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**Neuronal Glutamate Transporter EAAC1
Regulates Motor Activity and Anxiety in Mice**

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An honors thesis presented to the Department of Biological Sciences at University at Albany, SUNY in partial fulfillment to graduate from The Honors Program for Spring 2016.

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Abstract

Obsessive Compulsive disorder (OCD) is a neuropsychiatric disorder characterized by the onset of recurrent thoughts, anxiety, and repetitive motor behaviors. The molecular basis of OCD remains elusive, but recent meta-analysis and genome-wide association studies (GWAS) suggest the existence of a genetic association between polymorphisms in the gene coding for excitatory amino acid carrier 1 (EAAC1) and OCD. It is also known that the Cortico-Striatal-Thalamo-Cortical (CSTC) pathway shows patterned hyperactivity in patients with OCD. EAAC1 is the primary neuronal glutamate transporter in the brain and is abundantly expressed in the cortex and the striatum, two regions that are part of the CSTC pathway. It is currently unknown whether mice that do not express the transporter EAAC1 have a behavioral phenotype consistent with that of OCD in humans, which would make them useful to study the molecular basis of the disease. Through a variety of behavioral tests our research examines phenotypic differences between wild-type C57BL/6 mice and conventional EAAC1 knockout mice (EAAC1^{-/-}) to determine how EAAC1 regulates motor activity, anxiety, and coordinated information processing in the CSTC pathway. Our results suggest that the loss of EAAC1 expression is associated with the onset of motor hyperactivity and anxiety in mice of either sex. These behaviors are reminiscent of the repetitive behaviors and increased anxiety of patients with OCD. Taken together, these findings suggest that EAAC1^{-/-} mice may be a valuable model in which to determine the molecular mechanisms underlying hyperactivity in the CSTC pathway and OCD.

Keywords: OCD, EAAC1, motor activity, anxiety, CSTC, striatum, D1 and D2 MSNs

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Introduction

Obsessive compulsive disorder (OCD) is a severe, chronic anxiety disorder characterized by the presence of unwanted obsessions consisting of repetitive thoughts, feelings, ideas, or sensations. Individuals with OCD carry out ritualistic behaviors (compulsions) such as hand washing that provide temporary relief from these obsessions (American Psychological Association, 2013). OCD disrupts the lives of a significant proportion of the population; epidemiological studies conducted in several countries report lifetime prevalence rates of OCD in the general population to be between 1 and 9% depending on the age and sex of the populations studied (Mattina et al., 2016). Traditional treatment for OCD involves serotonin reuptake inhibitors and cognitive behavioral therapeutic (CBT) approaches, but the pharmacological approaches are effective only in 40-60% patients (60-80% when combined with CBT), leaving a large proportion of patients suffering with OCD unable to find relief from this disorder (Abramowitz et al., 2005; Bystritsky, 2004).

There are many variants of mice previously used for studying OCD including *Hoxb8*^{-/-}, *Slitrk5*^{-/-} and *Sapap3*^{-/-} mice. These mice can exhibit excessive compulsive grooming resulting in fur loss with 100% penetrance and are thus both difficult to work with and questionable in their validity as models of OCD aligning closer with a disorder called Trichotillomania (Greer et al., 2002). EAAC1^{-/-} mice, on the other hand, demonstrate fur loss with only a 30% penetrance and may pose a more valid model for studying OCD (Aoyama et al., 2006).

Though the etiology of OCD remains elusive, functional brain imaging has allowed researchers to delve deep into the brains of OCD patients to examine aberrations in neural circuits that control behavior. Over the past 25 years these approaches have consistently identified abnormal patterns of activity in a particular circuit that connects the cortex, thalamus, and striatum, named the Cortico-Striatal-Thalamo-Cortical (CSTC) pathway (Ting et al., 2011). Additionally, recent genome-wide association studies (GWAS) have identified a polymorphism in the gene *Slc1a1* in individuals with OCD (Grados, 2010). *Slc1a1* codes for excitatory amino acid carrier 1 (EAAC1), a peri-synaptic glutamate transporter that functions to limit the activation of extra-synaptic glutamate receptors, with strong expression in the areas of the CSTC pathway (Rothstein et al., 1994). I highlight one particular area of the CSTC pathway, i.e. the striatum, because the striatum receives excitatory glutamatergic input from the cortex, and is involved in phenotypic behaviors of OCD; the striatum coordinates the initiation of stereotyped actions and regulates anxious behaviors (Canales et al., 2000).

The striatum is composed of two types of medium spiny neurons (MSNs), D1 and D2 dopamine neurons. D1 receptor signaling in D1 neurons enhances glutamatergic signaling in striatonigral MSNs, whereas D2 receptor signaling in D2 neurons exerts the opposite effect in striatopallidal MSNs and long-term alterations in dopamine signaling produce profound and cell-type-specific reshaping of corticostriatal connectivity and function (Surmeier et al., 2007; Ena et al., 2011). Alterations in dopaminergic signaling in the striatum produced by a loss of EAAC1^{-/-} could produce functional changes in the striatum leading to the production of behaviors phenotypic of OCD.

It is currently unknown how mutations in EAAC1 lead to the synaptic changes in D1 and D2 signaling to cause hyperactivity in the CSTC pathway and the onset of behaviors phenotypic of OCD such as anxiety and repetitive motor behaviors. In order to study the synaptic environment of mice missing EAAC1 (EAAC1^{-/-}), we obtained a comprehensive behavioral analysis of EAAC1^{-/-} mice.

Methods

All experiments were performed in accordance with the Institutional Animal Care and Use Committee at SUNY at Albany. Male and female C57BL/6 (WT) and EAAC1^{-/-} mice (P14-35) were tested with a battery of behavioral tests to monitor the occurrence of anxious and compulsive motor behaviors. The behavioral experiments included: (1) flying saucer (FS); (2) marble burial (MB); (3) open field (OF); (4) elevated plus maze (EPM); and (5) grooming tests. All mice had *ad libitum* access to food and water and were maintained on a 12-hour light/dark cycle. The videos for each test were acquired using a Creative webcam (Model #VF0770 30 fps with 320 × 240 pixels) and saved as .wmv files. All behavioral apparatuses were cleaned with a 70% ethanol solution and left to air dry between each trial. Mice were tested in the light phase, between 9:00 AM and 1:00 PM. The mice were transferred to the testing room and let acclimate for 30 min before each test. Means and standard deviations of the listed parameters were calculated using Igor Pro (Wavemetrics, Inc., Portland, OR).

Flying Saucer Test

The Flying Saucer (FS) test is designed to measure overall level of mouse motor activity. We positioned a plastic flying saucer disk ($\varnothing=5.25''$) (Figure 4A) in the middle of the floor of a

mouse cage and connected it to an odometer (Model # SD-548B, Shenzhen Sunding Electron Co., Shenzhen, China) to monitor distance, speed, and time spent running. Each mouse was tested for 30 min before being returned to their home cage.

Marble Burial Test

The Marble Burial (MB) test is designed to measure motor activity and anxiety in mice by measuring the number of marbles that each mouse buries in a given time (Archer et al., 1987). The MB test was performed in plastic cages filled with 1" ½" of sawdust bedding. 24 glass marbles ($\varnothing = \frac{1}{4}$ ") were arranged in a 4 × 6 grid (distance from cage walls = 1/2"; distance between marbles = 1"½") on top of the sawdust bedding. One mouse was placed in each cage and covered with a cage lid. After 30 min, we returned the mice to their home cage and counted the number of marbles that were buried with sawdust. Marbles with $\geq 50\%$ of their top surface buried in sawdust were considered buried. At the end of each experiment, we soaked the marbles in a solution of 70% ethanol and let them air dry before using them for other experiments.

Open Field Test

The open-field (OF) test is a useful tool for measuring exploratory, locomotor, and anxiety-like behavior in mice. Rodents initially display a preference to walk close to walls, a behavior called thigmotaxis; however, the behavior gradually decreases over the duration of exploration (Simon et al., 1994). Anxious behavior is measured using three parameters: time spent and increased travelled within the central zone of the field (Adams and Geyer., 1985, Simon et al., 1994, Prut and Belzung, 2003). The OF test was performed in a white, opaque

Plexiglas box (L 18" × W 18" × H 15"). Each mouse was recorded for 15 min before being removed from the cage. AnyMaze (Stoelting, Wood Dale, IL) was used to analyze mouse behavior. For video analysis the apparatus was divided into 16 squares using a 4x4 grid and each square was allocated to one of three zones: corner, side, and center. Videos were analyzed for 10 min after the experimenter completely exited the recording frame. The parameters measured for the OF test were 'total time' spent in each section, 'total distance' traveled in each section, 'maximum speed', 'mean speed', 'number of entries' into each section, and 'time immobile'. The normalized values calculated in Igor Pro 6.32 (Wavemetrics, Lake Oswego, OR) are the ratios of the distance, number of entries, time, and time immobile spent in specific regions of the field and the total duration of the test. Each mouse was placed in the center of the field facing the top wall of the field. Entry into a zone was scored from the center of the body.

Elevated Plus Maze Test

In the elevated plus maze (EPM), mice are allowed to explore the apparatus for 15 min. Less anxious and more curious mice demonstrate an increased number of entries into the open arm (Walf et al., 2007). The EPM test was performed in a white, opaque Plexiglas apparatus built in a plus-shaped configuration (+). The apparatus consisted of two open arms (L 14" x W 2") across from each other which were perpendicular to two closed arms (L 14" x W 2" x H 6") with a center platform (L 2" x W 2"). Each mouse was placed in the center of the EPM initially facing the top open arm and recorded for 15 min. An arm entry was counted when more than 80% of the body was within the arm. Each video was analyzed off-line using AnyMaze (Stoelting, Wood Dale, IL). The analysis began when the experimenter was completely absent

from the recording frame and ended after 10 min. The following parameters were measured from the EPM test: 'time in open arm', 'time in closed arm', 'entries into closed arm', and 'entries into open arm.' Normalized time in the closed arm was calculated using the ratio of time spent in the closed arm and total duration of the test. Normalized time in the open arm was similarly calculated as the time spent in the closed arm over the total duration of the test. Mice were tested in the EPM at ages P14, P21, P28, and P35. Data from WT naïve (tested at P14, P21, P28, or P35) and WT control (tested at P14, P21, P28, and P35) groups were pooled together because naïve group exhibited similar exploration patterns as the WT control mice.

Grooming Test

Mice can spend over half of their awake-time grooming (Klueff et al., 2007). The grooming test was conducted to examine the frequency and duration of the mice's grooming behaviors. The grooming test was performed in a L 12" x W 7.5" x H 6" plastic mouse cage. We used a custom-made software (M-Track) for data acquisition (Reeves et al., 2016). We positioned a webcam 12" below the bottom of the cage to record the underside of the mice. The acquisition of each video file began once the mouse was placed in the center of the plastic cage and the experimenter was out of view. Each trial is recorded for duration of 12 min. Videos were analyzed starting with the first complete bout in frame until the last complete bout in frame for the total duration of grooming, the duration of each bout and the number of bouts as defined by grooming sessions separated by more than 6 s.

Results

Flying Saucer Test

To determine if EAAC1 has any effect on the overall level of motor activity in mice, we performed a series of experiments using the FS test (Figure 1). The FS test provides a measure for the general activity level of a mouse. In the FS test, the time spent running, distance traveled, average speed, and maximum speed of both WT and EAAC1^{-/-} mice increased progressively with age. However, none of these parameters changed significantly between WT and EAAC1^{-/-} mice in a paired t-test (time $p=0.126$; distance $p=0.362$; average speed $p=0.248$; maximum speed $p=0.934$; Figure 1B).

Marble Burial Test

To determine if EAAC1 has any effect on a more precise, repetitive motor behavior in mice, we performed the MB test. The MB test is a behavioral test that is commonly used to measure digging behaviors and anxiety in mice. In this test, increased digging/anxiety causes mice to bury a larger proportion of marbles. In our experiments, WT and EAAC1^{-/-} mice buried more marbles as they grew older from P14 to P35. Across all ages, in the MB test, EAAC1^{-/-} mice buried more marbles than WT mice (** $p=3.9e-11$; Figure 2A *top*). In a separate analysis, we showed that this leads to a higher proportion of EAAC1^{-/-} mice than WT mice burring a higher proportion of marbles (Figure 2A *bottom*). The pronounced digging behaviors of EAAC1^{-/-} mice is also observed when analyzing females (Figure 3A) and males separately (Figure 4A). The results obtained from this set of experiments are consistent with EAAC1 contributing to the regulation of digging behaviors and anxiety in mice.

Open Field Test

The OF test is another assay used to examine general motor activity in mice, but with more specificity as to when and where activity is taking place. Consistent with the FS data that suggest that EAAC1^{-/-} and WT mice have a similar level of overall activity, in the OF test, EAAC1^{-/-} and WT mice traveled the same distance in various fields of the apparatus (Figure 2B *right axis, white/red dots*). However, the ability to track more specific data in the OF test allowed us to see that EAAC1^{-/-} mice spent less time immobile compared to WT counterparts (**p=4.6e-3; Figure 2B *left axis, grey/pink histograms and Gaussian fits*). This trend is also observed when analyzing females (Figure 3B) and males separately (Figure 4B). Data collected from the OF test suggest that EAAC1^{-/-} display more motor activity and are consistent with EAAC1 regulating motor behavior.

Elevated Plus Maze Test

To determine if EAAC1 has any effect on the presence of anxiety in mice, we performed the EPM test (Figure 2C). The EPM test consisting of 4 arms (2 open and 2 closed) and provides a measure of rodent anxiety based off of rodents' aversion to open spaces. Anxious behavior is displayed by a mouse as more thigmotaxic behavior (more time spent in the closed arms) and less exploratory behavior (less time spent in the open arms). In the EPM, EAAC1^{-/-} mice spent less time in the open arm (*p=.019) and entered the open arm a lower number of times compared to WT mice (**p=.006). This trend is also observed between WT and EAAC1^{-/-} mice when analyzing females (Figure 3C) and males separately (Figure 4C). These findings are consistent with the hypothesis that EAAC1 contributes to the regulation of anxiety (Figure 1C).

Grooming Test

The Grooming test was performed to determine if EAAC1 is responsible for regulating repetitive motor behaviors such as grooming behavior. We found that although EAAC1^{-/-} mice groom more frequently compared to WT mice (**p=2.9e-8; Figure 5A). Each grooming episode is shorter in EAAC1^{-/-} compared to WT mice (**p=2.0e-4; Figure5B). Consequently, the total grooming time, obtained by multiplying the duration of each grooming bout by their frequency of onset, was reduced in EAAC1^{-/-} with respect to WT mice (*p=.029; Figure 5C). Similar results are also observed when analyzing females (Figure 5 *middle columns*) and males separately (Figure 5 *right columns*). These data suggest that through EAAC1^{-/-} mice groom for less time, they groom more frequently. These results suggest that EAAC1 plays a role in the regulation of repetitive motor behaviors.

Discussion

Taken together, the results of our behavioral assays strongly support the hypothesis that EAAC1^{-/-} mice exhibit increased anxiety and motor activity, which is consistent with the behavioral phenotype of patients with OCD. EAAC1^{-/-} mice displayed higher anxious behavior than WT mice in EPM test as EAAC1^{-/-} mice spent less time in the open arms and entered the open arms a lower number of times. EAAC1^{-/-} mice also display anxiety in the MB test. In the MB test, EAAC1^{-/-} mice displayed higher anxiety and motor behavior than their WT counterparts by burying a higher number of marbles.

Evidence from the OF test also shows increased motor behavior in EAAC1^{-/-} mice that is consistent with increased repetitive motor behaviors displayed as compulsions in OCD patients that relieve the displayed anxiety (American Psychological Association, 2013). A common

compulsion of individuals with OCD involves decontamination or washing procedures, especially compulsive hand washing (Jhung et al., 2014). Activity in the striatum is correlated the initiation of grooming in mice (Ahmari et al., 2013). The hyperactivity observed in the striatum in the OCD brain (Ting et al., 2011) is consistent with the increased frequency (increased bout initiation) and with decreased time spent in a bout that we observed in the grooming test.

Specifically, D1 receptor containing neurons' activity is linked to motor initiation and D2 receptor containing neurons' activity is linked to a cease of locomotor activity (Kravitz, et al., 2010). Recent findings from our lab indicate that glutamatergic transmission is up-regulated in D1-MSNs of EAAC1^{-/-} mice. Thus, D1 cells are more active and, by promoting initiation of motor behaviors in EAAC1^{-/-} mice, they lead to the onset of motor hyperactivity. Thus, the absence of functional EAAC1 protein in OCD allows glutamate spillover onto extra-synaptic receptors in the striatum, causing an imbalance in the activation of D1 and D2 receptor containing neurons and increased repetitive motor behaviors.

These findings suggest that EAAC1^{-/-} mice provide a valid new model to study the synaptic basis of OCD. The identification of the cellular and network mechanisms underlying OCD is likely to contribute to the development of more effective pharmacological treatment for patients that do not respond to currently available therapeutic approaches. In summary, we believe EAAC1^{-/-} mice represent an innovative research tool in which we can identify previously unknown mechanisms underlying the onset of a neuropsychiatric disorder like OCD

Figures

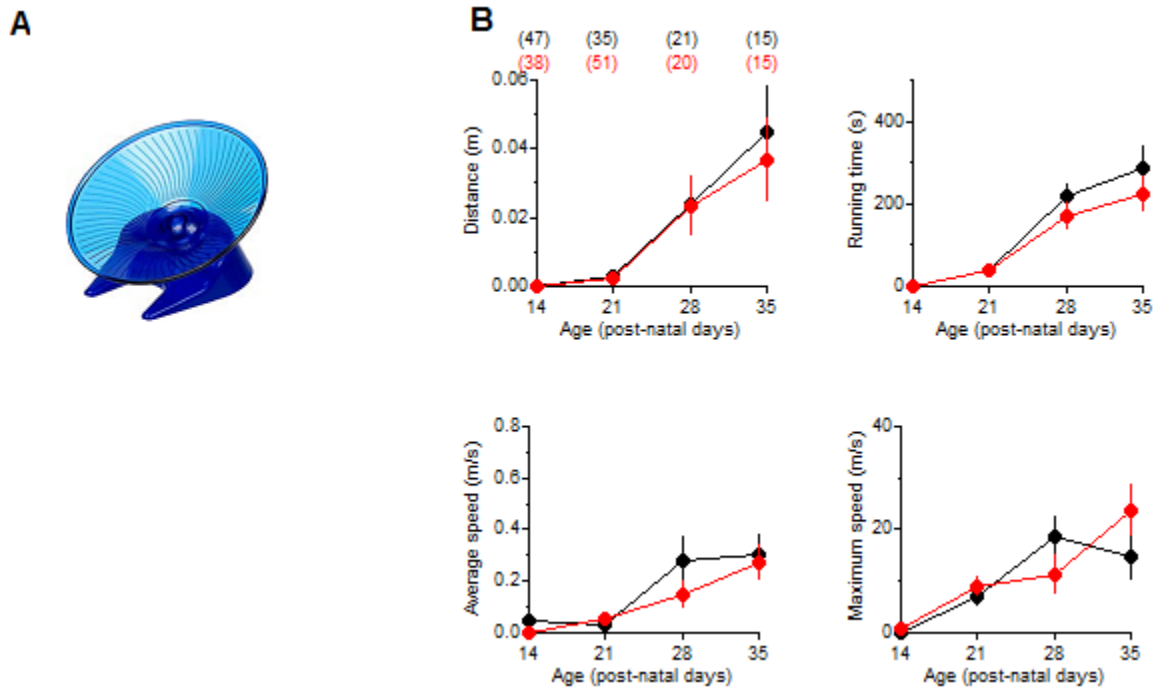


Figure 1. EAAC1^{-/-} mice show similar activity in the FS test

Figure 1A. Picture of the flying saucer with which mice were tested.

Figure 1B. Distance, time spent running, average speed, and maximum speed increase with age, but no difference is observed between WT and EAAC1^{-/-} mice. The numbers in parenthesis represents the number of animals tested for each age.

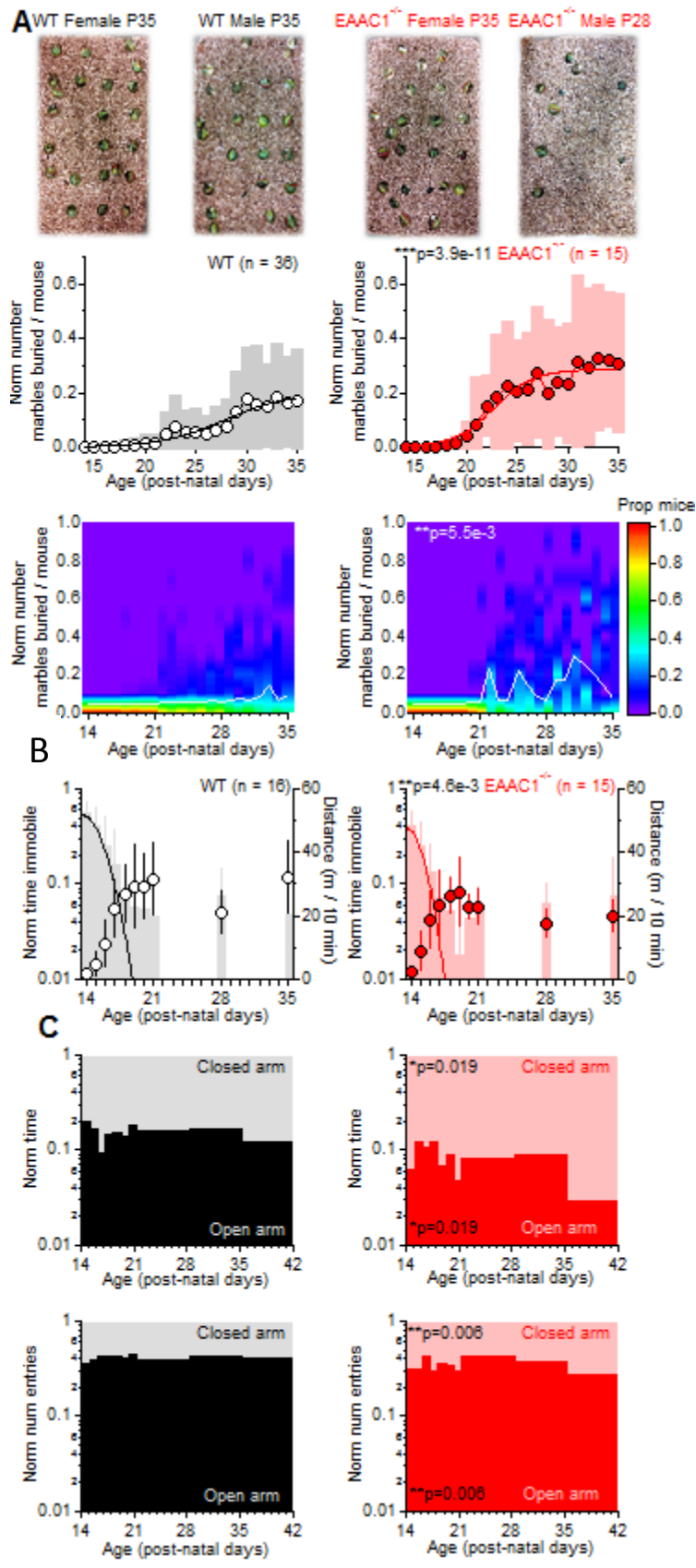


Figure 2. EAAC1^{-/-} mice of either sex show increased digging behaviors in the MB test.

Figure 2A. *Top*, in the marble burial test, EAAC1^{-/-} mice across ages P14-P35 consistently buried more marbles. In both WT (Gray) and EAAC1^{-/-} (Red), mice buried more marbles with increasing age. *Bottom*, the proportion of mice that buried a higher number of marbles (warmer color) was greater in EAAC1^{-/-} mice than WT mice.

Figure 2B. In the open field test, WT and EAAC1^{-/-} mice traveled the same distance across ages P14-P35. Compared to WT mice, EAAC1^{-/-} mice spent less time immobile.

Figure 2C. In the elevated plus maze test, EAAC1^{-/-} mice entered the open arm a lower number of times and spent less time on the open arm compared to WT mice.

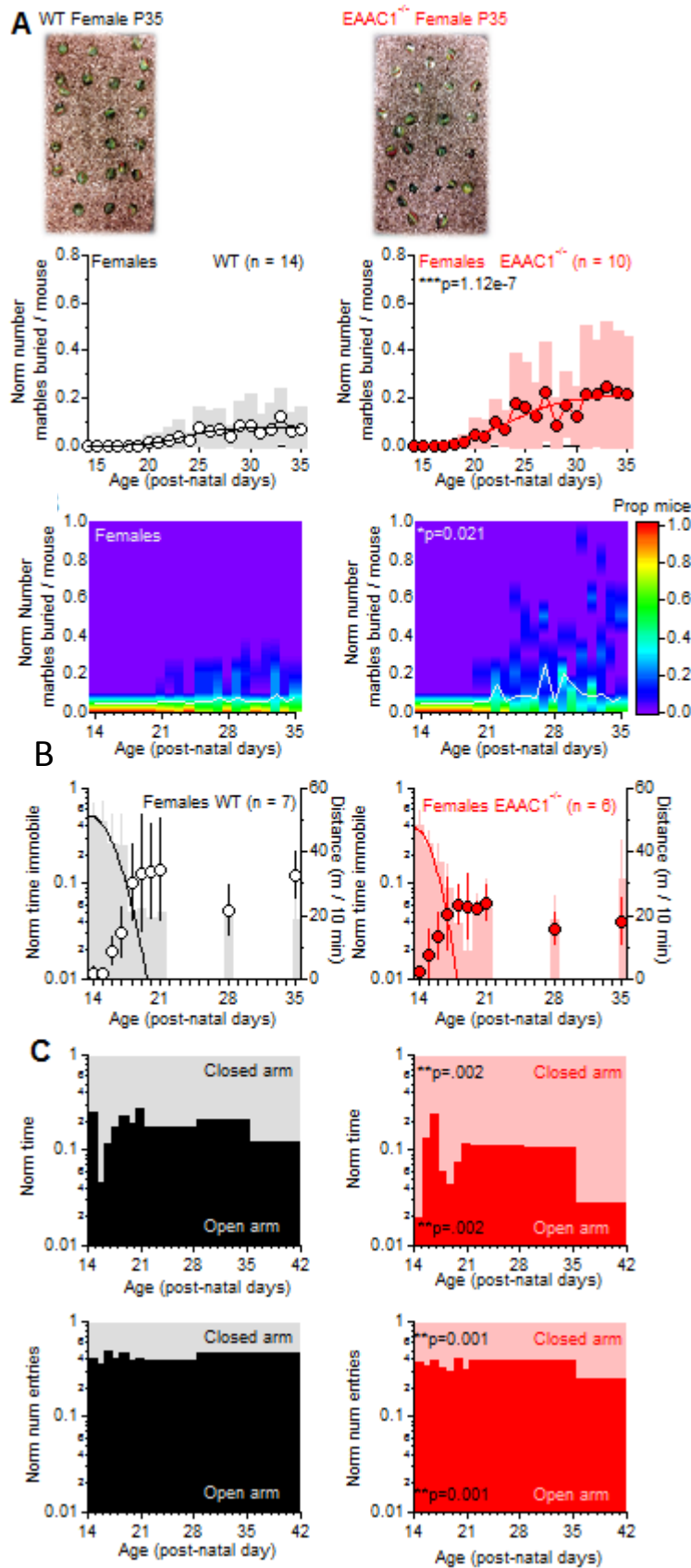


Figure 3. Results averaged from females demonstrate increased anxiety and motor behavior in EAAC1^{-/-} mice.

Figure 3A. *Top*, in the marble burial test, EAAC1^{-/-} mice across ages P14-P35 consistently buried more marbles. In both WT (Gray) and EAAC1^{-/-} (Red), mice buried more marbles with increasing age. *Bottom*, the proportion of mice that buried a higher number of marbles (warmer color) was greater in EAAC1^{-/-} mice than WT mice.

Figure 3B. In the open field test, WT and EAAC1^{-/-} mice traveled the same distance across ages P14-P35. Compared to WT mice, EAAC1^{-/-} mice spent less time immobile.

Figure 3C. In the elevated plus maze test, EAAC1^{-/-} mice entered the open arm a lower number of times and spent less time on the open arm compared to WT mice.

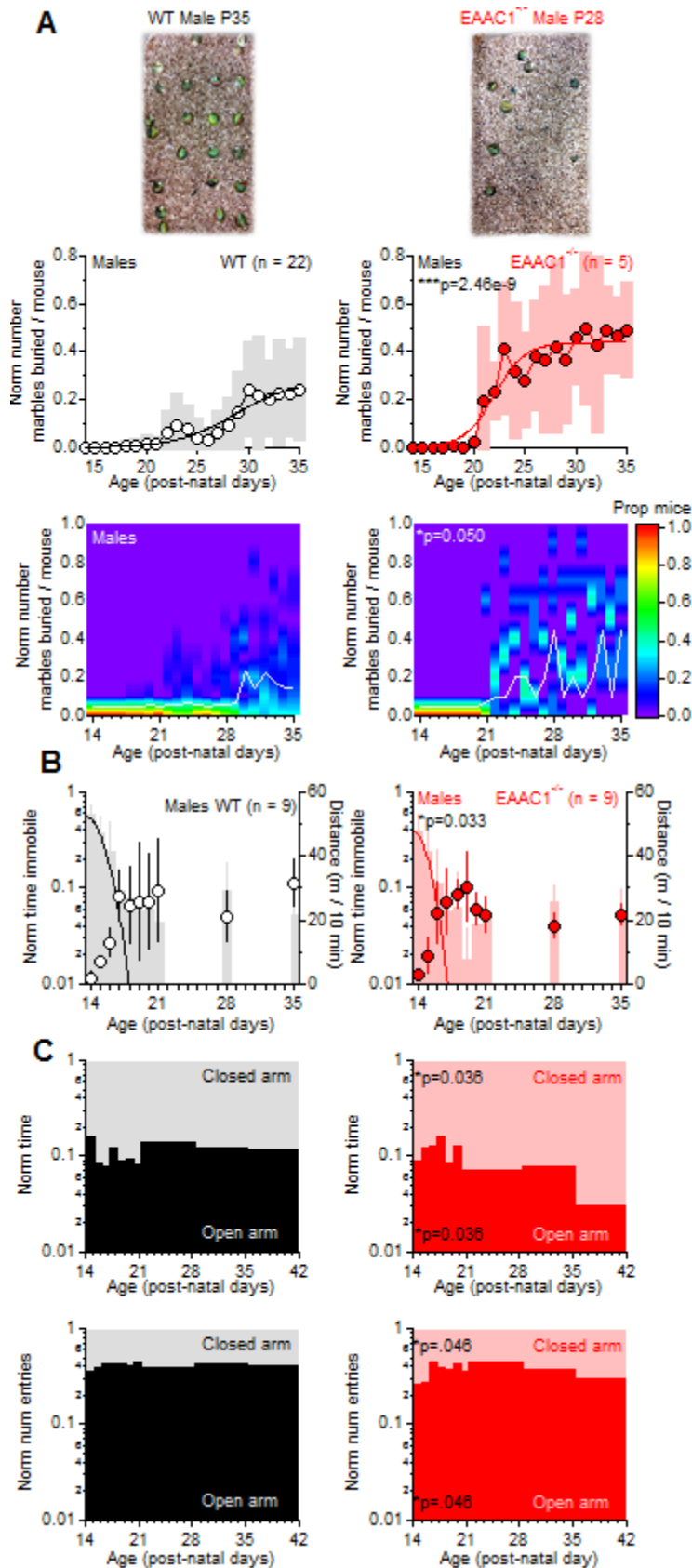


Figure 4. Results averaged from males demonstrate increased anxiety and motor behavior in EAAC1^{-/-} mice.

Figure 4A. *Top*, in the marble burial test, EAAC1^{-/-} mice across ages P14-P35 consistently buried more marbles. In both WT (Gray) and EAAC1^{-/-} (Red), mice buried more marbles with increasing age. *Bottom*, the proportion of mice that buried a higher number of marbles (warmer color) was greater in EAAC1^{-/-} mice than WT mice.

Figure 4B. In the open field test, WT and EAAC1^{-/-} mice traveled the same distance across ages P14-P35. Compared to WT mice, EAAC1^{-/-} mice spent less time immobile.

Figure 4C. In the elevated plus maze test, EAAC1^{-/-} mice entered the open arm a lower number of times and spent less time on the open arm compared to WT mice.

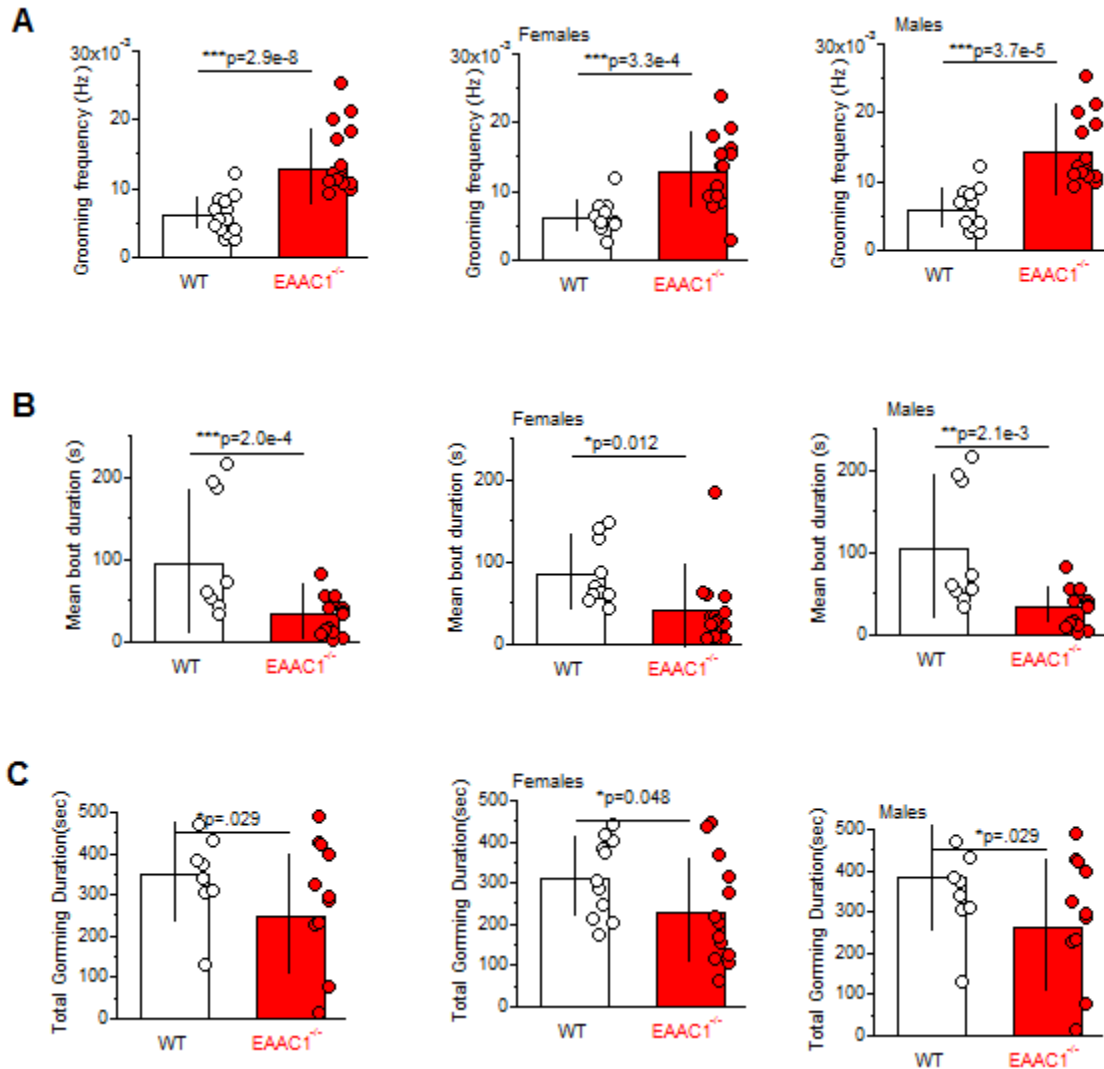


Figure 5. Results averaged from females demonstrate increased anxiety and motor behavior in EAAC1^{-/-} mice.

Figure 5A. In the marble burial test, EAAC1^{-/-} mice across ages P14-P35 consistently buried more marbles. In both WT (Gray) and EAAC1^{-/-} (Red), mice buried more marbles with increasing age. Additionally, the proportion of mice that buried a higher number of marbles (warmer color) was greater in EAAC1^{-/-} mice than WT mice.

Figure 5B. In the open field test, WT and EAAC1^{-/-} mice traveled the same distance across ages P14-P35. Compared to WT mice, EAAC1^{-/-} mice spent less time immobile.

Figure 5C. In the elevated plus maze test, EAAC1^{-/-} mice entered the open arm a lower number of times and spent less time on the open arm compared to WT mice.

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