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Targeted Mutations in the Na,K-ATPase Alpha 2 Isoform Confer Ouabain Resistance and Result in Abnormal Behavior in Mice

Tori L. Schaefer[1], **Jerry B Lingrel**[2], **Amy E. Moseley**[2], **Charles V. Vorhees**[1,*], and **Michael T. Williams**[1,*]

[1]Division of Neurology, Cincinnati Children's Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229-3039

[2]Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Abstract

Sodium and potassium-activated adenosine triphosphatases (Na,K-ATPase) are ubiquitous, participate in osmotic balance and membrane potential, and are composed of α, β, and γ subunits. The α subunit is required for the catalytic and transport properties of the enzyme and contains binding sites for cations, ATP, and digitalis-like compounds including ouabain. There are four known α isoforms; three that are expressed in the CNS in a regional and cell-specific manner. The α2 isoform is most commonly found in astrocytes, pyramidal cells of the hippocampus in adults, and developmentally in several other neuronal types. Ouabain-like compounds are thought to be produced endogenously in mammals, bind the Na,K-ATPase, and function as a stress-related hormone, however, the impact of the Na,K-ATPase ouabain binding site on neurobehavioral function is largely unknown. To determine if the ouabain binding site of the α2 isoform plays a physiological role in CNS function, we examined knock-in mice in which the normally ouabain-sensitive α2 isoform was made resistant ($\alpha2^{R/R}$) while still retaining basal Na,K-ATPase enzymatic function. Egocentric learning (Cincinnati water maze) was impaired in adult $\alpha2^{R/R}$ mice compared to wild type (WT) mice. They also exhibited decreased locomotor activity in a novel environment and increased responsiveness to a challenge with an indirect sympathomimetic agonist (methamphetamine) relative to WT mice. The $\alpha2^{R/R}$ mice also demonstrated a blunted acoustic startle reflex and a failure to habituate to repeated acoustic stimuli. The $\alpha2^{R/R}$ mice showed no evidence of altered anxiety (elevated zero maze) nor were they impaired in spatial learning or memory in the Morris water maze and neither group could learn in a large Morris maze. These results suggest that the ouabain binding site is involved in specific types of learning and the modulation of dopamine-mediated locomotor behavior.

Keywords

Na-K ATPase; ouabain; acoustic startle response; egocentric learning; dopamine; methamphetamine-induced locomotor activity

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*Correspondence to: Charles V. Vorhees, Division of Neurology (MLC 7044), Cincinnati Children's Research Foundation, 3333 Burnet Ave., Cincinnati, OH 45,229-3039, USA. charles.vorhees@cchmc.org or Michael T. Williams, Ph.D., Division of Neurology (MLC 7044), Cincinnati Children's Research Foundation, 3333 Burnet Ave., Cincinnati, OH 45229-3039, USA. michael.williams@cchmc.org.
Present address of Amy E. Moseley: Monsanto Company, 800 N Lindbergh Blvd, St Louis, MO 63167, USA.

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INTRODUCTION

Sodium and potassium-activated adenosine triphosphatases (Na^+, K^+-ATPases) are transmembrane proteins found in all mammalian cells that contribute to the membrane potential by pumping potassium in and sodium out of the cell (see Blanco and Mercer, 1998; Scheiner-Bobis, 2002). Mutations in the Na,K-ATPase genes have been implicated in psychiatric disorders (Goldstein et al., 2006, 2009) and mutations in the human α2 isoform are associated with familial hemiplegic migraine and sporadic hemiplegic migraine (De Fusco et al., 2003; de Vries et al., 2007; Estevez and Gardner, 2004) whereas mutations in the α3 isoform have been linked to Rapid-Onset Dystonia Parkinsonism (de Carvalho et al., 2004). In addition, altered Na,K-ATPase activity or reductions in protein can have effects on neuronal function and have been shown to disrupt neurotransmitter release and alter behavior in rodents (Erecinska and Silver, 1994; Ikeda et al., 2003; Moseley et al., 2007; Vaillend et al., 2002; Vatta et al., 2004).

The holoenzyme of the Na,K-ATPase is comprised of three subunits: α, β, and FXYD (γ is included in this group). The α subunit is required for catalytic and transport properties and contains the binding sites for cations, ATP, and of particular interest contains the cardiotonic steroid-binding site of the Na,K-ATPase which is often referred to as the ouabain binding site (Lingrel and Kuntzweiler, 1994; Pressley, 1996). The β subunit modulates K^+ and Na^+ affinity and acts as a chaperone to stabilize folding and deliver the enzyme to the plasma membrane (Chow and Forte, 1995; McDonough et al., 1990) while the FXYD subunit has a regulatory role in Na,K-ATPase function (Beguin et al., 1997).

Isoforms of the α subunits are encoded by four different genes (α1, α2, α3, and α4) (Shamraj and Lingrel, 1994; Shull et al., 1986). Each α isoform has a unique tissue distribution and sensitivity to cardiotonic steroids, including ouabain, that modulate the function of Na,K-ATPases when bound to the Na,K-ATPase ouabain binding site (Jewell and Lingrel, 1991; Segall et al., 2001). The α4 isoform is only found in the testis, while the remaining isoforms are found in brain and other tissues (Orlowski and Lingrel, 1988; Woo et al., 1999). In the brain, the α1 subunit is expressed ubiquitously and is relatively insensitive, having a low affinity for ouabain in rodents. In the brain, the α2 subunit is predominately expressed in astrocytes (Watts et al., 1991), pyramidal cells of the hippocampus in adults (McGrail et al., 1991), and in other neurons during early development (Moseley et al., 2003). The α2 subunit is sensitive to ouabain and binds with high affinity. The α3 subunit is found only in neurons in the brain and is also sensitive to ouabain and also binds with high affinity (McGrail et al., 1991).

It has been established that exogenous cardiotonic steroids, such as ouabain, bind to the α subinit of the Na,K-ATPase and inhibit ion transport (see Buckalew, 2005; Schoner, 2002), however it has recently been discovered that there are endogenous compounds, similar in composition to ouabain, that are found in the periphery as well as in the brain (Schoner, 2000). These endogenous ouabain-like compounds are thought to act as steroid hormones, to be released during stress, and can affect proliferation, differentiation, and migration of epithelial cells (Contreras et al., 2006). Taken together with the fact that the ouabain/cardiotonic steroid binding site of the α2 and α3 isoforms of the Na,K-ATPase are highly conserved throughout the animal kingdom, it is reasonable to anticipate that this site plays a physiological role and that an endogenous ligand occurs which interacts with this site and may influence CNS function and ultimately behavior. Our previous studies demonstrate that this site is required for ACTH-induced hypertension and alters exercise ability in mice (Lorenz et al., 2008; Radzyukevich et al., 2009). Others have demonstrated that ouabain-Na,K-ATPase binding at low concentrations activates several signal transduction cascades without inhibiting the enzyme sufficiently to observe any changes in intracellular Na^+ or K^+

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levels (Nesher et al., 2007). Ouabain has also been shown to up- and down-regulate the expression of multiple genes (Huang et al., 2004; Martin et al., 2004; McGowan et al., 1999). To better understand the importance of the Na,K-ATPase-ouabain binding site, we used knock-in mice previously described (Dostanic et al., 2003) in which the α2 subunit was genetically modified to be ouabain-resistant ($\alpha2^{R/R}$) while still retaining basal Na,K-ATPase enzymatic function and compared them to wild type α2 ouabain-sensitive mice ($\alpha2^{S/S}$).

We used the elevated zero maze, automated locomotor activity, and marble burying to assess amygdale-associated anxiety-related behavior. It was previously shown that the α2 subunit is present in the amygdala and piriform cortex during development, and $\alpha2^{\pm}$ mice show increased neuronal activity in these brain regions with increased anxiety-related behaviors as adults (Ikeda et al., 2003; Moseley et al., 2007). Because the α2 subunit is found in pyramidal cells of the hippocampus in adults (McGrail et al., 1991), we also assessed hippocampally-dependent spatial learning in the Morris water maze and compared it to route-based egocentric learning in the Cincinnati water maze. Egocentric learning is associated with the pre- and postsubiculum, entorhinal cortex, striatum, and other regions (Fuhs and Touretzky, 2006; McNaughton et al., 2006; Rondi-Reig et al., 2006; Sargolini et al., 2006; Whishaw et al., 1997; Witter and Moser, 2006). Dopamine-modulated behaviors (locomotor activity levels before and after stimulation with the dopaminergic agonist, methamphetamine, and acoustic startle with prepulse inhibition and habituation) were examined because ouabain has been shown to release dopamine (Boireau et al., 1998), and dopamine can regulate the activity of Na,K-ATPase in an organ-specific manner. Motor coordination was evaluated in a narrow bridges task since the α2 subunit is expressed in muscle (Sweadner, 1989).

MATERIALS AND METHODS

Animals

Male mice (60- to100-days old) on a mixed Swiss Black/129/Sv background containing a homozygous cardiac glycoside-resistant α2 Na,K-ATPase isozyme ($\alpha2^{R/R}$) and wild type (WT; $\alpha2^{S/S}$) littermates were developed as previously described (Dostanic et al., 2003) and transferred from the University of Cincinnati College of Medicine to Cincinnati Children's Research Foundation (CCRF) after weaning. All testing was done at CCRF and all procedures were approved by the CCRF Institutional Animal Care and Use Committee. Prior to testing, mice were acclimated to the CCRF vivarium (maximum of four animals per cage) for at least 1 week after removal from 30 to 40 days of quarantine and maintained on a 14/10 h light/dark cycle. Behavior was assessed during the light cycle and food and water were available ad libitum. Mice naïve to behavioral testing were used for each experiment and testing occurred in the following order.

Experiment 1—

$$N=21\ \text{WT}, 19\alpha2^{R/R.}$$

In Experiment 1, we characterized the behavioral phenotype of these mice by assessing anxiety-related behavior, motor control, sensorimotor gating, cognition, and drug-induced locomotor activity.

Elevated zero maze (EZM): On the first day of testing, anxiety-related behavior was assessed in the elevated zero maze as described previously (Shepherd et al., 1994) with minor modification (Moseley et al., 2007; Williams et al., 2003). Sessions were video recorded and later scored with ODLog (Macropod Software, Armidale, Australia). The EZM

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is a circular runway 105 cm in diameter with a 10 cm path width made of black Kydex and divided into four equal quadrants. Two opposite quadrants have black acrylic sidewalls (28 cm high; “closed quadrants”) and the remaining quadrants have no sides except for a 1.3 cm high clear acrylic curb to prevent animals from slipping off the edge. The runway is mounted 72 cm above the floor. Mice were placed in the center of one of the closed quadrants and behavior was recorded for 5 min with an overhead camera connected to a digital video recorder. The maze was dimly illuminated by a single halogen lamp; between animals the maze was cleaned with 70% ethanol. Dependent measures were: head dips, time in the open, and stretch-attends. Time in the open was defined as when an animal had both front paws and shoulders past the boundary between the open and closed quadrants extending into the open area.

Spontaneous locomotor activity: At least 1 h following completion of the elevated zero maze, locomotor activity was assessed for 1 h in chambers equipped with infrared sensors (41×41 cm^2; Accuscan Instruments, Columbus, OH) as previously described (Moseley et al., 2007). Total horizontal distance and peripheral and center distance (distance traveled in the designated region) were analyzed in 5-min intervals. Peripheral activity was movement within 10 cm of the walls and central activity was movement in the center 20×20 cm^2 zone of the arena.

Marble burying: Immediately after locomotor activity mice were moved to an adjacent room and tested in a defensive marble burying task as modified previously (Williams et al., 2007). Fifteen marbles (1.5 cm in diameter) were arranged in five rows of three using a template that spaced the marbles 4.5 cm apart, 4.5 cm from the long edge, and 3.5 cm from the short edge of a 16×27 cm^2 mouse cage containing wood chip bedding 5 cm deep. Animals remained in these cages covered with a filter-top lid for 30 min. Latency to begin bedding disruption (digging or burying) and the number of marbles visible at the end of 30 min were recorded. New cages and bedding were used for each animal and marbles were cleaned with a 70% ethanol solution between animals.

Acoustic startle response/prepulse inhibition (ASR-PPI): ASR-PPI is a test of startle reactivity and sensorimotor gating and was assessed 1–7 days following the previous test (Brunskill et al., 2005). Each mouse was placed in a sound-attenuating test chamber (San Diego Instruments, San Diego, CA) inside an inner cylindrical acrylic holder with sliding doors at each end. The inner holder had a piezoelectric force transducer mounted beneath it that was sensitive to the animal’s movements. Mice were placed in the acrylic holder for a 5-min acclimation period followed by a 4×4 Latin square sequence of trials of four types and repeated three times for a total of 48 trials: no stimulus, startle stimulus (SS) with no prepulse, 74 dB prepulse 1 SS, or 76 dB prepulse 1 SS. The intertrial interval (ITI) was 8 s. The interstimulus interval on prepulse trials was 70 ms from prepulse onset to startle stimulus onset. The startle signal consisted of a mixed frequency white noise burst of 120 dB SPL for 20 ms. The responses of each animal were recorded for 100 ms after startle stimulus onset and the responses recorded were peak amplitude (V_{max}), average response amplitude (measured in arbitrary units of mV of change), and latency to peak amplitude.

Morris water maze (MWM): Spatial learning and memory were assessed in the MWM using procedures described previously (Vorhees and Williams, 2006). Testing began 1–4 days following ASR/PPI and was performed in a 122 cm circular tank (Moseley et al., 2007). Animals were first tested in cued learning (submerged platform with cue protruding above the surface) that consisted of six trials on Day 1 in which the start position (west) and platform (east) were in fixed positions to teach the basic task characteristics (i.e., swimming, moving away from the perimeter, and climbing and remaining on the escape platform). On the next 5 days, two trials per day were given in which both the platform and start positions

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were moved randomly. On all cued trials, curtains were drawn around the maze to reduce distal cues, and the 10 cm diameter platform was submerged 1 cm below water with an orange ball positioned 7 cm above the surface of the water on a metal pole to mark its location. Latency to reach the cued platform was recorded with a time limit of 1 min trial^{-1}. Following cued learning, animals were tested in three phases of the hidden platform (submerged platform with no cue) version of the MWM. The acquisition (Phase 1, southwest quadrant position) and reversal phases (Phase 2, northeast quadrant position) were performed as previously described (Moseley et al., 2007) and consisted of four trials per day for 6 days followed by a 30-s probe trial on Day 7. The shift phase (Phase 3) required the animals to learn a third platform position located in the northwest quadrant of the maze. Each phase used a different sized platform (i.e., 10, 7, and 5 cm in diameter). During the hidden platform trials, video tracking software was used to record performance (Smart® software, SDI, San Diego, CA). On hidden platform learning trials (Days 1–6 of each phase), latency, cumulative distance, path length, and speed were recorded. During probe trials (removal of hidden platform), platform site crossings (crossovers), speed, average distance to the platform site, percent distance and time in the target quadrant, and mean search distance (MSD) were assessed. MSD was defined as follows, where target quadrant = $q1$, hence MSD = $\Sigma[(q1 - q2) + (q1 - q3) + (q1 - q4)] \div 3$ (Brown et al., 2000).

Startle habituation: Startle habituation was performed 1–3 days following MWM testing. The same apparatus described above for ASR/PPI was used. Each test session began with a 5-min acclimation period with no signal presented. At the end of the acclimation period, animals received 50 identical trials of startle stimulus with an 8-s intertrial interval. Ten blocks of five trials per block were analyzed for change in peak amplitude (V_{max}) across blocks measured in mV relative to a subtracted baseline of nonstartle-related movements within the chamber. The apparatus was cleaned with a 70% ethanol solution between animals.

Locomotor activity with methamphetamine challenge: Locomotor activity was reassessed 6–10 days following acoustic startle response habituation. The animals were placed in the locomotor chambers described above for 30 min to rehabituate them to the apparatus with no drug. They were then briefly removed and administered a subcutaneous injection of 1 mg kg^{-1} (+)-methamphetamine [(MA) HCl, calculated as the freebase, NIDA], and returned to the chambers for an additional 120 min.

Experiment 2—

$$N=11\ \mathrm{WT};10\alpha 2^{\mathrm{R/R.}}$$

In Experiment 2, we increased the difficulty of the Morris water maze and further explored the motor control and circadian rhythm of the $\alpha 2^{R/R}$ and WT mice.

Large Morris water maze: A larger MWM tank (210 cm diameter) was used to determine if increased search area would differentiate genotypes more clearly. The same cued learning and acquisition phase procedures were used in the large maze as described above with the addition of 4 more days of hidden platform testing. Probe trial learning was not assessed in this experiment. For this test, latency was recorded because we were unable to track mice in this apparatus. Following completion of cued and Phase 1 in the large MWM, mice were re-assessed in the smaller MWM for an additional 5 days because the data from the large tank revealed the mice were showing little improvement across days and were not approaching levels of performance of mice in the smaller maze.

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Narrow bridges: Narrow bridges began 1–5 days following completion of the MWM retest. Square wood beams (1 m in length) with cross sections of 25, 12, and 5 mm^2 and round wood dowels 28, 17, and 11 mm diameters were used. Beams were placed horizontally, 50 cm above the bench surface. One end was mounted to a narrow support and the other attached to an enclosed 20 cm^2 box. The starting point of the beam was illuminated with a 65 W floodlight and two ceiling lights. There were two phases: training and test. For training, the mice were trained to traverse the 12 mm^2 beam for three consecutive days with four trials per day. A 2-min maximum time limit was imposed for the first trial and a 1-min maximum for the remaining trials. The test phase occurred on Day 4 and each mouse received two consecutive trials (1 min limit/trial) on each of the square and round beams progressing from widest to narrowest. Latency to traverse each beam and the number of foot slips were recorded during the test phase.

Multiple-day activity testing: To test whether circadian rhythms were disrupted in the $\alpha 2^{R/R}$ mice we examined locomotor activity continuously for 3 days. The previously described locomotor activity chambers were fitted with water bottles, food containers, and bedding that did not interfere with movement detectors. Mice were allowed to habituate to the chamber and room conditions for 24 h before activity levels were recorded for an additional 72 h. Data were organized into 30-min intervals for a total of 144 intervals. The room was maintained on the same light/dark cycle as the housing room and mice were disturbed once per day to check food and water. Total distance was recorded.

Experiment 3—

$$N=31\ \mathrm{WT};30\alpha 2^{\mathrm{R/R}.}$$

In Experiment 3, we tested the learning ability of the $\alpha 2^{R/R}$ and WT mice in a route based, egocentric learning task to further assess a wider range of cognitive ability.

Cincinnati water maze: Prior to Cincinnati water maze (CWM) testing, animals underwent cued learning in the smaller MWM as described above to familiarize them to swimming and show them that escape was possible by climbing onto the platform. The CWM is a test of egocentric rather than allocentric learning (MWM) because distal cues are eliminated by testing animals in darkness with only infrared light so that the experimenter could see the animal on a closed-circuit monitor in an adjacent room. Mice were tested for 15 days. The maze was scaled for mice [~[1/4] the size of the maze for rats (Vorhees, 1987)] and is a 9-unit multiple-T maze with cul-de-sacs that branch from a central channel extending from the starting point to the goal where an escape ladder is located. The arms of the Ts and the channels are 8 cm wide and the walls are 25 cm high. The maze was filled with water to a depth of 12.5 cm and maintained at room temperature (21°C ± 1°C). Infrared light was provided by an infrared light emitter mounted above the maze to enhance image quality of the CCD camera. On each trial, an animal was placed in the maze at the start and allowed 5 min to find the goal. Two trials per day were given with a minimum 15-min intertrial interval. Animals not finding the goal within 5 min were removed without being shown how to find the goal. Errors and latency to escape were recorded by an observer while viewing the maze on a video monitor located in an adjacent room. An error was defined as a head and shoulder entry into one of the arms of a T. On early trials, many animals failed to find the escape within the time limit but succeeded after repeated days of testing. A few animals took longer to learn the path and sometimes these animals stopped searching and remained in one T for extended intervals. In order to correct for search failures, these animals were given a score equal to that of the animal making the most errors within the time limit +1.

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Statistical analysis: Data were analyzed using mixed linear ANOVA models (SAS Proc Mixed, SAS Institute, Cary, NC). The covariance matrix for each data set was checked using best fit statistics. In most cases the best fit was to the autoregressive-1 [AR(1)] covariance structure. Degrees of freedom were calculated using the Kenward-Roger method and do not match those obtained from general linear model ANOVAs and can be fractional. Measures taken repetitively on the same animal, such as trial, day, or interval, were repeated measure factors. Significant interactions were analyzed using slice ANOVAs at each level of the repeated measure factor. Genotype main effects (Gene) and interaction F-ratios are shown for clarity. Where two groups were compared with no repeated measure, *t* tests were used. Significance was set at $P \leq 0.05$.

RESULTS

Experiment 1

Elevated zero maze—There were no significant findings in the elevated zero maze for head dips, stretch attends, or time spent in the open (Table I).

Spontaneous locomotor activity—For total distance, there was only a gene × time interaction ($F(11,418) = 1.95$, $P < 0.03$). Slice effect tests showed that the $\alpha 2^{R/R}$ mice traveled less distance than WT controls from 0 to 20 min (Fig. 1A). Peripheral distance also only showed a gene × time interaction ($F(11,418) = 2.07$, $P < 0.02$). Slice effect tests showed that from 0 to 15 min the $\alpha 2^{R/R}$ mice traveled less in the periphery (Fig. 1B) than WT mice. No main effect of Gene or interaction was observed for center distance (Fig. 1C).

Marble burying—No significant effects were observed for latency to bedding disruption or the number of visible marbles after 30 min (Table I).

Acoustic startle response/prepulse inhibition—There was no significant gene main effect on startle amplitude ($F(1,35) = 2.93$, $P < 0.10$) nor any interaction of gene × prepulse (PP) ($F(2,70) = 2.08$, $P < 0.10$) ($\alpha 2^{R/R}$ *V*max: PP-0 = 312.2 ± 69.6; PP-74 = 73.9 ± 15.6; PP76 = 49.1 ± 11.6; WT: PP-0 = 493.3 ± 87.6; PP-74 = 127.6 ± 32.1; PP-76 = 64.4 ± 14.8 mV). There was a significant effect of Prepulse ($P < 0.0001$). Regardless of genotype, with lower dB prepulses there was an increased startle response (data not shown), which is the expected response of normal animals. There were no effects on average response amplitude or latency to peak response.

MWM-cued—The purpose of the cued phase is to ensure that mice swim normally, are not visually impaired, and are motivated to escape from the water. On Day 1 of the cued phase neither Gene nor Trial latency were affected but there was a gene × trail interaction ($F(4,108) = 2.54$, $P < 0.04$). Slice effect tests demonstrated that $\alpha 2^{R/R}$ mice took longer to reach the platform than WT mice on the 6th trial of day 1 [Means ± SEM: $\alpha 2^{R/R}$ = 34.89 ± 5.08 s; wild type 23.05 ± 4.85 s]. On Days 2–6, there was no significant main effect of gene or gene × trial interaction.

MWM-acquisition—The MWM is an established test of allocentric learning and reference memory (Morris et al., 1982, 1986). During acquisition there were no significant effects of gene or gene × day for latency (Fig. 2A), path length, or cumulative distance. For swim speed there was an effect of gene ($F(1,37) = 4.85$, $P < 0.03$), Day ($P < 0.0003$) and a gene × day interaction ($F(5,139) = 2.45$, $P < 0.04$). Slice effect tests demonstrated that on Days 3, 5, and 6, the $\alpha 2^{R/R}$ mice swam slower than WT mice.

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During the probe trial (platform was removed to assess memory), there was a significant effect of gene for percent distance in the target quadrant ($t(37) = 2.54$, $P < 0.02$), percent time in the target quadrant ($t(37) = 2.11$, $P < 0.04$), and MSD ($t(37) = 2.12$, $P < 0.04$). The $\alpha2^{R/R}$ mice had reduced percent distance ($\alpha2^{R/R}$ = 32.7% ± 3.1%; WT = 43.7% ± 2.9%) and percent time in the target quadrant ($\alpha2^{R/R}$ = 33.7% ± 3.3%; WT = 43.3% ± 3.1%), as well as lower MSD scores ($\alpha2^{R/R}$ = 3.5 ± 1.3; WT = 7.4 ± 1.2) than WT mice. There was no significant effect on crossovers, average distance to the platform site, or swim speed.

MWM-reversal—When the hidden platform was moved to the opposite quadrant, there were no significant effects of gene or gene × day for latency (Fig. 2B), path length, or cumulative distance.

During the probe trial, there were no significant effects on any measure of retention.

MWM-shift—During shift when the hidden platform was moved to the adjacent quadrant, there were no significant effects for latency (Fig. 2C) or path length. There was an interaction of gene × day for cumulative distance ($F(5,140) = 2.56$, $P < 0.03$). Slice effect tests did not demonstrate significant differences between $\alpha2^{R/R}$ and WT mice on any individual day.

During the probe trial, there were no significant effects on any measure.

Startle habituation—For maximum amplitude, there was a significant interaction of gene × block (five trials of startle stimuli per block: $F(9,342) = 2.97$, $P < 0.002$), but no main effect of gene. Slice effect tests showed that $\alpha2^{R/R}$ mice had lower startle amplitude on Blocks 1–4 compared to WT mice (Fig. 3). Slice ANOVA on blocks for each genotype showed that there was a block effect in the WT mice ($F(9,342) = 10.82$, $P < 0.0001$) indicative of habituation, but no block effect in the $\alpha2^{R/R}$ mice ($F(9,342) = 1.77$, $P < 0.08$), indicating that their response across days was flat.

Locomotor activity with methamphetamine challenge—There was no effect of gene or gene × interval in the prechallenge phase (rehabituation to the chamber). After MA, there was a main effect of gene ($F(1, 58.4) = 4.66$, $P < 0.04$) and interval ($P < 0.0001$) (Fig. 4). All groups showed MA-induced hyperactivity, however the $\alpha2^{R/R}$ mice traveled greater distance than WT in response to MA. There was no gene × interval interaction.

Experiment 2

Large MWM-cued—Neither gene nor trial were affected for latency on Day 1 or in separate analyses on Days 2–6 and there were no gene × day interactions (not shown).

Large MWM-acquisition—In the large tank there was an effect of Day ($P < 0.008$) for latency, but there was no effect of gene or gene × day interaction (Fig. 5A). Therefore, following testing in the large MWM, animals were tested in the original smaller tank for five additional days (Fig. 5B). There was no effect of gene or gene × day interaction, but there was an effect of Day ($P < 0.02$; not shown). Although the improvement across days was small, levels of performance in the smaller maze were dramatically better than in the large maze and approached those seen in the same maze in Experiment 1.

Multiple-day locomotor activity—Data were analyzed in 144, 30-min intervals over 3 days. There was no effect of gene or interaction of gene with interval or day. There was a main effect of Day ($P < 0.0002$) (not shown), because groups were more active during the dark phase and activity decreased across days.

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Narrow bridges—For crossing latency there was no effect of gene or gene-related interactions (not shown). There was a main effect of Trial for the 25, 12, and 5 mm square bridges, and the 28, 17, and 11 mm dowels ($P < 0.02$ or beyond). No gene or gene-related interactions for foot slips was seen. For example, on trial two when crossing the 5 mm^2 bridge, the number of foot slips was 0.6 ± 0.31 for the $\alpha2^{R/R}$ mice and 1.92 ± 0.74 for the wild type mice (Means ± SEM).

Experiment 3

MWM-cued—During cued training, there was no significant gene, trial, or gene × trail interaction on Day 1 or Days 2–6 (not shown).

Cincinnati water maze—There was a significant interaction of gene × day for latency ($F(14,826) = 1.75$, $P < 0.04$) and errors ($F(14,826) = 1.99$, $P < 0.02$). Slice effect tests showed that $\alpha2^{R/R}$ mice took significantly longer to find the escape than WT mice on Days 7 and 12 (Fig. 6A). For errors, the $\alpha2^{R/R}$ mice committed more errors on Days 7, 8, 11, and 12 (Fig. 6B).

DISCUSSION

Prevention of endogenous ligand signaling through the α2 Na,K-ATPase ouabain binding site in the $\alpha2^{R/R}$ mice resulted in deficits in egocentric learning (Cincinnati water maze), reduced acoustic startle amplitude, diminished startle habituation, and exaggerated locomotion following challenge with the dopaminergic agonist, methamphetamine. Previously it was determined that the knock-in conferring ouabain resistance did not affect Na,K-ATPase α1, 2, or 3 protein distribution, α1 or α3 ouabain binding, normal heart function, or physiological hemodynamics (Dostanic et al., 2003). Anxiety-like behavior, PPI, spatial learning, and reference memory were not altered in the $\alpha2^{R/R}$ mice compared to wild type mice. Furthermore, $\alpha2^{R/R}$ mice did not demonstrate motor deficits during the cued phase of the MWM, the 72 h activity test, or the narrow bridge test, and displayed minimal hypoactivity when initially placed in a novel environment, indicating that the aforementioned effects are selective and not part of a generalized or global CNS deficit. Considering that ouabain binding was abolished in skeletal muscle (Dostanic et al., 2003) and there appear to be no overt neuromotor changes, suggests that significant learning differences in the $\alpha2^{R/R}$ mice are not attributable to altered performance factors but are more likely to be changes in learning per se. It is important to remember that the enzymatic abilities of the enzyme were not altered, but only its ability to bind ouabain and other similar modulators.

Altered dopamine (DA) signaling in the $\alpha2^{R/R}$ mice may contribute to some of the effects observed in the current study. For example, reduced startle reactivity was observed in this study including a lack of habituation in the $\alpha2^{R/R}$ mice, and it has been shown that DA receptor antagonists have similar effects (Stevenson and Gratton, 2004). DA receptor function may be altered since $\alpha2^{R/R}$ mice over-responded to the DA-releasing effects of methamphetamine compared to WT animals. DA is known to regulate locomotion, some aspects of learning and memory, and acoustic startle reactivity (El-Ghundi et al., 2007; Goldman-Rakic, 1998; Missale et al., 1998). Interestingly, egocentric learning (CWM) appears to rely on striatal function which is also the presumptive region affected in the altered response to methamphetamine. In other tissues, it is well established that DA alters ion pump activity of the Na,K-ATPase via receptor-mediated second messenger activation thereby modulating its removal or insertion into the plasma membrane (Bertorello and Aperia, 1990; Ridge et al., 2002). It has also been shown that the Na,K-ATPase regulates D1 and D2 receptor function by means of protein–protein interactions (Hazelwood et al., 2008).

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Taken together, these data suggest that the ouabain binding site of the Na,K-ATPase plays a role in modulating the reciprocal regulation of Na,K-ATPase and DA functioning.

In addition to altered DA signaling, the mechanism by which alteration in the α2 Na,K-ATPase ouabain binding site results in impaired egocentric route-based (CWM) learning in the $\alpha2^{R/R}$ mice may be from altered development or functioning of navigational circuitry not involved in spatial learning. Disruption of this circuitry may occur since the α2 isoform is present within neurons early in development (Moseley et al., 2003), or it may be a direct effect of altered function of pyramidal cells in the hippocampus since the α2 isoform is expressed in these cells in adulthood (McGrail et al., 1991) and the hippocampus has overlapping roles in both spatial (MWM) and route-based learning (CWM). In the route-based CWM test, the $\alpha2^{R/R}$ mice showed significant intermittent increases in latency on 2 days and errors on 4 days of testing and there was a trend for latency to be increased from Days 7 to 15 and errors from Days 6 to 15, indicating a significantly slower rate of acquiring an accurate memory of the maze. During these intervals, $\alpha2^{R/R}$ mice performance plateaued at a level higher than that of WT controls indicating incomplete representation of the maze configuration long after WT mice reached asymptotic performance. The CWM is a task that requires egocentric learning which relies on self-movement cues for the animal to determine its position within an environment (Etienne and Jeffery, 2004). This is one of the first experiments to show that mice will perform this task and successfully use route-based navigation to find an escape in the absence of distal cues (infrared lighting was used so that no visible cues could be seen). This task may be useful for assessing genetic manipulations in mice in which disruptions of egocentric substrates are suspected, such as pre- and postsubiculum head-direction cells, entorhinal cortex grid and border cells (Solstad et al., 2008), some hippocampal cell types (Fuhs and Touretzky, 2006; McNaughton et al., 2006; Rondi-Reig et al., 2006; Sargolini et al., 2006; Whishaw et al., 1997; Witter and Moser, 2006), and striatal subregions that together constitute the egocentric circuitry (Cook and Kesner, 1988).

Slight decreases in locomotor activity were observed during the first 20 min in the $\alpha2^{R/R}$ mice compared to WT mice when placed in the locomotor activity chambers on the first day of testing. This hypoactivity was no longer evident when rehabituated to the same chambers prior to the methamphetamine challenge. This effect is unlikely to represent a pervasive deficit since it was transient and was no longer present when the mice were retested later.

The MWM data show that $\alpha2^{R/R}$ mice have deficits in the probe trial 24 h after the last acquisition trial, an effect not seen on the probe trials at the end of reversal or shift phases, indicating that this retention deficit was small and was overcome with the additional experience that occurred during the second and third phases of training. The increased latency in the MWM cued phase on Day 1, Trial 6 and the intermittent decreases in swim speed during acquisition in the $\alpha2^{R/R}$ mice along with the absence of significant findings in the hidden phases further supports the notion that these alterations were minor and did not affect the learning performance of $\alpha2^{R/R}$ mice. Even though the MWM is known to be a hippocampally-dependent behavior and some regions of the hippocampus are important in egocentric learning (CWM), we did not see deficits in both mazes, demonstrating specificity of the CWM effect. We previously showed that a deficit in one maze is not predictive of effects in the other, implying that the pathways and cell types important in each type of learning are distinct.

To increase the difficulty of the MWM we tested the mice in a 210-cm diameter tank and found that this increase in size prevented learning in both genotypes indicating that a tank of this size is too difficult for mice to learn. Others have shown the size of the MWM tank can influence results and that this may be strain specific (Van et al., 2006).

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The importance of the α2 Na,K-ATPase isoform in behavior was previously shown in $\alpha2^{\pm}$ mice that have a 50% reduction of α2 Na,K-ATPase protein (Ikeda et al., 2003; Moseley et al., 2007). $\alpha2^{\pm}$ mice exhibited decreased elevated zero maze time in the open; hypoactivity; and increased latency in the MWM. No effects on locomotor activity following MA challenge or on MWM probe trials were seen (they were not tested in the CWM). The data suggest that $\alpha2^{R/R}$ and $\alpha2^{\pm}$ mice have distinct phenotypes with little overlap in function. This is not completely surprising given that the $\alpha2^{\pm}$ mice have only half the enzyme present, whereas the $\alpha2^{R/R}$ mice have the full complement of enzyme activity.

Taking into account the differences between the $\alpha2^{\pm}$ mice and $\alpha2^{R/R}$ mice we suggest that the differences seen here between WT and $\alpha2^{R/R}$ mice may not be the result of ion transport but rather the influence of absent ouabain or other endogenous ligand Na,K-ATPase-mediated second messenger activation, perhaps involving DA. There are many mechanisms that exert control over Na,K-ATPase expression and ion transport activity including hormones and catecholamines (DA), intracellular sodium, and the β and FXYD Na,K-ATPase subunits. Beyond these it has been suggested that the main physiological role of endogenous ouabain or similar endogenous ligands may not be regulation of Na,K-ATPase ion transport (Nesher et al., 2007), but instead to modulate other cellular functions. Interestingly, the binding of low levels of ouabain to Na,K-ATPase have been shown to activate multiple signal transduction cascades, including Src-kinase/MAP-kinase and PKC independent of pump inhibition or altered ion transport (Aydemir-Koksoy et al., 2001; Haas et al., 2000; Xie and Cai, 2003; Xie and Xie, 2005). The RAS-Raf-Erk1/2 cascade has also been shown to be activated via ouabain binding to Na,K-ATPase (Akimova et al., 2005). Recently, signal transduction cascades activated by ouabain binding to the α subunit of the Na,K-ATPase were shown to be mediated by a nonpump-related pool of Na,K-ATPases (Liang et al., 2007). Although we do not know if any signal transduction cascades are altered in the $\alpha2^{R/R}$ mice, the implication that the Na,K-ATPases are involved in a variety of human neuropathophysiological functions and the absence of the ouabain binding site results in aberrant behavior suggests that this site may influence some of the aforementioned conditions and warrants further investigation.

Abbreviations

CWM	Cincinnati water maze
DA	dopamine
MWM	Morris water maze
Na,K-ATPase	sodium and potassium-activated adenosine triphosphatases

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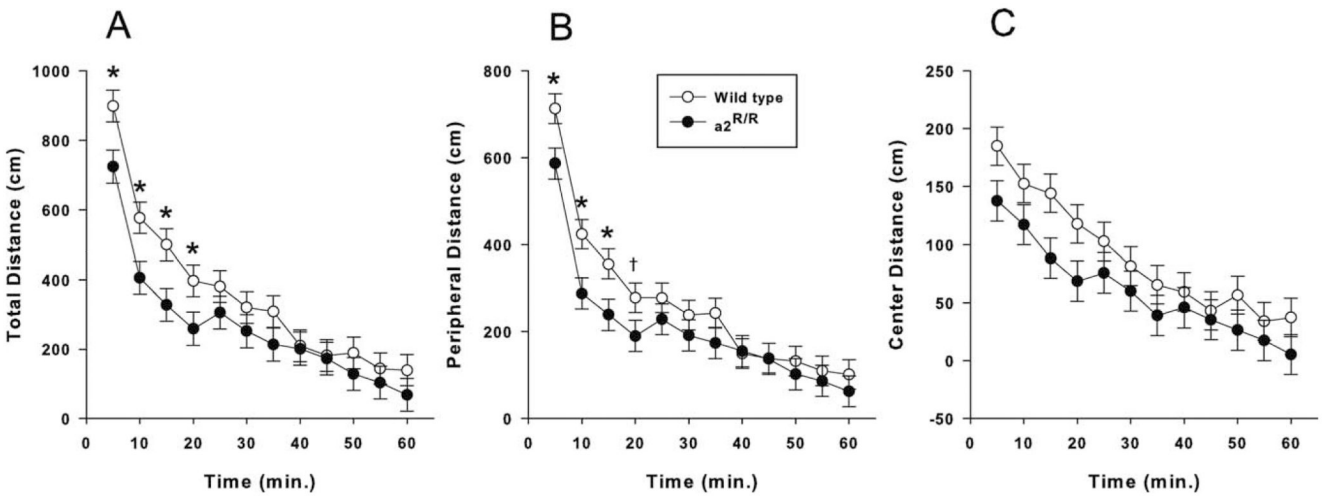

Fig. 1.

Spontaneous locomotor activity: (**A**) Total distance traveled (cm) was significantly decreased in the $\alpha 2^{R/R}$ mice vs. WT during the first 20 min. (**B**) Peripheral distance (cm) traveled was decreased in the $\alpha 2^{R/R}$ mice compared to WT during the first 15 min and there was a trend for decreased peripheral between 15 and 20 min. (**C**) Center distance (cm) was unaffected by genotype. n = 19–21 mice per genotype (males). (Mean ± SEM per 5-min interval): *$P < 0.05$; †$P < 0.10$.

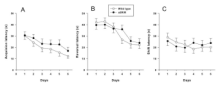

Fig. 2.

Morris Water maze: Latency (Mean ±SEM) across trials for each day; (**A**) Acquisition: latency was not affected by genotype. (**B**) Reversal: No differences between $\alpha 2^{R/R}$ and WT mice were observed. (**C**) Shift: No effect of genotype was observed. *n* = 19–21 male mice per genotype.

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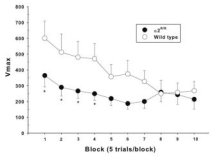

Fig. 3.

Acoustic startle habituation: $\alpha 2^{R/R}$ mice under-responded during the first four blocks compared to WT mice. There was no significant Block effect in the $\alpha 2^{R/R}$ mice; there was in WT mice. n = 19–21 male mice per genotype. (Mean ± SEM startle amplitude (mV) in blocks of five trials) *$P < 0.05$.

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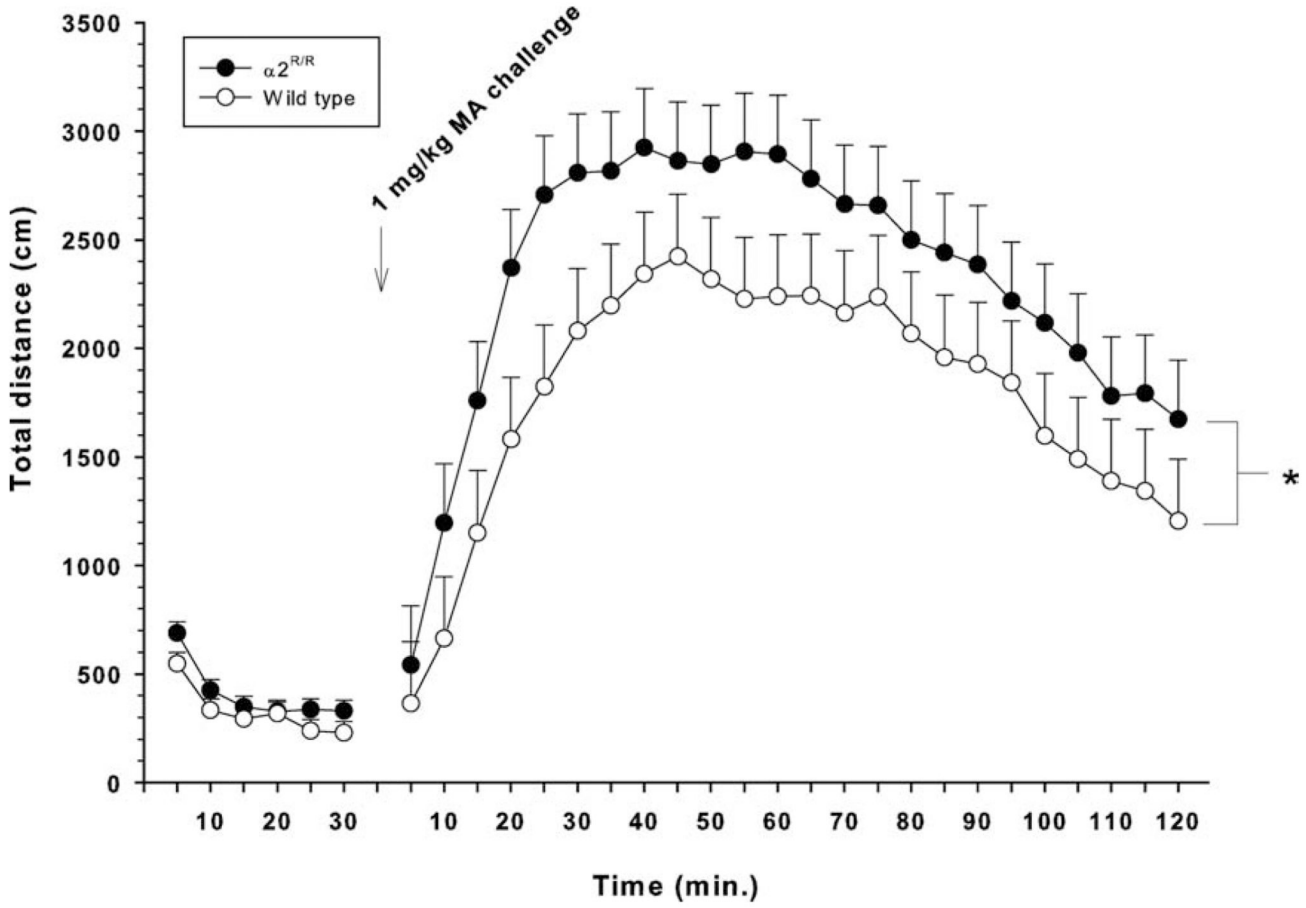

Fig. 4.

Locomotor activity with methamphetamine challenge: After 1 mg kg^{-1} MA challenge, $\alpha 2^{R/R}$ mice traveled further than WT mice. n = 19–21 male mice per genotype. (Mean ± SEM distance traveled (cm) before and after challenge) *$P < 0.05$.

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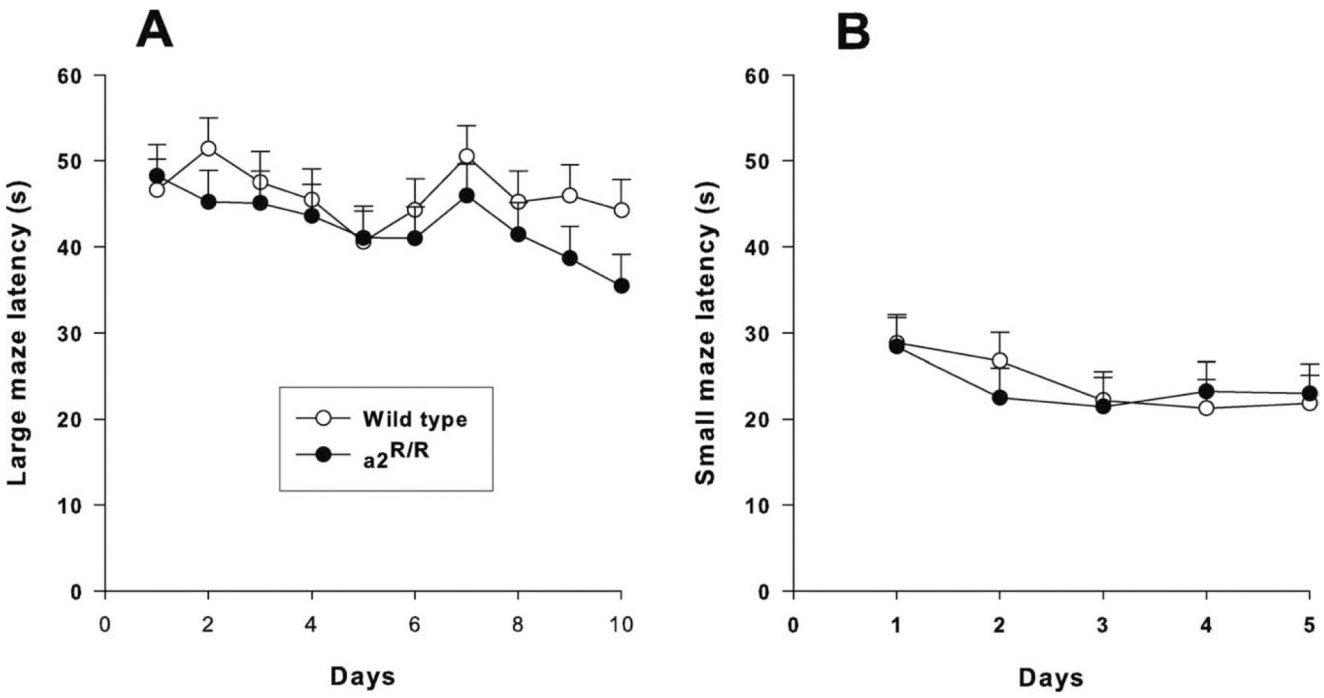

Fig. 5.

Large MWM acquisition (Mean ±SEM latency (s): (**A**) Performance in the 210-cm diameter MWM. There were no group differences in latency between genotypes. There was an overall day effect attributable to the fact that in the large maze performance was erratic, improving some days, remaining the same, or deteriorating, suggesting that the large maze was too difficult for the mice to learn effectively regardless of genotype. (**B**) Performance in the 122-cm diameter MWM: When animals were switched to the smaller maze, they performed significantly better but no differences in $\alpha 2^{R/R}$ and WT mice were observed. n = 10–11 male mice per genotype.

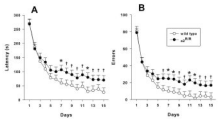

Fig. 6.

Cincinnati Water Maze: (**A**) Latency (s) to reach the escape platform was significantly increased on Days 7 and 12 and there was a trend for latency to be increased on Days 8–11 and 13–15 in $\alpha 2^{R/R}$ compared to WT mice. (**B**) Errors were significantly increased on Days 7, 8, 11, and 12 and there was a trend for errors to be increased on Days 6, 9, 10, and 13–15 in $\alpha 2^{R/R}$ compared to WT mice. Data are Mean ± SEM per day. n = 30–31 male mice per genotype. *$P < 0.05$; †$P < 0.10$.

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TABLE I

Elevated zero maze and marble burying

	Elevated zero maze			Marble burying	
	Time in open (s)	**Head dips**	**Stretch-attends**	**Latency to bury (s)**	**Marbles visible**
WT	102.6 ± 11.4	13.2 ± 1.7	9.3 ± 1.1	287.0 ± 100.1	6.4 ± 1.0
$A2^{R/R}$	86.0 ± 11.9	10.5 ± 1.7	10.7 ± 1.1	472.2 ± 105.2	6.8 ± 1.1

Data represent Mean ± SEM. For groups sizes see Methods.