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Neuronal Glutamate Transporters Modulate Reward-Based Behaviors in Mice

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1 Abstract

Obsessive-compulsive disorder (OCD) is a neuropsychiatric disorder characterized by uncontrollable, intrusive thoughts (obsessions), restlessness, and repetitive behaviors (compulsions). One of the genes associated with OCD encodes the neuronal glutamate transporter EAAC1. EAAC1 is expressed in the striatum, the main input nucleus of the basal ganglia, implicated with reward, which shows patterns of hyperactivity in patients with OCD. What remains unknown is how EAAC1 contributes to the different behavioral abnormalities of OCD. To address this question, we performed a set of behavioral tests in EAAC1$^{-/-}$ mice. Specifically, we asked how the execution of repetitive reward-based behaviors changed in these mice. Our findings show that the propensity to engage in this type of behavior is increased in EAAC1$^{-/-}$ compared to wild type mice and that this is due to an increased activity in neurons within the sensory cortex. Together, these findings indicate that the hyperactivity of striatal circuits might be driven by differences in sensory perception.

2 Acknowledgements

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4 Introduction

The persistence of intrusive thoughts, anxiety, and repeated execution of stereotyped movements are features characteristic of neuropsychiatric disorders such as OCD (Kessler et al.,2005). OCD is thought to affect 1-3% of the international population, and it currently lacks an effective route of treatment (Zike et al., 2017; Nestadt et al., 2010). Common approaches to treatment often include the use of serotonin reuptake inhibitors and cognitive behavioral therapy, but most experience the resurgence of symptoms later (Zike et al., 2017; Dougherty et al., 2004). Family-based linkage analyses identify the gene *Slc1a1*, which encodes neuronal glutamate transporter EAAC1, as a strong candidate gene for OCD (Arnold et al., 2006; Dickel et al., 2006; Samuels et al., 2011). One existing hypothesis suggests that the loss of function of EAAC1 causes increased extracellular glutamate and hyperactivity in the brain (Porton et al., 2013), though functional studies *in vitro* indicate that the regulation of ambient glutamate concentration in the brain relies primarily on glial and not neuronal glutamate transporters (Cavelier and Attwell, 2005; Le Meur et al., 2007; Bellini et al., 2018). This exclusively neuronal glutamate transporter has been identified in the striatum, neocortex, olfactory bulb, and hypothalamus, and its total tissue content is about 100 times lower than that of GLT-1, the most abundant glial glutamate transporter (Holmseth et al., 2012). The loss of EAAC1 has intensely described behavioral effects, including the induction of increased anxiety-like behaviors such as fidgeting, excessive grooming, and burying more marbles in a marble-burying test (Bellini et al., 2018). Despite these findings, the specific functional and structural implications of EAAC1 remain largely unknown.

The striatum is the main entry point of excitatory inputs to the basal ganglia, which are a group of subcortical nuclei that integrate and select for voluntary behaviors (Yin & Knowlton 2006). Neuroimaging studies have identified abnormal basal ganglia circuit function in patients with OCD

(Rosenberg et al., 1997; Radua & Mataix-Cols, 2009). EAAC1 is abundantly expressed in the striatum, and our previous data indicate that its loss is associated with increased anxiety and repetitive behaviors in mice (Holmseth et al., 2012; Bellini et al., 2018). In the striatum, projections are made to motor regions of the brain that control voluntary behaviors (Mink 1996). Excitatory inputs emerge from the direct (D1) pathway, while inhibitory inputs are processed by the indirect (D2) pathway. In the direct pathway, EAAC1 limits metabotropic glutamate receptor I (mGluRI) activation, thereby promoting D1 dopamine receptor (D1R) expression (Scimemi et al., 2009; Bellini et al., 2018). D1Rs are G_s coupled receptors that drive transcriptional changes through the cAMP/PKA pathway, and as a result, increase neuronal excitability that leads to GABAergic inhibitory output to the substantia nigra pars reticulata (Nishi et al., 2011; Bhatia & Saadabadi, 2019). Here, D1Rs exert control over motor functions, and this function has also been implicated in reward-based movements, cognition, and drug addiction (Nishi et al., 2011). Blocking mGluRIs in EAAC1$^{-/-}$ mice permits the bidirectional control of D1R expression, and cell-specific activation of mGluRI can recreate the altered locomotion like that in EAAC1$^{-/-}$ mice (Bellini et al., 2018). These data suggest that increased mGluRI activation drives repetitive OCD behaviors like those seen in OCD and adds to evidence implicating the increased activation of mGluRI with the onset of neuropsychiatric diseases like fragile X syndrome (Lohith et al., 2013). EAAC1$^{-/-}$ mice aged one year were found to have significant degeneration of dopaminergic neurons in the substantia nigra pars compacta, a region that functions as a pacemaker of dopamine release (Guzman et al., 2009; Berman et al., 2011). Together, altered dopaminergic innervation to the striatum seems to underly the onset of this behavioral phenotype with enhanced compulsivity. Here, we used behavioral approaches to support the notion that describes neuronal glutamate transporters as

modulators of reward-based behaviors and show that the loss of EAAC1 contributes to increased anxiety-like and repetitive movement executions in EAAC1$^{-/-}$ mice.

5 Materials and Methods

5.1 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.

5.2. Mice and genotyping. All mice were group-housed and kept under a 12 h light cycle (7:00 A.M. lights on, 7:00 P.M. lights off) with food and water available *ad libitum*. As originally described by Peghini et al. (1997), constitutive EAAC1 knock-out 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. To generate $EAAC1^{-/-}$ breeders, $EAAC1^{-/-}$ mice were back-crossed with C57BL/6J mice for >10 generations. Genotyping was performed on toe tissue samples of P7–P10 mice. Tissue samples were digested at 55°C overnight in a lysis buffer containing the following (in mM): 100 Tris base, pH 8, 5 EDTA, and 200 NaCl, along with 0.2% SDS and 100 g/ml proteinase K. DNA samples were then diluted in nuclease-free water (500 ng/l) and processed for PCR analysis. The PCR primers used for EAAC1 and $Ai9^{Tg/Tg}$ were purchased from Fisher Scientific. For all reactions, we used standard TaqDNA Polymerase (catalog #R2523; Millipore Sigma).

5.3 Behavioral tests. All mice had their home-cage water replaced with 2% citric acid water for at least three days before behavioral testing. By making their normal drinking water somewhat distasteful, receipt of pure water during a behavioral test can then be perceived as a reward (Reinagel, 2018). Therefore, we used a 7 μl water reward as incentive in all our reward-based experiments. Our behavioral setup was a custom 3D printed dual-sided chamber with each

chamber measuring 19 cm x 14.5 cm x 13 cm. Each behavioral test occurred between 9:00 A.M. and 8:00 P.M., and mice of either sex aged 2-6 months were sampled. Between sessions, the behavioral chamber was cleaned with 70% ethanol. Inside each was a lever and water valve controlled by custom code in MATLAB for the Bpod state machine. The custom code and B-pod work together to enact the parameters required for lever press, sequence, and timed sequence experiments. For 10 min in each behavior test, the mouse had free access to the lever and water valve.

5.3.1 Open-field experiments. To begin, we used the open-field behavior test to examine locomotion in mice housed singly or in a pair. We wondered whether social interactions may be occurring during lever press experiments, and to approach this curiosity, designed and 3D printed another custom behavioral chamber that lacked a lever and water valve. The removal of these two objects was an effort to reduce environmental enrichment and produce a raw model that depicts the baseline locomotive behavior of EAAC1$^{-/-}$ mice. Each behavioral test was recorded with a camera and later subjected to ezTrack for location tracking analysis. The total distance traveled throughout each paired and solo session was recorded.

5.3.2 Lever press experiments. After completion of the open-field test, the mice were introduced to an enriched environment with a lever that, upon being pressed once, yields a 7 μl water reward, as part of the fixed-ratio 1:1 (FR1) behavioral test. Here, we examined how EAAC1$^{-/-}$ mice perform reward-based behaviors. To acquire a temporal profile of these behaviors, we compared the times at which lever presses were occurring during each session. This analysis compared the total distances traveled, total number of lever presses, and the time at which they occurred. Here, we observed that EAAC1$^{-/-}$ mice exhibit altered reward-based behaviors as compared to WT mice. To further investigate the role of social interaction during the task, we employed ezTrack for location

tracking analyses. From this program, we compiled the locations of mice in sessions 1 and 9 from the FR1 test to create 2D Gaussian histograms that model localization. Additionally, these data provided us with the ability to calculate the distance between two mice during behavioral tests. Inter-mouse distances and total distances traveled were recorded to identify social interactions.

5.3.3 Sequence experiments. As a follow up, we asked whether increasing the requirement for a reward would change the frequency of lever pressing. If mice who are accustomed to a reward for each press, increasing the number of presses required for the reward may encourage or discourage repeated pressing. To investigate, we employed the fixed-ratio 8:1 (FR8) behavioral test, which requires 8 lever presses for the successful delivery of the water reward. Here, we analyzed the number of presses, rewards, average sequence duration, and average inter-sequence interval.

5.3.4 Timed sequence experiments. We then asked whether a time constraint on the reward would cause altered compulsivity. We hypothesized that a time restriction imposed on reward-based behaviors may increase compulsivity. To test this, we added a timer to the FR8 test, stating that, the 8 lever presses must occur within a span of 6 seconds or there is no reward and the counter resets. We accordingly called this test the timed FR8. When interpreting the results, we noticed that some mice understand and perform the task effectively while others do not appear to learn it at all. We then ran a cluster analysis to identify mice that behaved alike and created a correlative plot to depict the relative relatedness of performance between them. From the analysis, three unique groups of mice who perform the timed sequence task at different capacities were identified. Mice who did not learn the task were called non-performers, those who did performers, and those who learned the task but mostly in later sessions as improvers. Then, as before, we recorded the total number of lever presses as well as the times at which they occurred in sessions 1 and 9. Additionally, we calculated the average sequence duration and inter-sequence interval again to

uncover the temporal profile of repetitive reward-based movements by EAAC1$^{-/-}$ mice. After concluding the task, we selected two mice, one male WT non-performer and one female EAAC1$^{-/-}$ performer for whole-brain staining of c-Fos. To achieve this, mice were perfused, and preservation of tissue occurred 60-90 minutes following the end of the task, in accordance with evidence suggesting that c-Fos expression peaks 1-2 hours following stimulation (Gao & Ji, 2009). Then, we quantified c-Fos cell density (cells/mm^3) and across brain regions.

5.4 Data analysis and statistics. Since mice were tested in pairs for the FR1 behavioral test, we asked whether social interactions may be occurring during the test, and if they are, would they contribute to the executive action of lever pressing by either mouse. To reveal social interactions during the FR1, we employed ezTrack, an open-source python-based video analysis program that assesses a single animal's position throughout a continuous recorded video session (Pennington et al 2019). Recorded behavioral videos were first uploaded to the ezTrack Jupyter notebook in the batch-processing kernel and then specifications were made to parameters of the analysis, including the addition of regions of interest and exclusion, a scale for distance calculation (px to cm) and location tracking thresholds. The outcome of using ezTrack is frame-by-frame motion data and a time-binned summary report with x and y coordinates of the mouse. Following each use various heatmaps and motion traces are generated which allows the user to observe where the animal traveled throughout the session and determine if the analysis is accurate. Centers of mass are used to determine the position of the mouse throughout sessions. Experimental data were then imported into Igor Pro 6.37 for analysis and visualization. All data shown are presented as the mean ± the standard error of the mean (SEM). Kolmogorov-Smirnov statistical tests were used to examine and compare the timing at which lever presses occurred. ANOVA analyses of genotypes were

performed using IBM SPSS software, and $p<0.05$ (*$p<0.05$; **$p<0.01$; ***$p<0.001$) was considered statistically significant.

6 Results

6.1 Loss of EAAC1 does not induce hyperactivity

Our first experiment was the well-documented open-field test. Here, EAAC1$^{-/-}$ and WT mice were subject to nine, 10-minute sessions in which they could freely wander around a barren environment. To examine potential differences that may arise from social interactions, mice were tested singly and in pairs (another mouse was in the neighboring chamber; **Figure 1A**). Location tracking grouped analyses of paired and solo open-field tests showed that EAAC1$^{-/-}$ mice (n=18) travel similar distances to WT mice (n=18) in all sessions (**Figures 1B, C**). To further breakdown these behaviors, separate analyses that grouped mice by sex were conducted. Here, EAAC1$^{-/-}$ (n=9) and WT males (n=9) testing as a pair performed alike males performing individually (**Figures 1D, E**). There also appears to be a slight sex difference in locomotion between EAAC1$^{-/-}$ and WT males, though an increased sample size may minimize this finding. The same finding was also evident in females, who performed at a similar capacity regardless of whether they were in a pair or performing individually (**Figures 1F, G**). Overall, the baseline behaviors of EAAC1$^{-/-}$ during the open-field test mice appear to be alike those of WT mice. Within-group analyses revealed that both genotypes do adapt and change their behavior as successive open-field tests occurred and exploratory behavior decreased over time.

6.2 EAAC1 has sex-specific effects on reward-based behaviors

Since EAAC1 is expressed within the striatum, and the striatum is implicated in the execution of reward-based motor tasks, following the establishment of baseline locomotion, we tested 2–3-month-old male and female WT and EAAC1$^{-/-}$ mice in the FR1 behavioral task. In this, mice

endured nine, 10-minute sessions of exposure to a lever which would yield a 7μl water reward upon being pressed (**Figure 2A**). A comparison of the number of lever presses by both EAAC1$^{-/-}$ (n=71) and WT mice (n=71) found that the mutant mice had a lesser tendency to initiate reward-based movements with high statistical significance (***p=6.3e-35; **Figure 2B**, ***left***). In session one, however, both groups appear to press the lever in similar capacities (**Figure 2B**, ***center***). Our previous evidence suggests an acclimation to the behavioral suite is present as successive sessions occur, though it does not explain why by session nine, EAAC1$^{-/-}$ mice have reduced reward-seeking behavior despite the high accessibility of the reward (***p=2.0e-4; **Figure 2B**, ***right***). To investigate this, our next analysis separated EAAC1$^{-/-}$ mice and WT mice by sex. We again found that overall, EAAC1$^{-/-}$ males (n=33) pressed less than WT mice (***p=1.5e-15; **Figure 2C**, ***left***). In early sessions of behavioral testing, both groups of males exhibited similar performance in the task (**Figure 2C**, ***center***). By session nine, there is a pronounced difference in the number of lever presses performed by EAAC1$^{-/-}$ males as compared to those WT (***p=6.2e-5; **Figure 2C**, ***right***). EAAC1$^{-/-}$ females on average also pressed less than WT females, a statistically significant finding (***p=8.6e-21; **Figure 2D**, ***left***). The time course of female lever presses here resembled the same trend that was present in session one, with no noted difference in performance (**Figure 2D**, ***center***). Though by session nine, EAAC1$^{-/-}$ females had a lesser frequency of pressing as compared to WT females (***p=2.0e-4; **Figure 2D**, ***right***). Overall, it is apparent that both groups seek out rewards but that the as sessions progress, EAAC1$^{-/-}$ mice are less likely to partake.

We then asked whether social interactions were occurring during the lever press task, and if they implicitly would affect the reward outcome. To address this question, location tracking analyses were completed from video recordings of mice in the FR1 behavioral test. Using time-binned summaries that reported the x and y positions of animals during testing, we generated two-

dimensional Gaussian histograms modeling the propensity of WT and EAAC1 to spend time in specific locations of the behavioral chamber. In addition, we calculated inter-mouse distances in sessions 1 and 9 under the ideology that a small I.M.D. suggests for social interaction. As expected, WT and EAAC1$^{-/-}$ mice tended to localize in the proximity of the lever, assumedly due its unique function. There were no differences in the inter-mouse distances of either examined group or in their total locomotion, which means that both EAAC1$^{-/-}$ (n=50) and WT mice (n=46) socially interacted to a similar degree (**Figure 3A-F**). Segregating this analysis by sex revealed no differences between EAAC1$^{-/-}$ (n=26) and WT males (n=26) (**Figure 3G-L**), as well as between EAAC1$^{-/-}$ (n=24) and WT females (n=20) (**Figure 3M-R**). Therefore, we demonstrate that the quality of being housed singly or as a pair does not affect lever press reward-based behaviors during the FR1 test.

Following the closure of the FR1 behavioral test, WT and EAAC1$^{-/-}$ mice underwent another series of nine, 10-min behavioral sessions in the FR8 task. Here, we changed the requirement for a reward from 1 press to 8 presses. The time required to complete eight subsequent lever presses is referred to as a sequence. Upon the eighth press, no matter the length of time spanning from the first, a 7 µl water reward was offered (**Figure 4A**). This change was made to investigate how repetitive reward-based movements may differ in modulation from singular movements. We created two representative raster plots depicting the time course of presses by a WT (Willow) and EAAC1$^{-/-}$ mouse (Kobe) in which individual lever presses were represented as hashes on the plot from each of the nine sessions (**Figure 4B**). When comparing EAAC1$^{-/-}$ (n=30) and WT mice (n=31) in the FR8 test, there was no difference found in the number of lever presses or rewards by either group (**Figure 4C**). Though, the complexity of reward-based behaviors is again evident as during the first session EAAC1$^{-/-}$ mice both pressed and received rewards at an alternate pace than

WT mice (***p=1.1e-8; ***p=4.6e-6; **Figure 4D**). Contrary to our previous findings, which suggest that differences in behavior are most present in earlier behavioral sessions, here, we found that by session nine, reward-based behaviors were more alike between our mutant strain and WT mice (**Figure 4E**). We then characterized EAAC1$^{-/-}$ and WT by the average time it took for them to complete a sequence, referred to here as a sequence duration, and by the length of time between successive sequences (inter-sequence interval). Our data indicate that there is no difference between EAAC1$^{-/-}$ and WT mice in the time required to complete a sequence, nor in the time it takes to initiate the next (**Figure 4F**). We found that sequences vary dramatically by length in both mouse groups and that some mice are more apt to repeatedly complete sequences than others, who may receive only one in an entire 10-min session. Therefore, we are inclined to believe that an increased sample may better illustrate differences present between EAAC1$^{-/-}$ and WT mice in the temporal profile of reward processing during the FR8 behavioral test.

Next, to examine the possibility of sex-specific modulation of EAAC1, we completed the same analyses as described before but compared each sex to itself specifically. In all sessions, WT males (n=19) outperformed EAAC1$^{-/-}$ males (n=17) in both the number of presses and rewards, though neither measurement had statistical significance (**Figure 5B**). In session one, the timeline of lever presses and reward receipt by EAAC1$^{-/-}$ mice was comparatively reduced (***p=1.1e-8; ***p=2.0e-4; **Figure 5C**). By session nine, the same trend was evident, though in a slightly lesser capacity (***p=6.2e-5; ***p=1.7e-3; **Figure 5D**). Though the average sequence duration of EAAC1$^{-/-}$ males appeared to be longer, this was found to not be of significance, and neither was the average inter-sequence interval (**Figure 5E**). Female EAAC1$^{-/-}$ mice (n=13) also pressed like female WT mice (**Figure 6B**). A deeper analysis revealed that EAAC1$^{-/-}$ females engage in reward-based behaviors less than WT females, with fewer lever presses and rewards received (***p=6.2e-

5; ***p=4.6e-6; **Figure 6C**). This trend is reversed in session nine, where female mutant mice began to press more often and received rewards more often than WT females (***p=5.6e-8; ***p=2.6e-7; **Figure 6D**). As in males, there was no finding to indicate variability of the average sequence duration or inter-sequence interval by either genotype (**Figure 6E**). From these data, the modulation of EAAC1 appears to be complex and possibly dependent upon sex. In the FR1 behavior test, EAAC1$^{-/-}$ mice repeatedly performed less than WT mice in their frequency of lever pressing and receipt of reward. However, when we increased the requirement for reward by launching the FR8 test, EAAC1$^{-/-}$ males continued to press less, while EAAC1$^{-/-}$ females specifically began to press more than WT females. Though, significant differences are present in the timeline when the lever presses occur, and not in the cumulative count, suggesting that the decision to press consecutively may be managed by EAAC1. These data suggest that EAAC1 differentially modulates reward-based behaviors and that it may drive compulsivity.

6.3 Loss of EAAC1 affects the timing of reward-based behaviors

As previously described, findings thus far have implicated EAAC1 in producing altered temporal profiles of reward processing in EAAC1$^{-/-}$ mice. We then wanted to determine if increased presses were reward-based or compulsive and to approach this, we implemented a time-sensitive task. To further characterize mutant behavior, after being tested in the FR8, mice were subject to the timed FR8 in which 8 presses must occur within 6 seconds for a reward (**Figure 7A**). A correlative plot was used to demonstrate the variability of performance within the sample, with increasing relatedness being represented by increasing color intensity (**Figure 7B**, ***left***). We categorized WT (n=31) and EAAC1$^{-/-}$ mice (n=21) based on their performance during the timed FR8 test (**Figure**

7B, *center*), and split them into three groups: non-performers, improvers, and performers (**Figure 7B, *right***). Imposing the timer led to an increased rate of lever pressing by EAAC1$^{-/-}$ mice compared to WT mice, a rate that was maintained long enough for EAAC1$^{-/-}$ mice to outperform WT mice in session one of the task (***p=1.1e-5; ***p=4.5e-3; **Figure 7C, *top left***). In this task, eight presses are not causation for reward unless they occur rapidly (within 6 seconds). This time restriction did not limit the ability of EAAC1$^{-/-}$ mice to earn rewards, as they were received more often and in a much higher quantity (***p=2.3e-6; ***p=1.1e-5; **Figure 7C, *top center***). We also recorded the number of unrewarded presses, a value that reflects the number of lever presses that did not contribute to the completion of a sequence. Both EAAC1$^{-/-}$ and WT mice performed a similar number that was relatively high in session one (**Figure 7C, *top right***). Through session nine, EAAC1$^{-/-}$ mice continued to press at higher rates, even with the WT time. course of pressing becoming more refined. ***p=8.4e-12; **Figure 7C, *bottom left***). The total number of rewards earned by both groups increased drastically, though EAAC1$^{-/-}$ earned them at a higher rate (***p=2.6e-7; **Figure 7C, *bottom center***). We presumed that WT and EAAC1$^{-/-}$ mice had learned the task as they had significantly reduced numbers of unrewarded presses in comparison to the evidence from session one. EAAC1$^{-/-}$ mice had fewer unrewarded presses throughout session nine, relative to those that were rewarded, compared to WT mice (***p=1.8e-5; **Figure 7C, *bottom right***). In this task, EAAC1$^{-/-}$ mice on average pressed more than WT mice (**Figure 7D, *left***). Though, the significance lies in the number of rewards received, indicating that lever presses of mutants were more likely to contribute to the completion of a sequence than those of WT mice. (***p=6.1e-3; **Figure 7D, *center***). Supporting the hypothesis that the loss of EAAC1 drives compulsivity, as consecutive sessions occurred, our knockout strain performed fewer and fewer unrewarded presses (*p=0.02; **Figure 7D, *right***).

To further investigate how EAAC1 impacts reward-based behaviors in a sex-specific manner, we compartmentalized the previous analysis and generated a correlative plot that depicts the relatedness of only WT (n=13) and EAAC1$^{-/-}$ males (n=9) (**Figure 8B**, ***left***). Males had vast differences in their reward-based behaviors, with some mice exhibiting an enhanced understanding of the timed sequence task (**Figure 8B**, ***center***). Here, most WT males were non-performers, which means that they were not receiving many (if any) rewards. Variety was present more in the mutant sample as there were two performers (**Figure 8B**, ***right***). Here, we noticed an increased propensity to seek out rewards by EAAC1$^{-/-}$ males in session one, as they received them at a higher rate and overall, more often. Additionally, the presses by our mutant strain in this first session were more likely to contribute to the completion of a sequence, as indicated by their relatively reduced number of unrewarded presses compared to WT mice (***p=1.8e-5; **p=4.6e-3; **Figure 8C**, ***top***). By session nine, we saw the previously described refinement of the task by both groups. EAAC1$^{-/-}$ males continued to press at rates higher than WT males, and though the number of rewards between the groups was insignificant, the presses of knockout mice were far more likely to be in sequence, and they, therefore, performed less unrewarded presses (***p=2.6e-7; ***p=4.6e-6; ***p=1.7e-3; **Figure 8C**, ***bottom***). Comparing the means of the total number of lever presses performed by males of both genotypes revealed no difference (**Figure 8D**, ***left***). There were also no findings to suggest that the average number of rewards received by EAAC1$^{-/-}$ males specifically vary from the norm by WT males (**Figure 8D**, ***center***), nor was there regarding the average number of unrewarded presses (**Figure 8D**, ***right***). In summary, EAAC1$^{-/-}$ males performed uniquely compared to WT males, such that they were more likely to press in the first session. By session nine, this trend is abolished, and there are largely no differences present. We then illustrated WT (n=18) and

EAAC1$^{-/-}$ females (n=12) relatedness in terms of task performance (**Figure 9B**, ***left***). The number of rewards between clusters varies widely (**Figure 9B**, ***center***). There were no female performers, though there were comparatively more improvers than seen in males (**Figure 9B**, ***right***). In session one, mutant females vastly outperformed WT mice in the timed sequence task, pressing more earlier in the session and recording more total lever presses (***p=4.6e-6; ***p=1.8e-5; **Figure 9C**, ***top left***). This translated into the receipt of many more rewards by EAAC1$^{-/-}$ females (***p=2.0e-9; ***p=8.4e-12; **Figure 9C**, ***top center***). Our knockout strain comparatively performed many more unrewarded presses, which we considered an element of learning as refinement had not yet occurred (**Figure 9C**, ***top right***). In session nine, both WT and EAAC1$^{-/-}$ females continued to press the lever albeit some more. However, the rate at which mutant mice pressed was much higher, leading to a drastic difference present between females in the total number of lever presses (***p=1.5e-13; **p=1.7e-3; **Figure 9C**, ***bottom left***). The rate at which rewards were received was therefore higher, as was the total count (***p=1.8e-14; ***p=2.6e-7; **Figure 9C**, **bottom center**). Compared to the data from session one, refinement is now present as both female groups experienced at least a two-fold decrease in the number of unrewarded presses. EAAC1$^{-/-}$ females specifically pressed the lever with more intent of sequence completion, but there lacked significance in the total number (*p=2.5e-2; **Figure 9C**, ***bottom right***). These findings describe a compulsive behavioral phenotype driven by the loss of neuronal glutamate transporter EAAC1 in time-sensitive tasks.

Next, we sacrificed one female EAAC1$^{-/-}$ and WT mouse who had completed nine sessions of the timed sequence task to conduct immunohistochemistry. Mice who exceeded in performance during the task were ideal for this analysis, as we considered enhanced performance to reflect increased knowledge of the task. Here, we quantified c-Fos cell density (cells/mm^3) in coronal

slices and found vast differences in expression (**Figure 10A**). A differential heatmap revealed that c-Fos expression in the EAAC1$^{-/-}$ performer brain varied drastically from the expression found in the WT non performer (**Figure 10B**). Though, c-Fos expression overall was similar in density between groups (**Figure 10C**). With the knowledge that c-Fos expression varies widely across brain regions, we decided to first quantify the cell densities (mm^3) of regions with the highest expression in our control mouse. A comparison to the mutant strain revealed that c-Fos expression within the postrhinal area L6b and barrel cortex L4 is reduced during the timed sequence task (**Figure 10D**). Next, we did the same quantification but for the EAAC1$^{-/-}$ female performer and found that c-Fos expression within multiple areas of the sensory cortex and direct tectospinal pathway is increased (**Figure 10E**). Lastly, we compared relative cell densities across regions and found that various areas of the primary somatosensory cortex had increases in c-Fos expression. More specifically, the mouth L2/3 and mouth L4 areas had the highest increase in relative cell density (**Figure 10F**). On the contrary, regions with the largest decrease in expression included the medial mammillary nucleus and the perirhinal area among many others (**Figure 10G**). During the execution of repetitive reward-based tasks, there is an increase in neuronal activation in the primary somatosensory cortex. Our data suggest that enhanced compulsivity may be driven by altered neuronal activity within the primary somatosensory cortex, which indicates that differences in sensory perception may shape reward-based behaviors.

7 Discussion

7.1 EAAC1 shapes the time course of stereotypical behaviors

In the FR1 behavior test, EAAC1$^{-/-}$ mice were effectively outperformed by WT mice, who as sessions subsequent occurred, seemed to undertake the task of pressing more often. Only in early sessions (1-4) did EAAC1$^{-/-}$ perform like WT mice, indicating that EAAC1$^{-/-}$ were less likely to engage in reward-based behaviors. By the last session, both WT and EAAC1$^{-/-}$ mice demonstrated knowledge of the task by their comparatively enhanced performance to earlier sessions, but EAAC1$^{-/-}$ mice were slower to press. This finding is particularly surprising as previous work with EAAC1 describes its loss to drive hyperactivity. Heatmaps revealing the localization of the mutant and WT strain in sessions one and nine illustrate a propensity of both to linger in the proximity of the lever. This means that, despite sitting in front of the lever most of the time, EAAC1$^{-/-}$ mice were less likely to indulge in acquiring a relatively straightforward reward. An alternate interpretation of this localization paired with relatively lackluster reward-seeking behaviors may be that EAAC1$^{-/-}$ mice are exhibiting anxiety-like behaviors that cause a reduced interest in the lever.

In the FR8 behavior test, the trend of reduced lever pressing as subsequent sessions occur was extinguished, and instead, EAAC1$^{-/-}$ mice performed in a similar capacity to WT mice all throughout except in session one. Here, at any given point within the 10-minute session, they were less likely to press the lever than WT mice. A closer inspection of males alone revealed that, compared to females, they are less motivated to seek reward, especially by session nine. In all sessions, EAAC1$^{-/-}$ females were more likely to press than WT females, further suggesting the interplay of dopaminergic and glutamatergic modulation in compulsivity.

To more closely examine how the time course of reward-based behaviors develops in mice, we employed a time-sensitive task that required all lever presses in a sequence to be within 6 seconds for the delivery of a reward. Here, EAAC1$^{-/-}$ mice vastly outperformed WT mice in all sessions, with an increased propensity to press at any given moment evident in all nine sessions. This effect is attributed mostly to the females of the sample, who exhibited, compared to EAAC1$^{-/-}$ males, an even greater frequency of initiating and completing reward-based behaviors. This finding is surprising: we are the first to put forth evidence suggesting that the loss of EAAC1 causes sex-specific effects. It is possible that an increased sample size may reduce this effect. The first session depicts learning as both groups performed a relatively high number of unrewarded presses. Though, as subsequent sessions occurred, the gap in performance did gradually close between strains. However, at no point did WT mice remotely perform at the same caliber as EAAC1$^{-/-}$ mice considering the total number of rewards received by either. Here, we describe mechanisms of EAAC1 by which time sensitivity may cause altered impulsivity.

7.2 Implications of neuronal glutamate transporters in neuropsychiatric disorders

The loss of EAAC1 is associated with a distinct behavioral phenotype that has enhanced anxiety-like behaviors and altered voluntary movement selection. Previous work revealed that this effect can be driven by cell-specific activation of mGluRIs in the direct pathway (Bellini et al., 2018). Increased expression and activation of these mGluRI have been identified in patients with autism spectrum disorders and intellectual disabilities, denoting these receptors as a common denominator present among neuropsychiatric disorders (Boer et al., 2008; Maccarrone et al., 2010; Lohith et al., 2013; D'Antoni et al., 2014). Here, our finding that EAAC1$^{-/-}$ mice perform better in the timed

sequence task suggests that the altered activation of mGluRI increases compulsive behaviors. Under a time-constraint that likely drove enhanced compulsivity, EAAC1$^{-/-}$ mice effectively received awards and at a pace beyond WT mice. In this work, we characterized neuronal glutamate transporter EAAC1 and examined its control over repetitive reward-motivated voluntary behaviors, but more fundamentally, revealed mechanisms that treatments of the future for neuropsychiatric diseases may target. Though, the imminent complexity of this treatment should be noted as neuropsychiatric disorders like OCD are likely to require pharmacological manipulations that simultaneously control glutamatergic and dopaminergic transmission in the brain.

7.3 Future directions

The experiments presented in this thesis characterize a novel behavioral phenotype of EAAC1$^{-/-}$ mice with enhanced compulsivity. EAAC1$^{-/-}$ mice were more compulsive in the timed sequence behavioral test and were found to have increased c-Fos expression in the primary somatosensory cortex. This high expression of c-Fos is indicative of nonspecific neuronal activity and has previously been associated with several behaviors that are in response to acute stimuli (Velazquez et al., 2015). Therefore, we are led to hypothesize that the impulsive initiation of reward-seeking behaviors is partially dependent upon activity in this region. Channelrhodopsins, a protein family of light-sensitive ion channels, can drive the entry of cations into neurons and therefore drive neuronal activation. We are interested in introducing excitatory and inhibitory opsins into WT and mutant mice, respectively, to see if their characteristic behavioral phenotype is reversible and dependent upon sensory processing. We hypothesize that the increased neuronal activity present

in the sensory cortex of EAAC1$^{-/-}$ mice reflects differences in sensory perception that cause striatal circuit hyperactivity. Future experiments are required to enhance our understanding of the mechanisms responsible for the sensory processing of repetitive reward-based behaviors. At their conclusion, we will have a more complete understanding of EAAC1 at molecular, cellular, and behavioral levels.

8 Figures

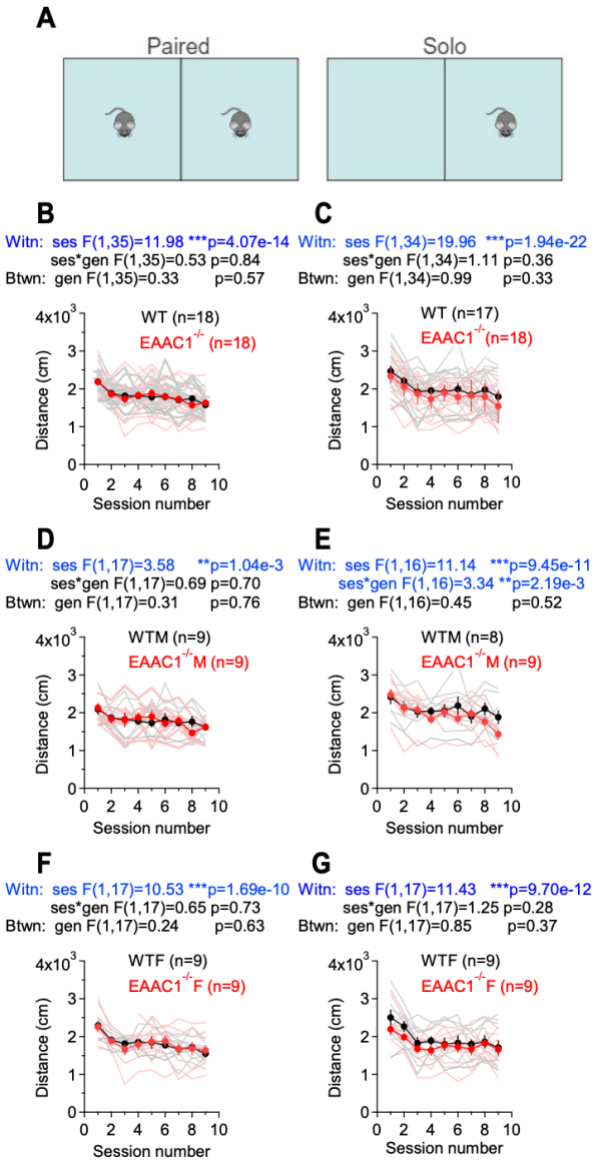

Figure 1. EAAC1$^{-/-}$ and WT mice travel similar distances. (A) Experimental setup. **(B)** Total distance traveled by paired EAAC1$^{-/-}$ (n=18) and WT mice (n=18). **(C)** Total distance traveled by singly housed EAAC1$^{-/-}$ (n=18) and WT mice (n=17) **(D)** Total distance traveled by paired male EAAC1$^{-/-}$ (n=9) and WT mice (n=9). **(E)** Total distance traveled by singly housed male EAAC1$^{-/-}$ (n=8) and WT mice (n=9). **(F)** Total distance traveled by paired female EAAC1$^{-/-}$ (n=9) and WT mice (n=9). **(E)** Total distance traveled by singly housed female EAAC1$^{-/-}$ (n=9) and WT mice (n=9).

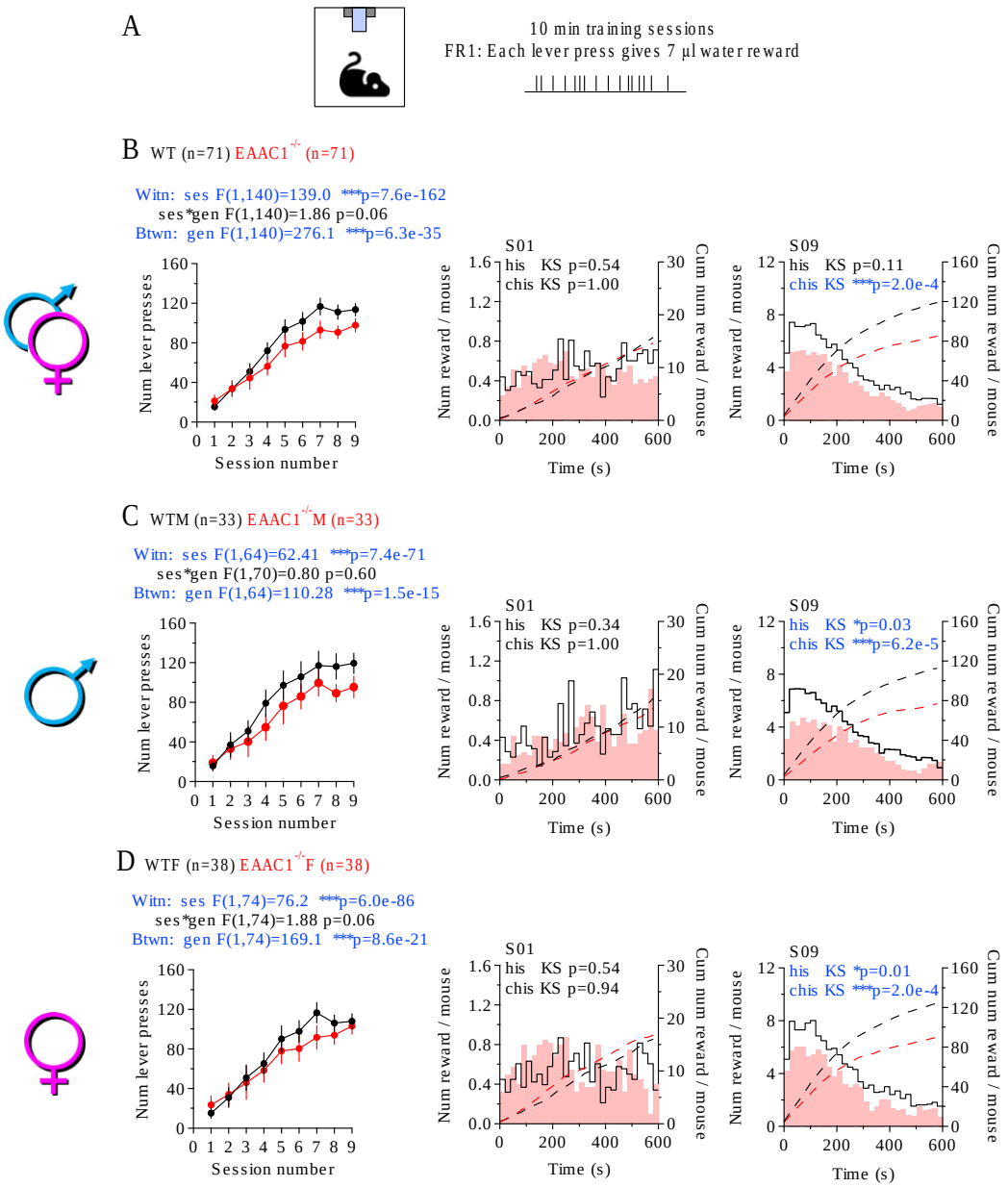

Figure 2. The loss of EAAC1 causes reduced reward-based behaviors in the FR1 test.(A) Lever press (FR1) experimental setup. **(B)** The number of lever presses (left) and rewards in session 1 (center) and session 9 (right) by WT (n=71) and EAAC1$^{-/-}$ mice (n=71). **(C)** The number of lever presses (left) and rewards in session 1 (center) and session 9 (right) by male WT (n=33) and EAAC1$^{-/-}$ mice (n=33). **(D)** The number of lever presses (left) and rewards in session 1 (center) and session 9 (right) by female WT (n=38) and EAAC1$^{-/-}$ mice (n=38).

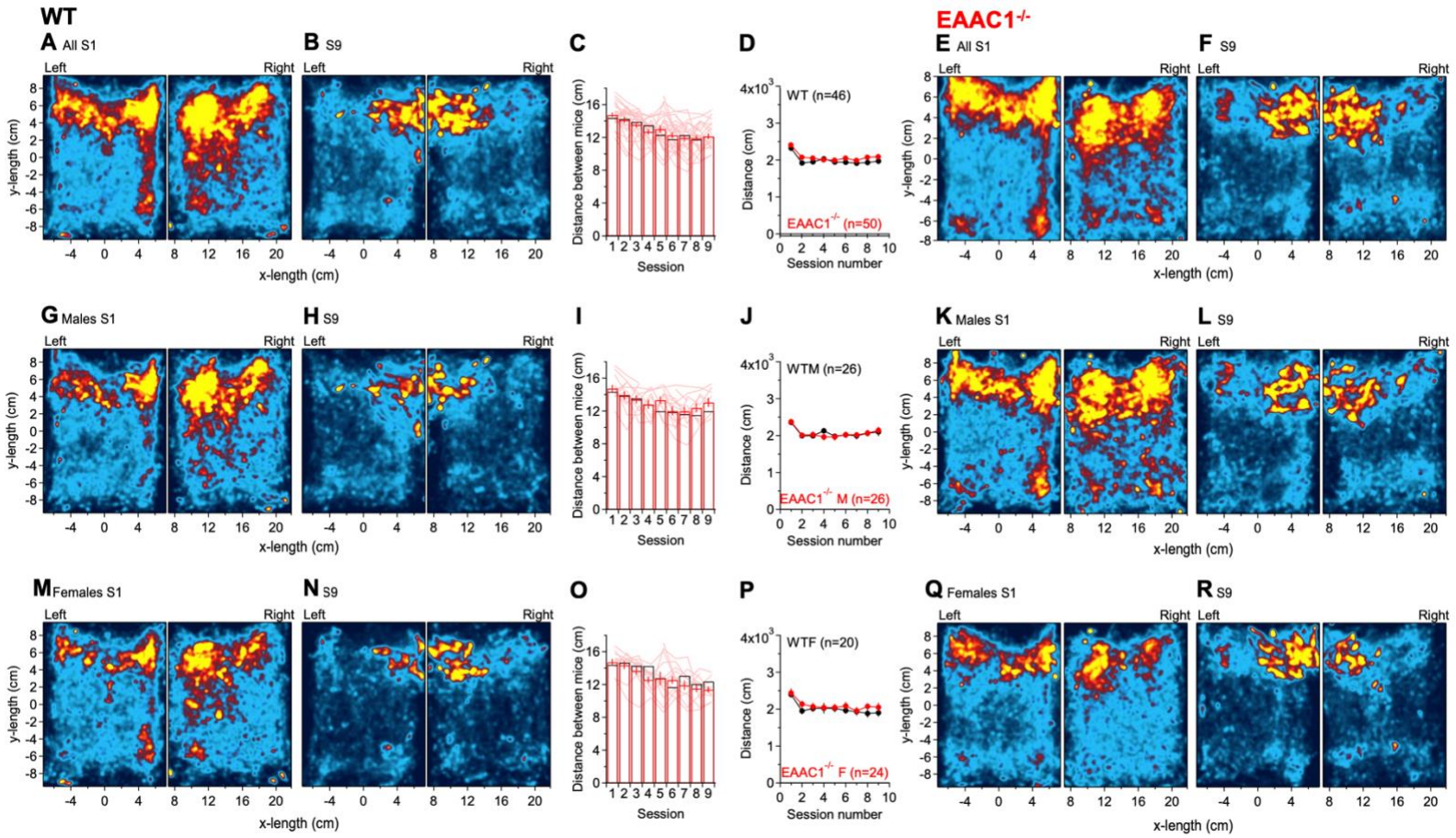

Figure 3. The loss of EAAC1 does not affect social interactions. (A-F) The localization, inter-mouse distance, and distance traveled by EAAC1$^{-/-}$ (n=50) and WT mice (n=46) during the FR1 behavioral test. **(G-L)** The localization, inter-mouse distance, and distance traveled by male EAAC1$^{-/-}$ (n=26) and WT mice (n=26) during the FR1 behavioral test. **(M-R)** The localization, inter-mouse distance, and distance traveled by female EAAC1$^{-/-}$ (n=24) and WT mice (n=20) during the FR1 behavioral test.

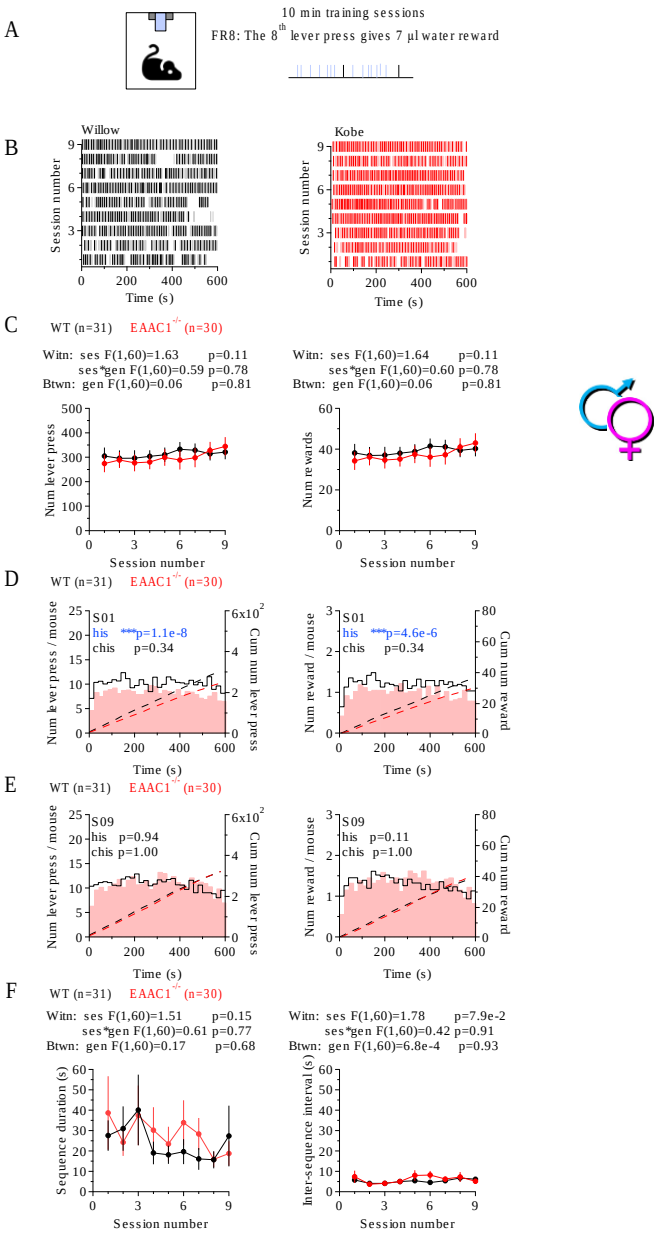

Figure 4. EAAC1$^{-/-}$ mice perform like WT mice in the sequence test. (A) Sequence (FR8) experimental setup. **(B)** Representative raster plots of a WT mouse, Willow (left), and an EAAC1$^{-/-}$ mouse, Kobe (right). **(C)** The average number of lever presses (left) and rewards (right) in sessions 1-9 by EAAC1$^{-/-}$ (n=30) and WT mice (n=31). **(D)** The time course of lever presses (left) and receipt of rewards (right) in session 1 by EAAC1$^{-/-}$ (n=30) and WT mice (n=31). **(E)** The time course of lever presses (left) and receipt of rewards (right) in session 9 by EAAC1$^{-/-}$ (n=30) and WT mice (n=31). **(F)** The average sequence duration (left) and average inter-sequence interval (right) of EAAC1$^{-/-}$ (n=30) and WT mice (n=31).

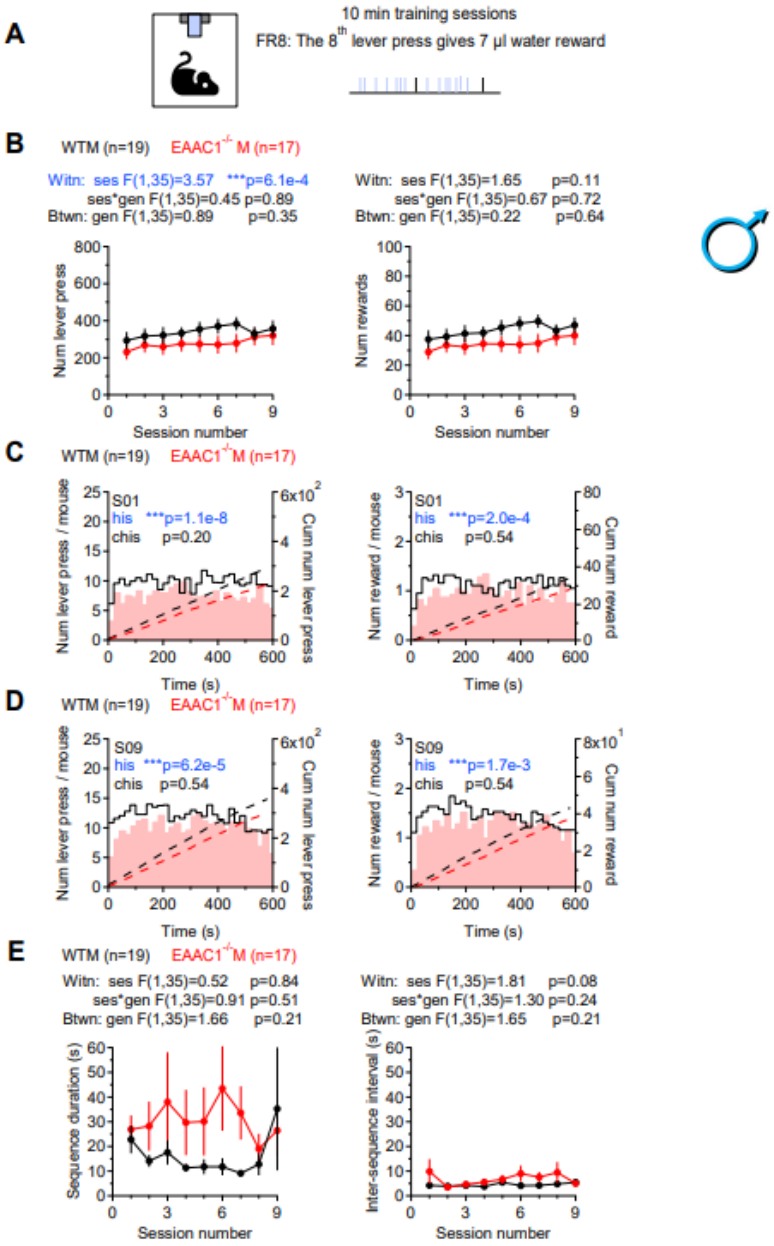

Figure 5. EAAC1$^{-/-}$ males press the lever like WT males. (A) Sequence (FR8) experimental setup. **(B)** The average number of lever presses (left) and rewards (right) in sessions 1-9 by male EAAC1$^{-/-}$ (n=17) and WT mice (n=19). **(C)** The time course of lever presses (left) and receipt of rewards (right) in session 1 by male EAAC1$^{-/-}$ (n=17) and WT mice (n=19). **(D)** The time course of lever presses (left) and receipt of rewards (right) in session 9 by male EAAC1$^{-/-}$ (n=17) and WT mice (n=19). **(E)** The average sequence duration (left) and average inter-sequence interval (right) of male EAAC1$^{-/-}$ (n=17) and WT mice (n=19).

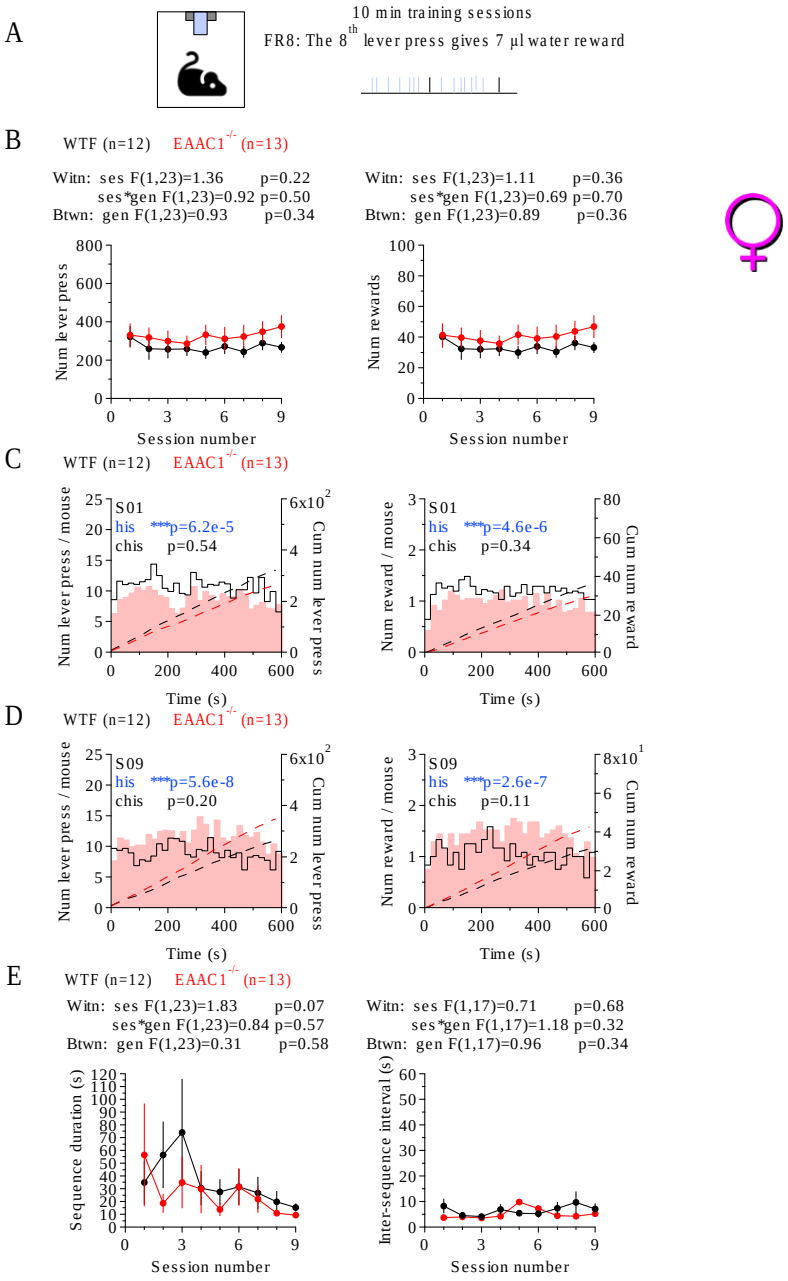

Figure 6. EAAC1$^{-/-}$ females press faster than WT females. (A) Sequence (FR8) experimental setup. **(B)** The average number of lever presses (left) and rewards (right) in sessions 1-9 by female EAAC1$^{-/-}$ (n=13) and WT mice (n=12). **(C)** The time course of lever presses (left) and receipt of rewards (right) in session 1 by male EAAC1$^{-/-}$ (n=17) and WT mice (n=19). **(D)** The time course of lever presses (left) and receipt of rewards (right) in session 9 by female EAAC1$^{-/-}$ (n=13) and WT mice (n=12). **(E)** The average sequence duration (left) and average inter-sequence interval (right) of female EAAC1$^{-/-}$ (n=13) and WT mice (n=12).

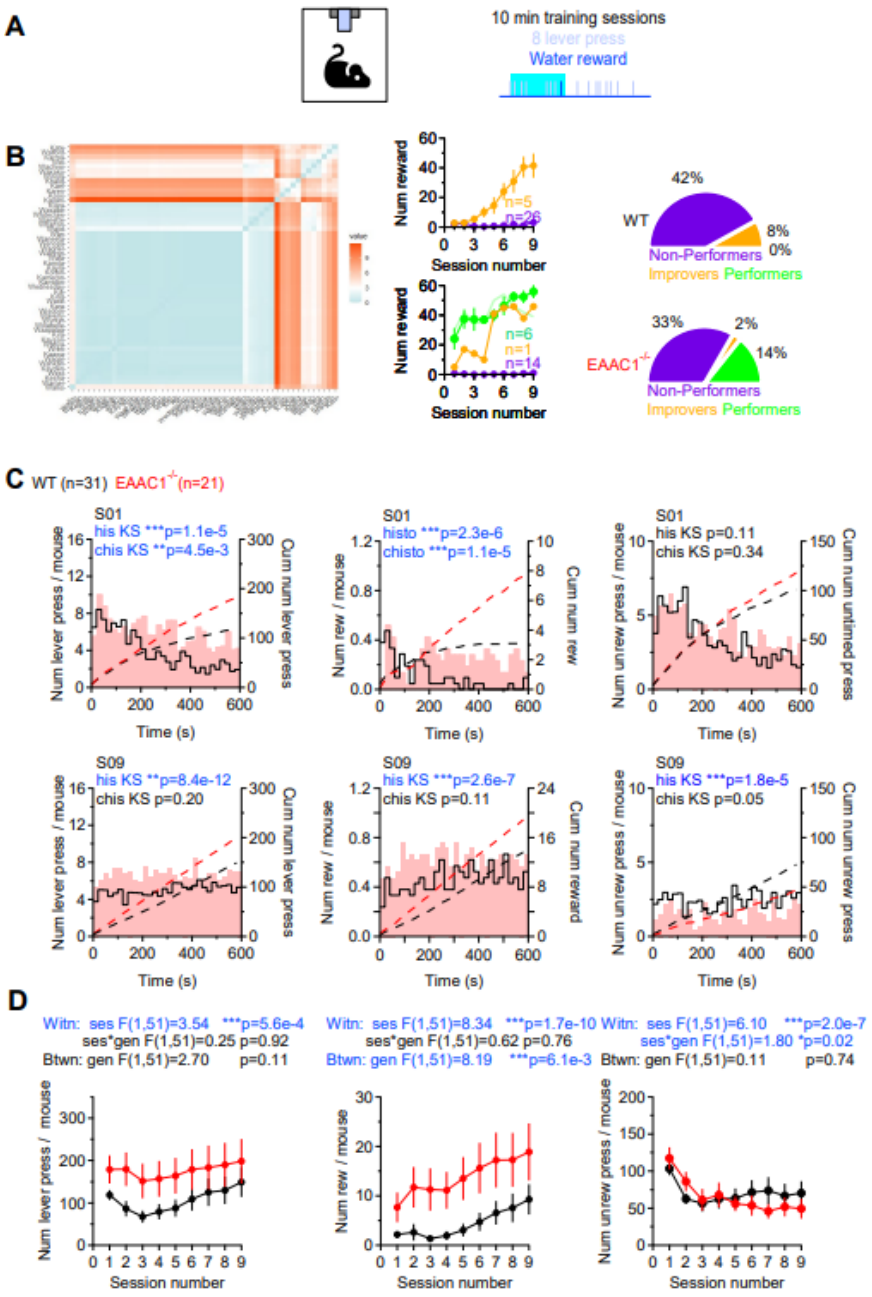

Figure 7. The loss of EAAC1 induces compulsivity in the time-sensitive sequence test. **(A)** Timed sequence (timed FR8) experimental setup. **(B)** Correlative plot relating mice based on their lever press frequencies (left). Cluster analyses of lever press data revealed three distinct groups of mice that range vastly in performance (center, right). There were no WT performers. **(C)** The time course of lever presses, rewards, and unrewarded presses by EAAC1$^{-/-}$ (n=21) and WT mice (n=31) in session 1 (top) and in session 9 (bottom). **(D)** The average number of lever presses (left), rewards (center), and unrewarded presses (right) by EAAC1$^{-/-}$ (n=21) and WT mice (n=31).

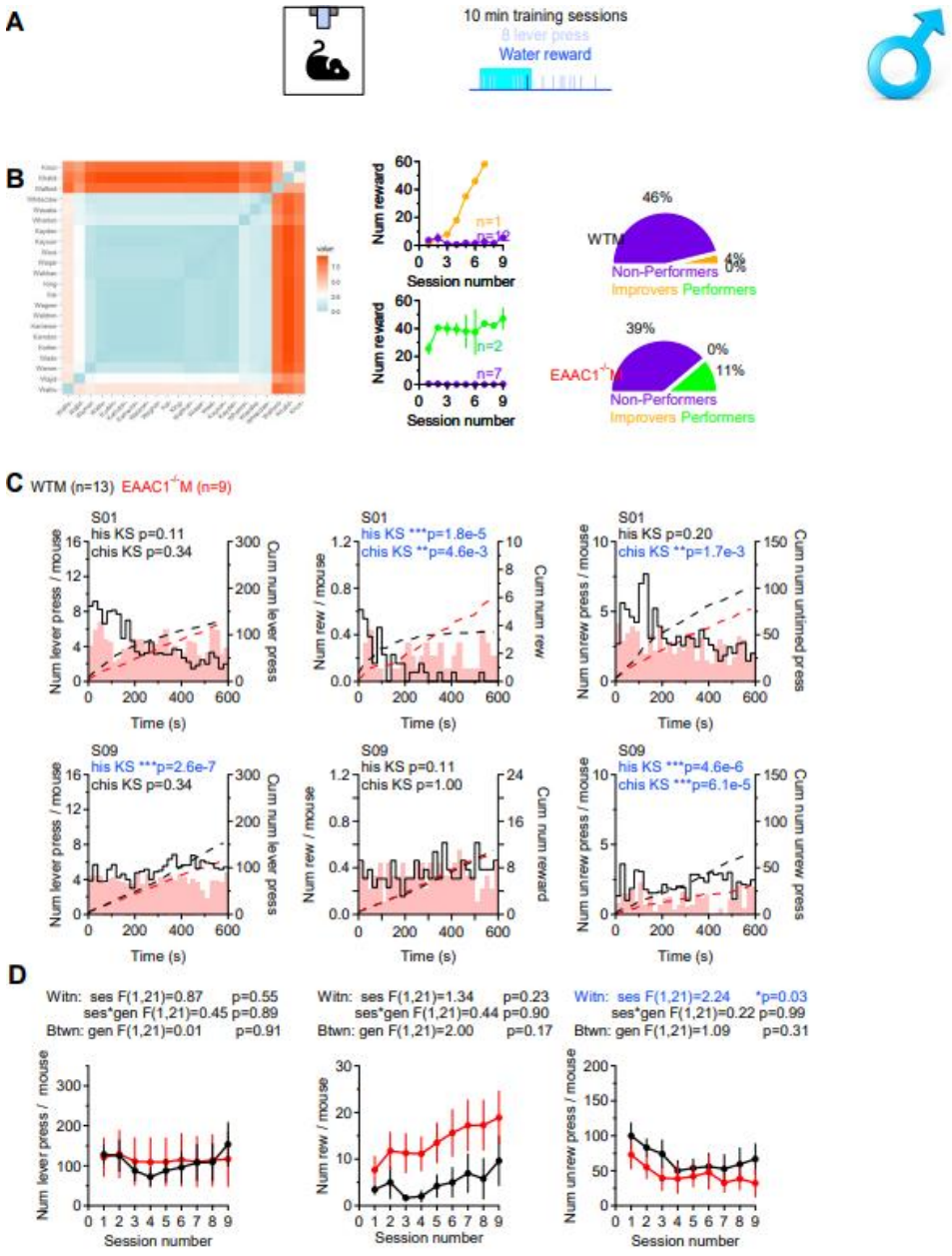

Figure 8. EAAC1$^{-/-}$ males complete sequences faster than WT males. (A) Timed sequence (timed FR8) experimental setup. **(B)** Correlative plot relating mice based on their lever press frequencies, with increasing color intensity (left). Cluster analyses of lever press data revealed three distinct groups of mice that range in performance (center, right). There were no WT performers. **(C)** The time course of lever presses, rewards, and unrewarded presses by male EAAC1$^{-/-}$ (n=9) and WT mice (n=13) in session 1 (top) and session 9 (bottom). **(D)** The average number of lever presses (left), rewards (center), and unrewarded presses (right) by male EAAC1$^{-/-}$ (n=9) and WT mice (n=13).

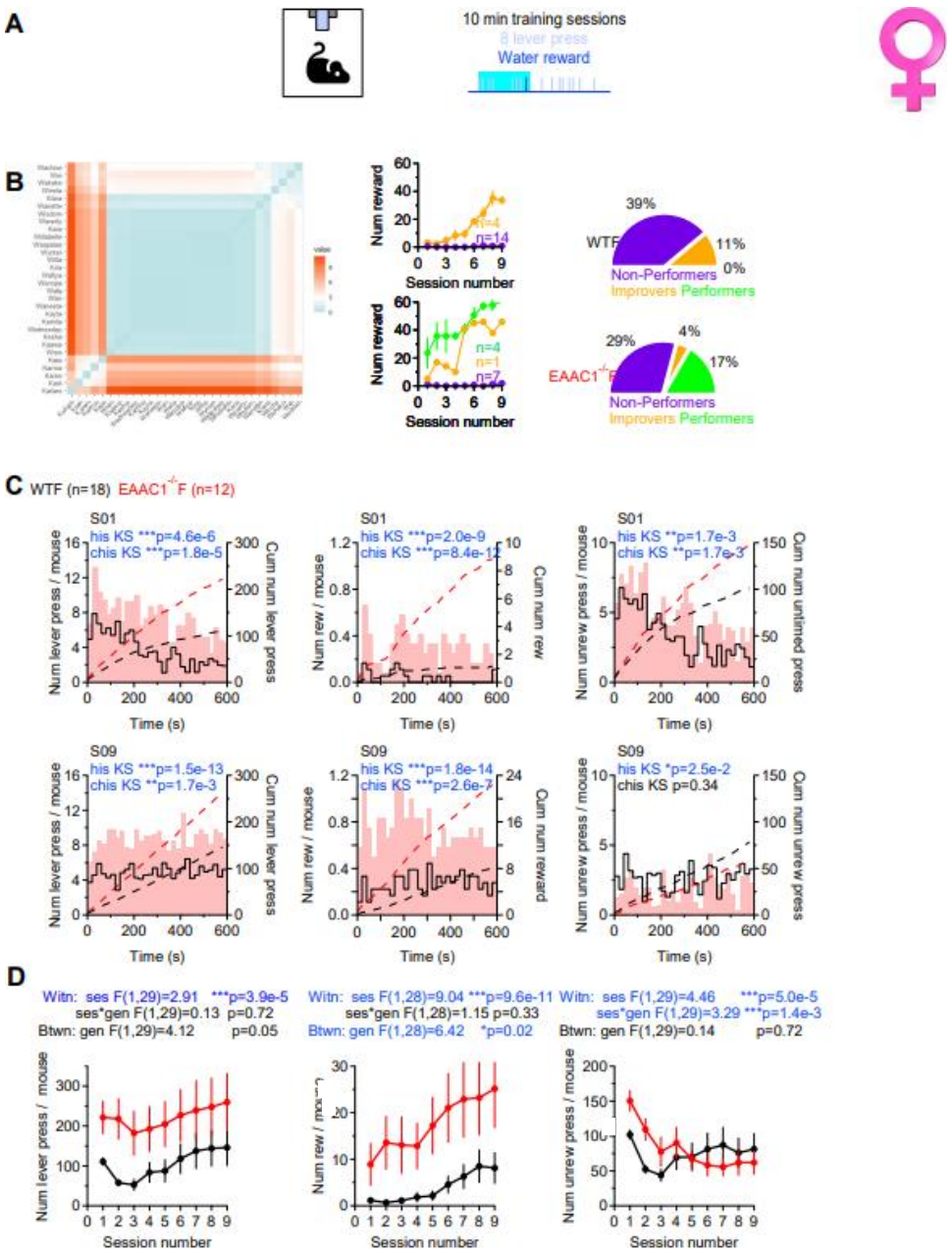

Figure 9. EAAC1$^{-/-}$ females press and receive much more rewards than WT females. (A) Timed sequence (timed FR8) experimental setup. **(B)** Correlative plot relating mice based on their lever press frequencies (left). Cluster analyses of lever press data revealed three distinct groups of mice that range in performance (center, right). There were no WT performers. **(C)** The time course of lever presses, rewards, and unrewarded presses by female EAAC1$^{-/-}$ (n=12) and WT mice (n=18) in session 1 (top) and session 9 (bottom). **(D)** The average number of lever presses (left), rewards (center), and unrewarded presses (right) by female EAAC1$^{-/-}$ (n=12) and WT mice (n=18).

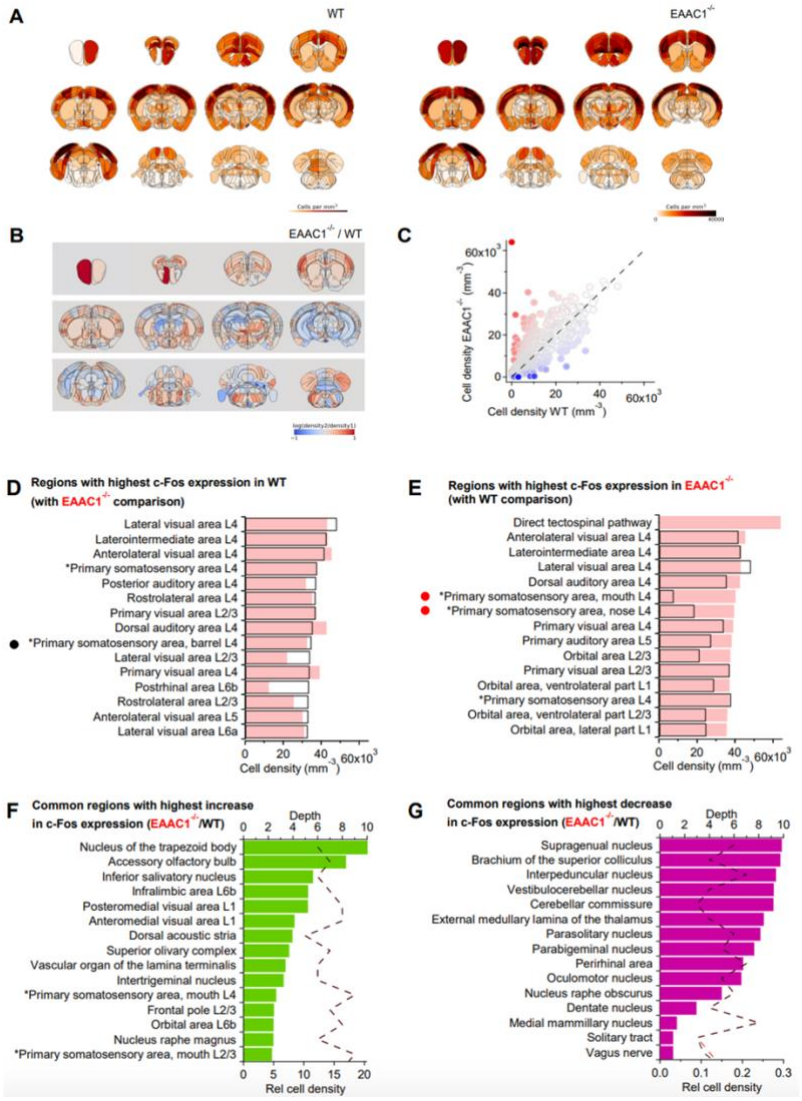

Figure 10. Neuronal activation within the sensory cortex of EAAC1$^{-/-}$ mice is increased during the timed sequence behavior test. (A) Coronal slices depicting the density (cells/mm^3) of c-Fos expression across brain regions of a WT male non performer (left) and an EAAC1$^{-/-}$ female performer (right). **(B)** A differential heatmap displaying brain regions that have particularly enhanced c-Fos in either the WT (blue) or mutant brain (red). **(C)** A comparison of c-Fos cell density (cells/mm^3) in EAAC1$^{-/-}$ and WT mice. **(D)** An overall quantification of c-Fos expression across WT brain regions. **(E)** An overall quantification of c-Fos expression across EAAC1$^{-/-}$ brain regions. **(F)** A depiction of the brain regions with increases in c-Fos cell density (cells/mm^3). **(G)** A depiction of the brain regions with decreases in c-Fos cell density (cells/mm^3).

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