 250 μm thick striatal slices using a vibrating blade microtome. Following slicing, the slices were kept in a submersion chamber at 36°C from a minimum of 30 minutes up to 4 hours. The recording solution contained 119 mM NaCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 26.2 $NaHCO_3$, 1.0 mM NaH_2PO_4, 22 mM glucose, 300 mOsm, at pH 7.4. We were able to identify and obtain whole-cell patch clamp recordings of various MSNs. The internal solution that was used to record these cells was 120 mM KCH_3SO_3, 10 mM EGTA, 20 mM HEPES, 2 mM MgATP, 0.2 mM NaGTP, 5 mM QX-314Br, 5.0 mM NaCl,

290 mOsm, at pH 7.2. In using trans-cardial perfusions, we were able to observe more in depth how the cells of the striatum may have functioned in live tissue.

5. Immunohistocytochemistry

In order to visualize the expression of various genes, including the Cre recombinase-reporter protein Ai9, mice of either sex, aged P25-P28 were used for immunolabeling. Our experiments were performed on mice that expressed Cre recombinase in either D1- or D2-MSNs, as well as expressed the red fluorescent reporter protein, Ai9. We were able to visualize Cre recombinase expression in D1- and D2-MSNs of the striatum using a fluorescent antibody conjugated with Alexa Flour 488 and used a confocal microscope for imaging.

6. Lever Press Experiments

We decided to use lever press experiments since compulsive lever pressing behaviors are typically attributed to the dysregulation of circuits in the striatum (Ahmari et al., 2013). In our experiments, we used operant learning of lever press behaviors and we compared WT mice and EAAC1$^{-/-}$ mice. Both groups of mice underwent nine, 10 min long fixed ratio (FR1) training sessions in which each lever press was rewarded with 70 μl of water. Note that water was a reward for these mice as the water in their home cage contained 2% citric acid for a week prior to the experiment. The timing of the lever presses was recorded using a B-pod state machine and custom made software written in MATLAB2019. We visualized the mice as they were performing this task using a camera positioned above the behavioral arena. Our experiments compared 17 WT mice (n=17) to 18 EAAC1$^{-/-}$ mice (n=18) of mice from both sexes. We conducted similar experiments using D1$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice and A2A$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice as well. Both these mouse lines consisted of a cohort of 10 mice (5 male and 5 female) and were each compared to a cohort of 33 WT mice (16 male and 17 female).

7. Statistical analysis

Data are presented as mean ± SEM unless otherwise stated. All experiments were performed on multiple mice of either sex. Statistical significance was determined by student's paired or

unpaired t test, as appropriate (IgorPro 6.36). Differences were considered significant at $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Comparisons between multiple groups were performed using an analysis of variance (ANOVA) test.

Results

1. Genotyping

After crossing various mice strains, we were able to confirm mouse genotypes using tissue collection, PCR, and gel imaging. In all the gels, we ran a DNA ladder in the leftmost line, as a baseline indicator of band lengths, and a control in the second leftmost lane, as an indicator of the presence of the gene of interest. In the EAAC1 gel, we ran three controls, a WT, an $EAAC1^{-/-}$, and an $EAAC1^{+/-}$(het) control. The mouse pup M80 expressed the same band length as the $EAAC1^{-/-}$ band, at 680 bp, indicating that this mouse pup did not express EAAC1 in any of its cells (see **Figure 2A**). The mouse pup M80 was born from a litter derived by crossing F849 ($D1^{tdT}$:$EAAC1^{-/-}$) with M845 ($EAAC1^{-/-}$). The expression of $EAAC1^{-/-}$ was consistent with theoretical estimates of genes expressed given the parental genotypes.

To determine expression of Cre recombinase in the D1-MSNs of mice, we used a control for $D1^{Cre/+}$ which generated a band at a band length of 340 bp. The sample from mouse, M10, also expressed a band at 340 bp (see **Figure 2B**). This demonstrates that mouse pup M10 will express Cre recombinase in all of its D1 neurons, and more specifically, in all of its D1 MSNs. The mouse pup M10 was generated from a cross between F881, ($D1^{Cre/+}$:$Ai9^{Tg/Tg}$:$EAAC1^{tm1c/tm1c}$) and M758 ($D1^{Cre/+}$:$Ai9^{Tg/Tg}$:$EAAC1^{tm1c/tm1c}$), the expression of $D1^{Cre/+}$ in the pup was consistent with theoretical estimates.

To determine Ai9 expression, we ran a control of $Ai9^{Tg/Tg}$ which generated band lengths at 297 bp and 196 bp, and the mouse pup being examined here, F10, expressed bands at both band lengths (see **Figure 2C**). This indicated that mouse pup F10 will express the florescent Cre recombinase reporter protein, Ai9, in all of its Cre recombinase-expressing cells. The mouse pup F10 was generated by crossing F829 ($D1^{Cre/+}$:$Ai9^{Tg/Tg}$:$EAAC1^{-/-}$) with M875, ($D1^{Cre/+}$:$Ai9^{Tg/Tg}$:$EAAC1^{-/-}$).

We next looked at expression of Cre recombinase in D2-MSNs, using an A2A mouse line. The control band length for $A2A^{Cre/+}$ was at 350 bp, and the mouse pup F30 also expressed a band at 350 bp as well (see **Figure 2D**). This indicated to us that the mouse pup will express Cre recombinase in all of its D2-MSNs, and more specifically, for our purposes, in all of its D2-MSNs. The mouse pup F30 was generated by crossing F813 $A2A^{Cre/+}$:$Ai9^{Tg/Tg}$, and M950

$A2A^{Cre/+}$:$Ai9^{Tg/Tg}$, thus the expression of $A2A^{Cre/+}$ expression was consistent with theoretical estimates.

In the following gel, $EAAC1^{tm1c/tm1c}$, the control band lengths are expressed at 269 bp, and 384 bp, and the mouse being typed for, mouse #687 (this is a unique three-digit number that we use on ear tags to identify breeders), also had bands at these two band lengths (see **Figure 2E**). Mice that express the floxed EAAC1 gene have a gene that is flanked in between two lox P sites, and when this is expressed with Cre recombinase in either $D1^{Cre/+}$ or $A2A^{Cre/+}$ mice, it can act to knockout EAAC1 expression in D1- or D2-MSNs, respectively. The breeder #687 was generated by a cross between F533 ($D1^{tdT}$), and M505, (WT).

The final gene we typed for was the tdTomato gene, whose control expresses a band length of 750 bp, and again we see breeder #687 expressed a band at this length as well (see **Figure 2F**). This means that breeder #687 will express bright right tdTomato florescence throughout the D1 neurons. The breeder #687 was generated by a cross between F533 ($D1^{tdT/+}$) and M505, (WT).

Using PCR allowed us to validate a myriad of different mice models with varying expressions of EAAC1. We have seen that we can analyze the genome of each mouse generated and from here we were able to image the brain for a more in depth look at the localization of Cre recombinase expression in the striatum specifically, and we were ultimately able to study more in depth the results of these genes on mice behavior using lever press experiments.

2. Confocal Laser Scanning Microscopy Imaging

Using confocal microscopy, we were able to visualize different sections and slices of mouse brains. Specifically, we wanted to examine the expression of Cre recombinase in the striatum, so we decided to use coronal slices to best visualize the expression of the Cre recombinase reporter protein, Ai9. We imaged the brain of a mouse of genotype $D1^{Cre/+}$:$Ai9^{Tg/0}$ (see **Figure 3A**). A mouse of this genotype will express Ai9 in all of its D1-MSNs, which is exemplified by the fluorescence seen in the dorsolateral striatum (DLS). There is also some fluorescence in the cortex as some of its neurons also express D2 receptors. We also imaged another mouse brain, of genotype $A2A^{Cre/+}$:$Ai9^{Tg/0}$ (see **Figure 3B**). This mouse should express red fluorescence in all of its D2-MSNs, and we see in this image that this mouse expresses Ai9 in

all of its D2-MSNs as indicated by the fluorescence seen in the ventral medial striatum (VMS). Again, there is some fluorescence in the outer portions of the cortex, but this is indicating the expression of Cre recombinase in cortical neurons expressing D2 receptors, in addition to the D2-MSNs of the striatum. Using confocal microscopy to visualize Ai9 and subsequently Cre recombinase expression was critical as it allowed us to validate the expression of these genes.

3. Lever Press Tests

After generating and validating the expression of the varying expression of various genes in the mice, we were able to conduct lever press experiments in an attempt to study the behaviors of the striatum and how they may be associated with varying EAAC1 expression. In this experiment, we studied the number of lever presses in controlled experiments and compared EAAC1 WT and EAAC1$^{-/-}$ mice. The mice underwent nine, 10 min long training session in which lever presses were rewarded with 70 µl of water (see **Figure 4A**). The reward system was a fixed ratio schedule; the mice would receive the water reward after a correct number of responses (lever presses).

We examined a cohort of 17 WT mice and compared them to 18 EAAC1$^{-/-}$ mice (see **Figure 4B**). In comparing the overall number of lever presses of lever presses from sessions one through nine, we found no statistical significance in the total amount of lever presses completed between WT and EAAC1$^{-/-}$ mice. When we compared the data obtained from the first 10 min session to the final 10 min session, it is clear that the EAAC1$^{-/-}$ mice began pressing the lever before the WT mice. In the first session, the EAAC1$^{-/-}$ mice pressed the lever significantly faster than the WT mice, as well as in the ninth session, the EAAC1$^{-/-}$ mice pressed the lever significantly faster than the WT mice. Since the differential expression of EAAC1 was the only changing variable between the WT and EAAC1$^{-/-}$, this indicated to us that the varying expression of EAAC1 may be implicated in the timing of reward-based behaviors. EAAC1

We also were interested to investigate the differences in lever press behaviors of the two strains of Cre/+ mice (D1$^{Cre/+}$and A2A$^{Cre/+}$). To examine the D1$^{Cre/+}$ mice, we conducted a similar series of lever press experiments in which the mice underwent a series of nine, 10 min long training sessions in which each lever press was rewarded with 70 µl of water (see **Figure 5A**). We studied a cohort of 10 D1$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice composed of 5 males and 5 females, and compared

them to a cohort of 33 WT mice, which was composed 16 males, and 17 females. In comparing the lever presses across all sessions, we were able to determine that the $D1^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice pressed the lever less than the WT mice, though this difference was not significant (see **Figure 5B**). Although the results from the first session did not significantly exhibit this difference, by the ninth session, $D1^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice pressed the lever much slower, and at a much lower rate than the WT mice. These results indicated to us that the lack of EAAC1 in D1-MSNs may be implicated in the decrease of lever press behaviors, in the context of timing and overall number of presses. This again implicates EAAC1 in the timing and overall presence of reward-based behaviors.

We conducted the same lever press experiments for $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice and again compared these to the WT mice. We used the same training setup as the previous two experiments, the mice underwent nine, 10 min long training sessions in which each lever press was rewarded with 70 μl of water (see **Figure 6A**). Similar to the previous experiment, we studied a cohort of 10 $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice composed of 5 males and 5 females, and compared them to a cohort of 33 WT mice, which was composed 16 males, and 17 females. In comparing the lever presses across sessions, we were able to determine that across all sessions, the $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice pressed the lever significantly less overall, and much slower than the WT mice (see **Figure 6B**). In the first session, the $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice pressed the lever significantly slower than the WT mice, this significant decrease in the number of lever presses was seen again in session 9. In comparison to the $D1^{Cre/+}$: $EAAC1^{tm1c/tm1c}$, the difference between the overall number of presses to WTs was much more significant for the $A2A^{Cre/+}$: $EAAC1^{tm1c/tm1c}$ mice. This indicated to us that the lack of EAAC1 in D2-MSNs is also implicated in the timing, and overall presence of reward-based behaviors.

Discussion

1. EAAC1 is Implicated in the Regulation of Striatal-Controlled Behaviors

Up to this point, we know that EAAC1 is involved in glutamate uptake and when knocked out, in the striatum specifically, it contributes to the prevalence of compulsive behaviors, specifically, the timing of them. Genetics studies have determined that EAAC1 arises from the *Slc1a1* gene (Bellini et al., 2018), but there is still much to be discovered regarding EAAC1. Here, we have determined that the lack of EAAC1 can contribute to the increase in compulsive behaviors seen in mice, but what remains unknown is how EAAC1 expression is reduced. Recent studies have implicated the presence of early life stressors, like neonatal maternal separation, in the reduction of EAAC1 expression in adulthood (Kim et al., 2020). These researchers observed that EAAC1$^{-/-}$ mice who were exposed to early life stressors exhibited impulsive-like behaviors. This is consistent with our data and implicates environmental stressors in the expression of compulsive behaviors.

Another interesting area of study is how altered EAAC1 expression can affect other brain regions. Our studies implicate the lack of EAAC1 in the striatum in timing changes of reward-based behaviors, but it would be interesting to see whether the lack of EAAC1 affects neuronal populations in other regions like the hippocampus. Previous studies have implicated the lack of EAAC1 in hippocampal and cortical neuronal death following ischemia, but more recent studies have indicated that properly functioning EAAC1 is necessary for proper hippocampal neurogenesis (Choi et al., 2018). Aside from its behavioral effects, EAAC1 may be a critical regulator of the maintenance of neuronal populations; this is a huge finding as it implicates EAAC1 in a broader context. Still, there are many discoveries yet to be made about EAAC1's complete function and its contribution to behavior.

2. EAAC1 Affects the Timing of Reward-Based Behaviors

In our experiments, we decided to use lever press in order to measure striatal activity, since the striatum is implicated in controlling motor output and even has been implicated in compulsive behaviors as well (Kravitz & Krietzer, 2014). In our lever press experiments, we observed that the EAAC1$^{-/-}$ mice learned to lever press faster than the WT mice, indicating that the presence or lack

of EAAC1 expression affects the timing of reward-based behaviors. We also were able to determine that knocking out EAAC1 in D1- or D2-MSNs can differentially affect lever press behaviors, which further indicated to us the EAAC1 is implicated in the presence and timing of reward-based behaviors. Lever press behaviors are thought to be controlled by various reward pathways in the striatum (Ahmari et al., 2013). We are cognizant of the fact that using lever press analysis is not only a measure of behaviors, but a measure of learning as well. These two are so closely intertwined, and our data may be measuring a variance of learning as well in addition to a variance of behavior. Our future studies may investigate whether the variance in behavior seen here is specifically a difference of behavior or rather a difference in learning. Taken together, this indicates that differential EAAC1 expression affects reward pathways in the brain, and thus can affect the prevalence of reward-based behaviors. Our findings about the function of EAAC1 are consistent with previous studies (Canales & Graybiel, 2000), that suggest striatal function is altered by glutamate and dopamine transmission, which affects the repetitive nature of behavior. Our findings that EAAC1 affects the timing of reward-based behaviors suggest that EAAC1$^{-/-}$mice care a great deal about the present reward, but it would be interesting to investigate whether the EAAC1$^{-/-}$ mice would prioritize pressing the lever for a water reward over other items, such as instead of going toward a presented food reward. Previous studies have shown that in addition to frontal cortex and the amygdala, the striatum is involved in the reward process (Bermudez, and Schultz, 2014). Perhaps the mechanism underlying the change in the timing of the lever presses (in EAAC1$^{-/-}$ mice) implicates the reward pathway; in other words, perhaps EAAC1 plays a critical role in the striatal reward pathway. This has yet to be investigated.

3. How Can EAAC1$^{-/-}$ Mice Provide Insights into OCD?

OCD is a complex neuropsychiatric disorder, associated with multiple mutations in multiple genes (Alonso et al., 2015). Perhaps because of its complex nature, at present, there is no animal model of OCD. Our data should not be interpreted to suggest that EAAC1$^{-/-}$ mice provide a valuable model OCD, but rather they provide a valid model to study the contribution of EAAC1 to this disease. Other mutant mouse lines that can also aid our understanding of OCD include Hoxb8 mutant mice, specifically Hoxb8neo and Hoxb81ox. These mice show OCD-like behaviors through excessive grooming, but it is unclear to which extent these findings can be translatable

and informative about OCD in humans (Greer & Capecchi, 2002). Other mouse lines used to study OCD rely on mutations that lead to increased extracellular concentration of dopamine (e.g. the dopamine transporter knockdown by (Zhuang et al., 2001). A previous optogenetics study implicated the activation of D1-MSNs in movement initiation, whereas the D2-MSNs were implicated in the termination of motor activity (Kravitz & Kreitzer, 2010). Since D1- and D2-MSNs are implicated in the movement pathway, the next logical step would be to investigate how they contribute to the onset of dysregulated motor behaviors, such as compulsive or other reward-based behaviors. From our findings, we know that when EAAC1 function is knocked out in the striatum, there is a change in the timing of reward-based behavioral phenotypes. We also saw that mice lacking EAAC1 expression in either D1- or D2-MSNs had differential effects on the timing and presence of the reward-based behaviors. Though the mechanism underlying this is still unknown, it is likely due to an increase in glutamatergic transmission in D1-MSNs, though studies on this are yet to be completed. As we continue to study the differential expression of EAAC1 in the striatum, it will continue to provide important insights into how EAAC1 affects certain OCD-like behaviors.

Generating a valid mouse model of OCD is critical as we continue to search for therapies and treatments for people living with OCD. Human genetic studies can only go so far in defining the gene expression underlying OCD in the human brain. Currently, there are about 60 candidate genes that have been reported to be implicated in the prevalence of OCD (Hemmings & Stein, 2006). Though genetic studies are a good first step to identify molecular origins of OCD, little can be done in the human brain to define the mechanism underlying these behaviors, which is why creating a valid mouse model of OCD is so important.

Valid mouse models of OCD are imperative to creating targeted therapies for treating OCD in humans. Current therapies for treating OCD include cognitive behavioral therapy and the use of serotonin reuptake inhibitors, but treatments like these only reduce symptoms for about 50% of patients (Bellini et al., 2018). Not only would creating a new mouse model of OCD open the door for testing new potential treatments, but it would also open the possibility of creating a treatment that targets EAAC1 expression. Either way, OCD patients would benefit immensely from more specific and more effective treatments.

4. Future Directions

Our new understanding of EAAC1's role in the timing of reward-based behaviors is crucial in adding to the current knowledge of how OCD affects the cortico-limbic striatal network (Wood & Ahmari, 2015). New areas of study could investigate the role of other transporters in the striatum and how they may interact with EAAC1 and contribute to OCD-like behaviors as well. Though the mechanism underlying EAAC1's effects on reward-based behaviors is still relatively undefined, we have been able to determine that differential EAAC1 expression does alter reward-based behaviors. Human studies have also implicated the frontostriatal network in the prevalence of compulsive behaviors seen in OCD (Pinto et al., 2019), though the mechanism underlying this effect is yet to be discovered.

Our findings in this paper contribute significantly to the current understanding of the role of EAAC1 in regulating the activity of striatal circuits, and more specifically how it can be implicated in OCD-like behaviors. We hope to continue investigating the role of EAAC1 in various populations of neurons, and we also hope to investigate the extinction response of the mice in our current experiments. There is still much to be discovered regarding how EAAC1 affects the timing of reward-based behaviors, but our findings indicate that $EAAC1^{-/-}$ mice may nevertheless be a valuable model to study OCD in mice.

Appendix

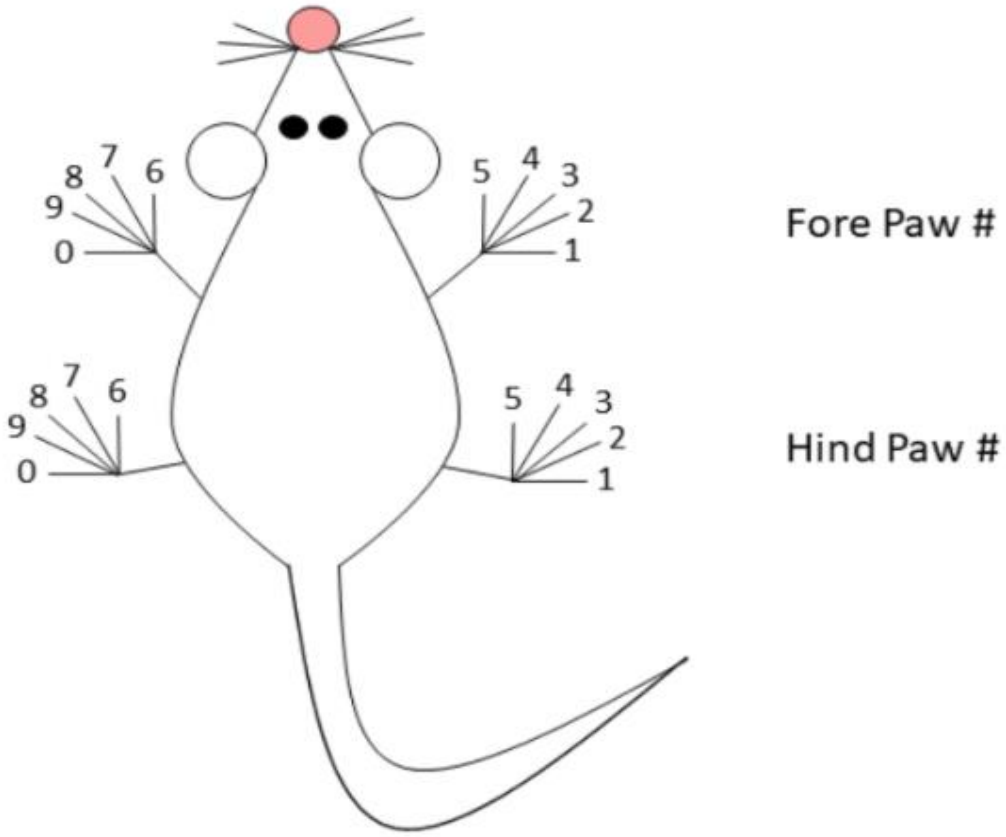

Figure 1 *Mouse toe clipping layout*

Note. Schematic representation of toe labeling in mice.

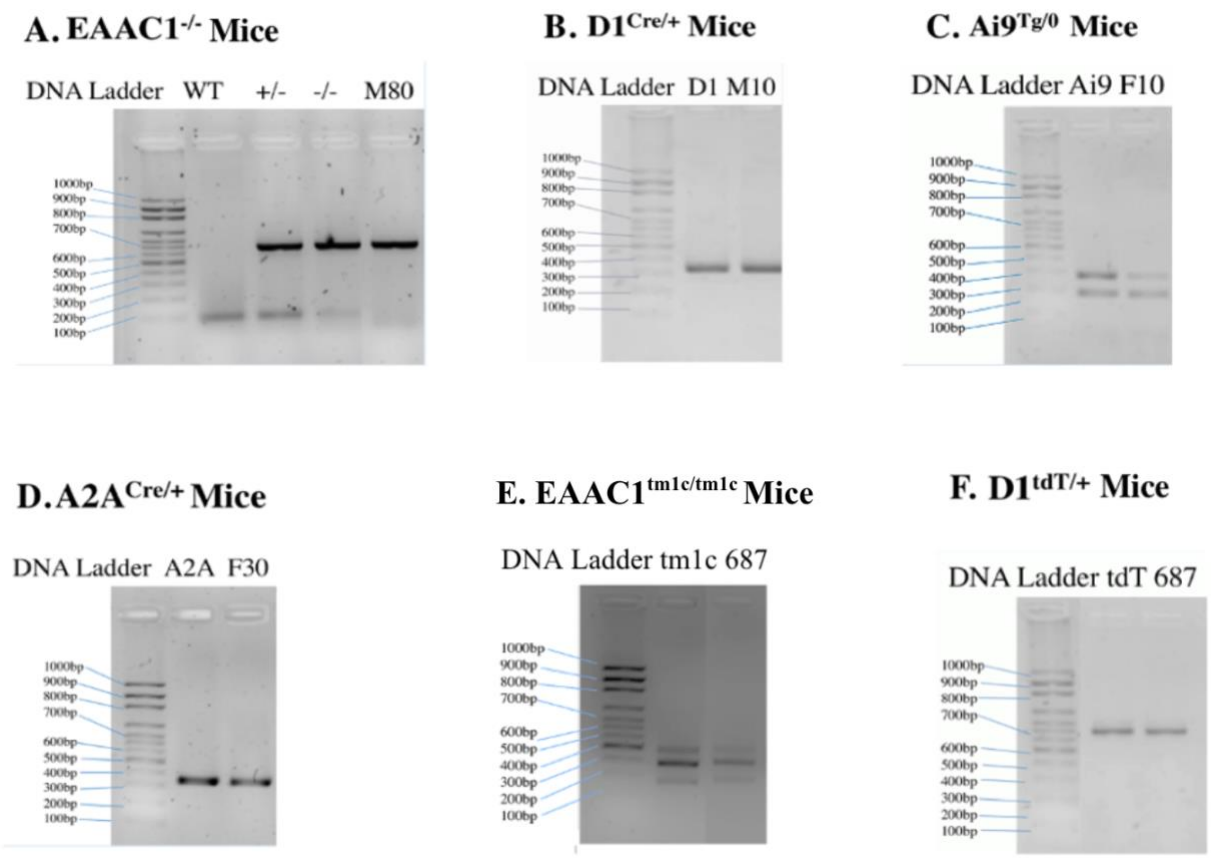

Figure 2 *PCR results*

Note. PCR results obtained from various crossings of mice. Each gel has DNA ladder ran in the leftmost lane and a control ran in the second leftmost lane. In each gel, the mouse pup/breeder being genotyped expressed the gene of interest. **A**, The samples are being typed for EAAC1 expression, against a WT, heterozygote (+/-), and EAAC1$^{-/-}$ (-/-) controls. **B**, These samples are being typed for D1$^{Cre/+}$ expression, **C**, These samples are being typed for Ai9 expression. **D**, These samples are being typed for A2A$^{Cre/+}$ expression. **E**, These samples are being typed for EAAC1$^{tm1c/tm1c}$. **F**, These samples are being typed for D1$^{tdT/+}$ expression.

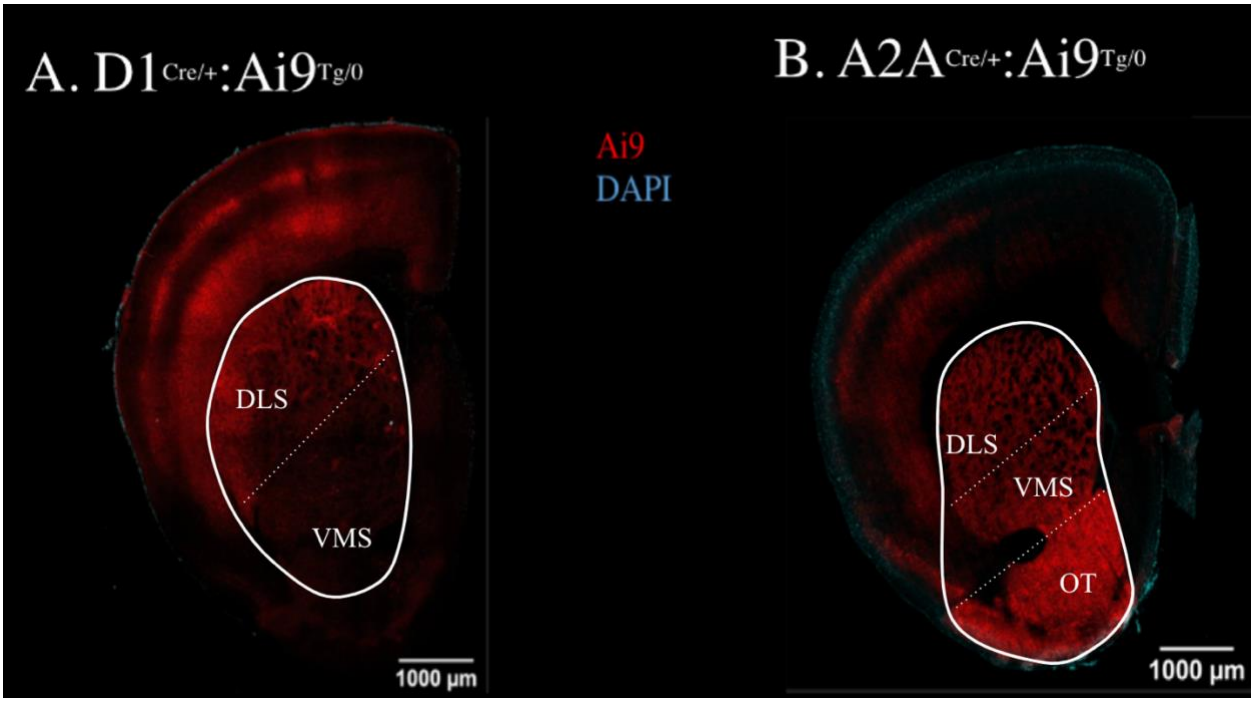

Figure 3 *Coronal sections of mouse brains*

Note. Images of these coronal sections were obtained using confocal microscopy. **A**, The mouse of genotype $D1^{Cre/+}$:$Ai9^{Tg/0}$ expressed Cre recombinase in all of its D1-MSNs, as indicated by the red fluorescence along the dorsolateral striatum (DLS). The striatum is outlined in white, the DLS and the ventral medial striatum (VMS) are outlined as well. **B**, The mouse of genotype $A2A^{Cre/+}$:$Ai9^{Tg/0}$ expresses Cre recombinase in all of its D2-MSNs, as indicated by the red fluorescence of the VMS. The striatum is outlined in white, the DLS, VMS, and the olfactory tubercule (OT) are outlined as well.

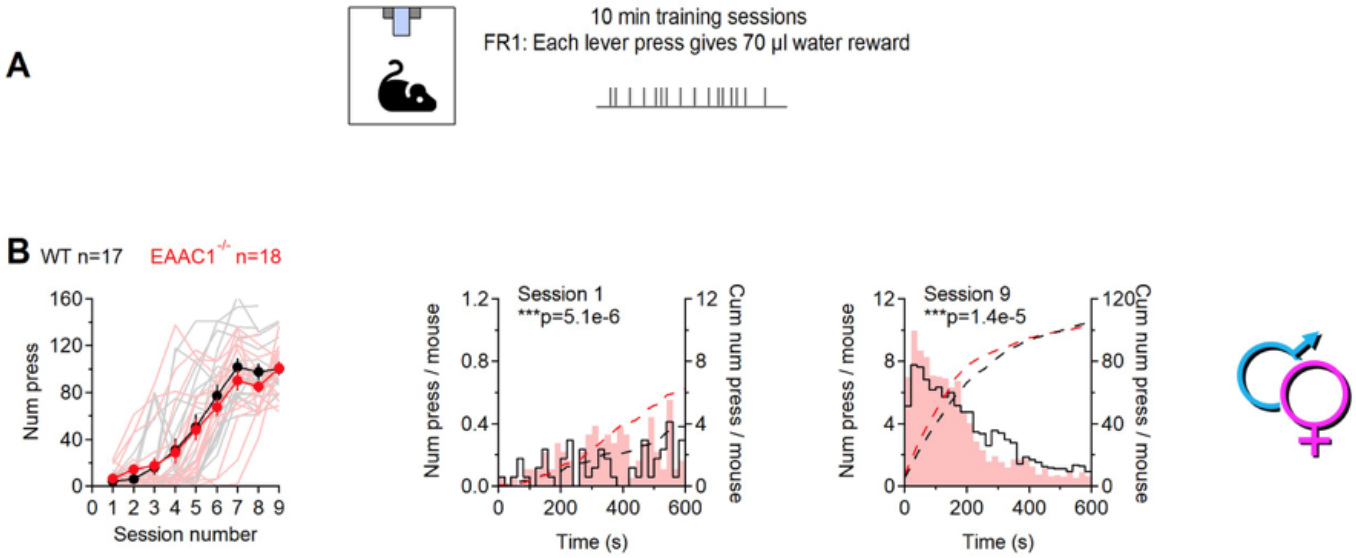

Figure 4 *Lever press analysis of WTs and EAAC1$^{-/-}$*

Note. **A,** Data obtained from lever press experiments of nine, 10 min training sessions, in which each lever press was rewarded with 70 µl of water reward. **B**, In comparing the WT and EAAC1$^{-/-}$ mice, there was no variation in the total number of lever presses (left), but in session 1 (middle) and session 9 (right) the EAAC1$^{-/-}$ mice press the lever significantly faster than the WT mice.

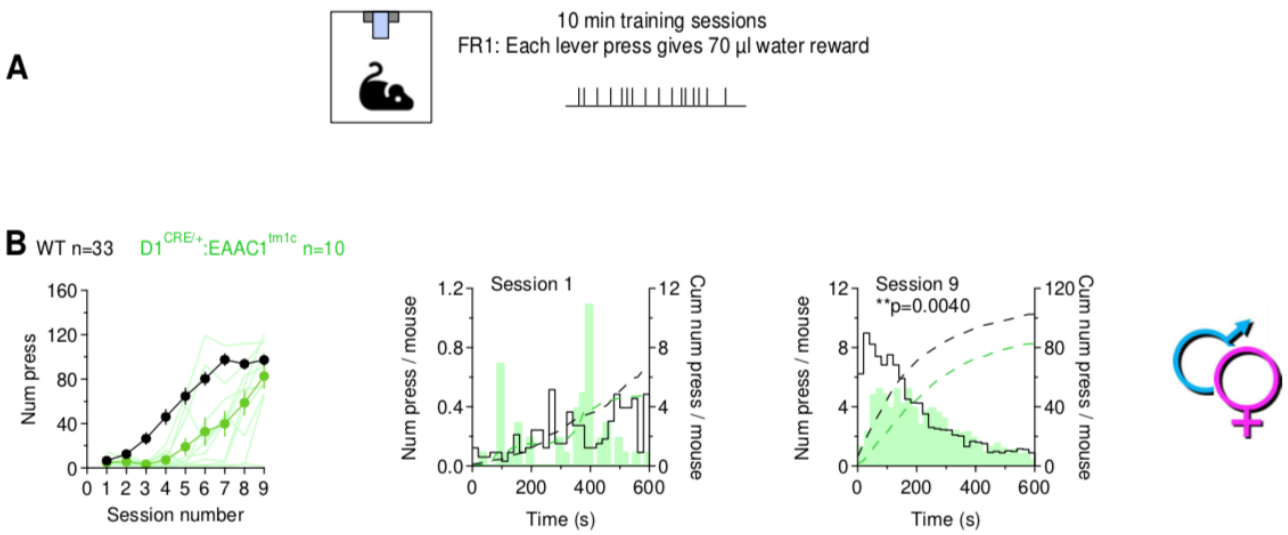

Figure 5 *Lever press analysis of D1$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$*

Note. **A,** Data obtained from lever press experiments of nine, 10 min training sessions, in which each lever press was rewarded with 70 µl of water reward. **B**, In comparing the WT and D1$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice, there was no significant decrease in the timing and overall quantity of lever presses exhibited by these mice (left). The difference in number of presses was not significant in the first session (middle), but the D1$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice pressed the lever significantly less in the ninth session (right) than the WT mice.

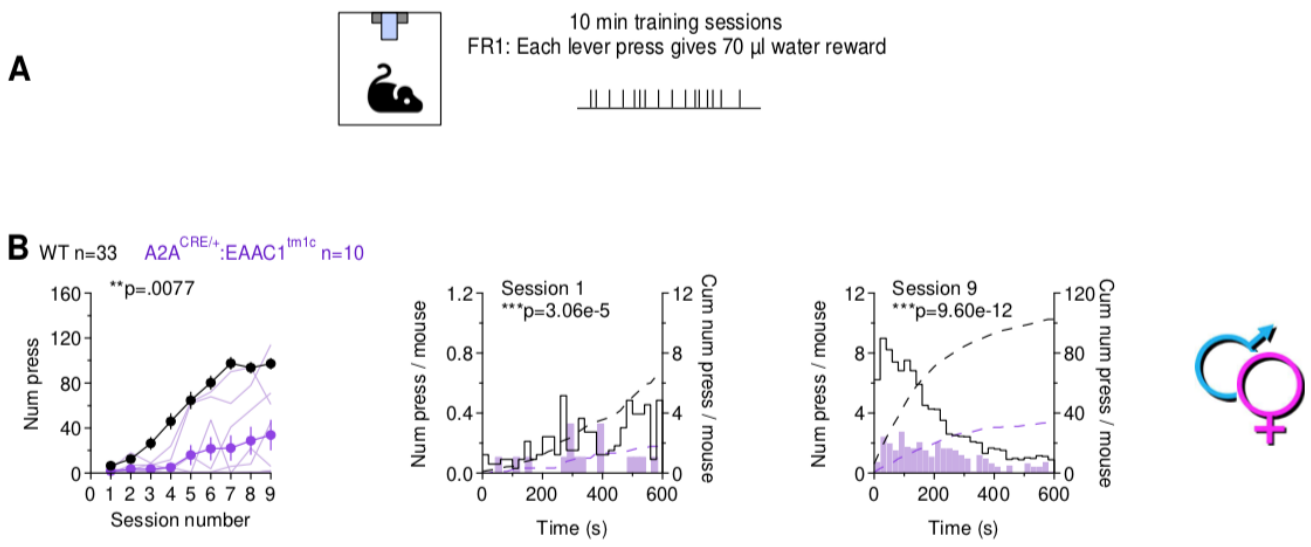

Figure 6 *Lever press analysis of A2A$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$*

Note. **A,** Data obtained from lever press experiments of nine, 10 min training sessions, in which each lever press was rewarded with 70 µl of water reward. **B**, In comparing the WT and A2A$^{Cre/+}$: EAAC1$^{tm1c/tm1c}$ mice, there was a significant difference in the timing and overall number of presses between the mice across all sessions (left). This significant decrease in the number of presses was also seen in session 1 (middle) and session 9 (right).

Table 1 *Primer sequences*

Primer Name	**Primer Sequence (5’ to 3’)**
D1_F	5’ - GCT ATG GAG ATG CTC CTG ATG GAA - 3’
A2A_F	5’ - CGT GAG AAA GCC TTT GGG AAG CT - 3’
Cre_R	5’ -CGG CAA ACG GAC AGA AGC ATT - 3’
D1-tdT_F	5’ - CTT CTG AGG CGG AAA GAA CC - 3’
D1-tdT_R	5’ - TTT CTG ATT GAG AGC ATT CG - 3’
Ai9_WT_F	5’ - AAG GGA GCT GCA GTG GAG TA - 3’
Ai9_WT_R	5’ - CCG AAA ATC TGT GGG AAG TC - 3’
Ai9_mut_F	5’ - CTG TTC CTG TAC GGC ATG G - 3’
Ai9_mut_R	5’ - GGC ATT AAA GCA GCG TAT CC - 3’
Neo_F	5’ - CTG TGC TCG ACG TTG TCA CTG - 3’
EAAC1_R	5’ - GAG AGC AGC AGC CAG TGA TTC - 3’
tm1a_F	5’ - TAC CCC AGT GAC TCA TCA GA - 3’
tm1a_R	5’ - CAT GGT GTT TAC CAG CGT GA - 3’

Note. Primer sequences used for mouse genotyping, all of which was obtained through BLAST. These primer sequences were essential for PCR and genotyping processes.

Table 2 *Gene product lengths*

Gene	**Base Pair Length (bp)**
D1Cre	340
A2ACre	350
tdTomato	750
Ai9_0	297
Ai9_Tg	196
EAAC1_WT	94
EAAC1_KO	680
tm1c_WT	269
tm1c_tm1c	384

Note. Product length for each of the genes tested in the PCR and gel electrophoresis analysis. The length of a band in an electrophoresis gel lane can be determined by comparing it to reference bands in the DNA ladder lane.

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