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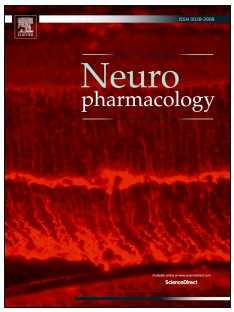

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Effects of the synthetic neurosteroid ganaxolone on seizure activity and behavioral deficits in an Angelman syndrome mouse model

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Abstract

Angelman syndrome (AS) is a rare neurogenetic disorder characterized by severe developmental delay, motor impairments, and epilepsy. GABAergic dysfunction is believed to contribute to many of the phenotypic deficits seen in AS. We hypothesized that restoration of inhibitory tone mediated by extrasynaptic $GABA_A$ receptors could provide therapeutic benefit. Here, we report that ganaxolone, a synthetic neurosteroid that acts as a positive allosteric modulator of synaptic and extrasynaptic $GABA_A$ receptors, was anxiolytic, anticonvulsant, and improved motor deficits in the Ube3a-deficient mouse model of AS when administered by implanted mini-pump for 3 days or 4 weeks. Treatment for 4 weeks also led to recovery of spatial working memory and hippocampal synaptic plasticity deficits. This study demonstrates that ganaxolone ameliorates many of the behavioral abnormalities in the adult AS mouse, and tolerance did not occur to the therapeutic effects of the drug. The results support clinical studies to investigate ganaxolone as a symptomatic treatment for AS.

1. Introduction

Angelman syndrome (AS) is a rare neurogenetic disorder characterized by developmental delay, speech and motor impairments, easily provoked laughter, and epilepsy (Clayton-Smith and Laan, 2003; Williams et al., 2010). AS is associated with maternal deletions of human chromosome 15q11-13, resulting in loss of function of the E3 ubiquitin ligase Ube3a (Kishino et al., 1997; Matsuura et al., 1997). Deletion of this chromosomal region also often involves disruption of the $GABA_A$ receptor subunit gene *GABRB3*, and epilepsy is more prevalent in patients with this deletion (Minassian et al., 1998; Røstergaard and Balslev, 2001). Altered $GABA_A$ receptor function may underlie the epileptic, behavioral, and cognitive abnormalities in AS, whether or not *GABRB3* is affected (Ciarlone and Weeber, 2016). Decreased tonic inhibition has been reported in the Ube3a-deficient AS mouse model, and administration of a selective extrasynaptic $GABA_A$ receptor agonist improves the abnormal firing properties of Purkinje neurons in cerebellar brain slices from these animals and ameliorates motor abnormalities when administered in vivo (Egawa et al., 2012). Additionally, Ube3a loss in GABAergic neurons in mice leads to cortical hyperexcitability and enhanced seizure susceptibility (Judson et al., 2016). Moreover, ratios of $GABA_A$ receptor α_5/α_1 subunit expression in the AS human cortex are decreased compared to age-matched controls, consistent with a relative reduction in extrasynaptic $GABA_A$ receptors inasmuch as α_5 subunits are mainly found in extrasynaptically (Caraiscos et al., 2004) whereas α_1 subunits are synaptic (Roden et al., 2010). These various lines of converging evidence suggest that deficient tonic inhibition mediated by extrasynaptic $GABA_A$ receptors is a critical determinant of diverse clinical manifestations in AS.

Certain endogenous neurosteroids, such as the progesterone metabolite allopregnanolone, are potent positive modulators of synaptic and extrasynaptic $GABA_A$ receptors (Reddy, 2010). These neurosteroids exhibit anxiolytic and anticonvulsant actions, while other neurosteroids have

been shown to enhance cognition as demonstrated by improved rodent performance on learning and memory tasks such as the foot-shock active avoidance, passive avoidance, and visual discrimination tests (Engel and Grant, 2001; Flood et al., 1992; Isaacson et al., 1995; Meziane et al., 1996). Neurosteroids may also facilitate cellular phenomena believed to be related to learning and memory such as hippocampal prime-burst potentiation (Diamond et al., 1996) and long-term potentiation (Yoo et al., 1996). Ganaxolone, the 3β-methyl synthetic analog of allopregnanolone, is also a positive allosteric modulator of synaptic and extrasynaptic $GABA_A$ receptors (Carter et al., 1997; Nohria and Giller, 2007) Unlike allopregnanolone which is devoid of oral bioavailability, ganaxolone can be administered orally to obtain meaningful systemic exposures (Monaghan et al., 1997; Nohria and Giller, 2007; Reddy, 2010; Reddy and Kulkarni, 2000). Ganaxolone exhibits protective activity in various seizure models in mice and rats including chemoconvulsant, 6 Hz electroshock, and kindling models (Carter et al., 1997; Gasior et al., 2000; Reddy and Rogawski, 2010a). Importantly, there is no tolerance to the seizure protection conferred by neurosteroids including ganaxolone allowing them to be used chronically in the treatment of epilepsy (Reddy and Rogawski, 2000). In limited human clinical trials in adult and pediatric patients, ganaxolone has shown indications of efficacy and was well tolerated (Bialer et al., 2013; Monaghan et al., 1997; Nohria and Giller, 2007; Reddy and Rogawski, 2010b).

In this study, we sought to evaluate the effects of 3 day and 4 week continuous ganaxolone treatment on behavior, neurological function and seizure susceptibility of AS mice. We found that AS mice demonstrate significant improvements is these diverse domains, whether studied at the early or late time point. The results support clinical studies of chronic ganaxolone in the treatment of AS.

2. Materials and Methods

2.1 Animals

UBE3A$^{tm1Alb/J}$ null mutation AS mice, described previously (Jiang et al., 1998), were purchased from the Jackson Laboratory. Wild-type (WT) and AS mice were obtained through breeding of heterozygous female mice with WT males to produce maternal-deficient AS offspring and age-matched, wild-type littermate controls. Animals were housed with a standard 12-hour light/dark cycle and supplied with food and water *ad libitum* at the University of South Florida, and were housed in groups of three to four per cage. Experiments were performed on 12-14 week-old male and female mice. All animal testing procedures and care followed the NIH guidelines and were approved by the University of South Florida's Institutional Animal Care and Use Committee (Approval ID number A4100-01).

2.2 Ganaxolone administration

Ganaxolone (ScinoPharm, Taiwan Limited, Tainan, Taiwan) was administered via a subcutaneous mid-scapular osmotic pump (Alzet) at 5mg/mL dissolved in aqueous 30% 2-hydroxypropyl-β-cyclodextrin (BCD) (Sigma-Aldrich) resulting in ~150 nM serum concentration. This estimation was calculated via pharmacokinetic analysis ((maintenance dose rate) = CL * Css) using specific drug information from the Ganaxolone Investigator's Brochure, including the Vdss (6.5 L/kg) and clearance in the mouse (77.0 mL/min/kg). We determined 6.5 µg of ganaxolone using a solution that is 5 mg/mL needed to be delivered to the mouse in order to obtain a 150 nM concentration, and ~5000 ng/hr needed to be administered at 1 µL/hr using the osmotic mini-pumps. Short-term and chronic experiments were completed at 3 days and 4 weeks post-implantation, respectively.

Prior to implantation, all mice were treated with 10mg/kg s.c. carprofen (Rimadyl®, Butler Schein) to suppress post-surgical pain, and carprofen was administered every 12 hours for the first 48 hours following surgery. Osmotic pump implantation was performed under continuous isoflurane anesthesia, and pumps were placed subcutaneously between the scapulae following an incision to ensure continuous drug release.

2.3 Behavioral testing

Open field behavior was assessed to determine general locomotor activity and anxiety. Mice were placed in an acrylic chamber (40cm x 40cm x 27cm) and were allowed to explore for 15 minutes. ANY-Maze animal activity system (Stoelting Co.) was used to monitor movement and distance traveled.

Elevated plus maze (EPM) was used to assess anxiety levels. The EPM consisted of four arms: two (30 cm x 5 cm) open, well-lit arms and two (30 cm x 5 cm x 15 cm) enclosed arms facing each other. Each arm attached to a common open square center platform (4.5 cm). Mice were placed in the center platform and allowed to explore for 5 min. A digital camera (XV-BP330, Panasonic) was used to monitor activity, and ANY-Maze animal activity system (Stoelting Co.) was used to record and analyze behavior. Total time spent in open arms versus closed arms was measured, and anxiety levels were determined by comparing percentage of time spent in the open arms.

Rotarod was used to assess motor coordination, motor learning, and stamina. Mice were placed on a 3 cm diameter rod with an initial rotation of 4 rpm and accelerated to 40 rpm over a maximum of 5 min (Ugo Basile, Italy). Mice were tested for latency to fall off the rod for four trials per day over two consecutive days.

Wire hang test was used to measure subacute muscle function and fatigue. A horizontal wire (2 mm in diameter, 40 cm in length) was suspended above a padded table. The animal was allowed to cling in the middle of the wire with its forepaws for one 60 s trial, and latency to fall was recorded.

Hind limb clasping was used as a marker for neurological dysfunction, including certain ataxias. The clasping test evaluated the animal's hind limb response during tail suspension 10 cm above their home cage. If the hind limbs were consistently splayed outward, away from the abdomen, the mouse was assigned a score of 0. If one hind limb was retracted toward the abdomen, the animal received a score of 1. If both hind limbs were partially retracted toward the abdomen, it received a score of 2. The animal received a score of 3 if the animal's hind limbs were entirely retracted and touching the abdomen.

Y-maze spontaneous alternation task

Each animal was placed in a Y-maze and allowed to habituate to the maze environment for 5 minutes. The next day the mice were placed into the center of the maze and allowed to move freely through the maze for 5 minutes. Spontaneous alternation (entering all three arms sequentially without repetition) was calculated as follows: number of triads containing entries into all three arms/maximum possible alternations (the total number of arms entered – 2) x 100. Chance performance is 50%.

2.4 Audiogenic seizures

For audiogenic seizure testing, mice were habituated to a sound attenuation chamber for 60 sec and exposed to sound stimulation (115 dB) for 60 sec or until tonic or clonic episodes occurred (Ciarlone et al., 2016). An occurrence of sound-induced seizure was defined as tonic, clonic, or

tonic-clonic seizures during sound stimulation. Animals were tested only once. Seizure testing was carried out between 1:00 PM and 6:00 PM to limit effects of diurnal variation on results.

2.5 Pentylenetetrazol-induced seizures

Pentylenetetrazol (PTZ, Sigma-Aldrich) dissolved in phosphate-buffered saline was injected intraperitoneally at a single convulsive dose of 60 mg/kg to test susceptibility to generalized convulsive seizures (Hill-Yardin et al., 2015). Animals were placed into chambers and monitored for 30 min after the injection. Behavioral responses were recorded using a video camera and seizure activity was classified according to the following scale (Ishisaka et al., 2013): 0) no abnormality; 1) exploring, sniffing, and grooming ceased, becoming motionless; 2) head-nodding, facial and forelimb clonus; 3) myoclonic jerks of the head and neck, with brief twitching movements, or repetitive movements with head-bobbing or tail rigidity; 4) forelimb or forelimb and hind limb clonus, reciprocal forepaw padding, hind limb abduction, continuous rearing, and falling, Straub tail response; 5) tonic convulsions; 6) death. The highest seizure score was recorded during each minute, and total scores were calculated as the sum of the minute-by-minute scores.

2.6 Extracellular recordings

Following behavioral testing, a cohort of mice was euthanized and the hippocampi dissected out to be used in hippocampal LTP experimentation as previously described (Trotter et al., 2013). The brain was rapidly dissected and placed in ice-cold, oxygenated cutting solution containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 5 glucose, 0.6 ascorbate, 7 $MgCl_2$, and 0.5 $CaCl_2$. Hippocampal slices (400 μm) were prepared on a vibratome and allowed

to equilibrate in a 50% cutting saline and 50% artificial cerebrospinal fluid solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$. Slices were maintained in this solution with constant 95% O_2/5% CO_2 perfusion for 10 min before being transferred to the brain slice recording chamber supported by nylon mesh or maintained in a holding container. Slices were recovered for a minimum of 1 h before recording. The recording chamber was held at 30° ± 0.5°C with a ACSF flow rate of 1 ml/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum radiatum in hippocampal area CA1 via glass microelectrodes filled with artificial cerebrospinal fluid (resistance 1–4 MΩ). Responses were generated by stimulation of Schaffer collaterals arising from the CA3 region. Stimulating electrodes consisted of formvar-coated nichrome wire, which was used to deliver biphasic stimulus pulses (1–15 V, 100 μs duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Molecular Devices), was via the Digidata 1322A interface (Molecular Devices) and a stimulus isolator (model 2200; A-M Systems). Signals were amplified using a differential amplifier (model 1800; A-M Systems), filtered at 1 kHz, and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited ~50% of the maximum fEPSP response as determined from the input–output curve. The input–output relationship was determined by stimulating slices from 0 to 15 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation (PPF), which was induced by stimulating slices at half-max intensity with sequential pulses spaced at 20 ms intervals from 20 to 300 ms. LTP was induced by a theta-burst stimulation (TB-stim) protocol, which consisted of five trains of four pulse bursts at 200 Hz separated by 200 ms, repeated six times with an intertrain interval of 10 s. For analysis, the last 10 minutes of recording was averaged and compared.

2.7 Statistical analysis

All data is represented as the mean ± SEM. Data was analyzed using Student's t-test or ANOVA followed by Tukey's Multiple Comparison test, set at a significance of $p < 0.05$ (GraphPad Prism software).

3. Results

3.1 Short-term ganaxolone administration decreases anxiety and improves motor deficits in AS mice

To evaluate general anxiety, percent time spent in the open arm of the EPM was analyzed by two-way ANOVA with genotype and treatment as factors. AS mice spent significantly less time in the open arm compared to WT controls and AS treated animals (Figure 1A). We found a significant interaction of group and treatment ($F_{(1,42)} = 5.20$, $p < 0.05$; Bonferroni post-hoc tests: WT vs. AS $p < 0.05$, AS vs. AS GNX $p < 0.05$).

Following the 3 day ganaxolone treatment, AS control mice demonstrated significant deficits in rotarod performance compared to WT controls, and this deficit was significantly improved by ganaxolone treatment (Figure 1B, repeated measures ANOVA $p < 0.001$; WT and AS GNX vs. AS $p < 0.001$). AS mice also demonstrated a significant hind limb clasping phenotype compared to WT controls, which was ameliorated with ganaxolone treatment (Figure 1C). A two-way ANOVA revealed a significant effect of genotype ($F_{(1,17)} = 8.66$, $p < 0.01$; Bonferroni post-hoc tests: WT vs. AS $p < 0.01$; AS vs. AS GNX $p < 0.05$). All WT and WT GNX mice were able to hang for the maximum trial time of 60 seconds, while 20% of AS and 75% of AS GNX mice reached the maximum trial duration.

3.2 Short-term ganaxolone treatment decreases audiogenic seizure frequency and latency

Following audiogenic stimulation, we observed seizures in 63% of the AS mice, whereas no seizures were observed in WT animals (data not shown). AS mice treated for 3 days with ganaxolone demonstrated a 45% reduction in seizure activity compared to AS controls when tested (Figure 1D, $p < 0.05$ Fisher's exact test). AS treated animals also demonstrated a significant increase in latency to seize (50.94 sec) compared to AS controls (28.02 sec) (Figure 1E, $p < 0.01$).

3.3 Ganaxolone decreases anxiety and improves motor coordination

General locomotor activity was examined in the open field test, as measured by overall distance traveled in 15 minutes. There was no significant difference in general locomotion in mice treated with ganaxolone for four weeks (Figure 2A). Anxiety was measured in the elevated plus maze as percent time spent in the open arm of the maze. AS GNX mice spent significantly more time in the open arm compared to AS controls (Figure 2B). A two-way ANOVA revealed a significant interaction of group and treatment ($F_{(1,36)} = 6.32$, $p < 0.05$; Bonferroni post-hoc tests: AS vs. AS GNX $p < 0.05$). AS mice were more anxious than WT controls, similar to reports of patients with AS (Clayton-Smith, 2001; Thibert et al., 2013).

Following 4 weeks of ganaxolone administration, we observed significantly improved motor coordination and motor learning in AS GNX mice compared to AS controls as demonstrated by an increased latency to fall off the rotarod (Figure 2C, repeated measures ANOVA $p < 0.0001$; WT vs. AS and AS GNX $p < 0.001$; AS vs. AS GNX $p < 0.001$; WT vs. WT GNX $p < 0.05$). Neurological and motor improvement was also observed by a significantly decreased hind limb clasping score in AS treated mice compared to AS BCD animals (Figure

2D). A two-way ANOVA revealed a significant effect of genotype ($F_{(1,63)} = 65.84$, $p < 0.0001$) and treatment ($F_{(1,63)} = 4.79$, $p < 0.05$) (Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS GNX $p < 0.001$; WT vs. WT GNX $p < 0.05$).

3.4 *Long-term ganaxolone treatment recovers spatial working memory and LTP deficits*

Spontaneous alternation behavior, which is regarded as a measure of spatial working memory, was investigated next. AS mice displayed significantly impaired working memory when measured four weeks post osmotic pump implantation, whereas the AS GNX group performed to WT levels (Figure 3A). A two-way ANOVA revealed a significant interaction of group and treatment ($F_{(1,26)} = 6.32$, $p < 0.01$; Bonferroni post-hoc tests: WT vs. AS $p < 0.05$; AS vs. AS GNX $p < 0.05$). The total number of entries into the arms of the maze was not significantly different between all experimental groups, demonstrating that general locomotor activity was not affected by ganaxolone in this task (Figure 3B).

Using a TB-stim LTP protocol, the extent of LTP, calculated by averaging the slope values of fEPSPs recorded between 50 and 60 minutes after stimulation, was calculated. The level of potentiation was significantly lower in slices from AS mice (92.1 ± 0.61) compared to those from WT animals (131.2 ± 1.06) (Figures 4C and 4D, ANOVA, Bonferroni post-hoc tests, $p < 0.001$). Chronic ganaxolone treatment recovered the LTP impairment in area CA1 of AS GNX mice (Figures 4C and 4D, 125.6 ± 0.75, $p < 0.001$) compared to AS controls. There were no changes observed in baseline synaptic transmission (input-output relationship, Figure 4A) or short-term synaptic plasticity (paired-pulse facilitation, Figure 4B).

3.5 Long-term ganaxolone treatment attenuates seizure activity

Audiogenic seizures were observed in 67% of AS mice, while only 20% of AS mice treated with ganaxolone for 4 weeks exhibited audiogenic seizures (Figure 5A, $p < 0.05$ Fisher's exact test). No seizures were observed in the WT group (data not shown). AS mice treated with ganaxolone for 4 weeks also demonstrated a significant increase in seizure latency (50.88 sec) compared to AS BCD mice (29.47 sec) (Figure 5B, $p < 0.05$). PTZ-induced seizure severity was significantly increased in AS BCD mice compared to WT controls and AS-treated mice (Figure 5C, repeated measures ANOVA $p < 0.001$). Total seizure scores for AS mice recorded during the 30-minute time period were also significantly higher than WT controls and AS GNX-treated mice, suggesting ganaxolone significantly reduces chemically-induced tonic-clonic seizure activity in AS mice (Figure 5D, $p < 0.001$). A two-way ANOVA revealed a significant effect of genotype ($F_{(1,27)} = 21.44$, $p < 0.0001$) and treatment ($F_{(1,27)} = 10.94$, $p < 0.01$), with a significant interaction of group and treatment ($p < 0.01$) (Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS GNX $p < 0.001$). These results are consistent with previous reports demonstrating that ganaxolone protects against chemically-induced seizures (Gasior et al., 2000; Reddy and Rogawski, 2000).

4. Discussion

One of the most devastating effects of reduced neuronal *UBE3A* in humans is seizure susceptibility, with >80% of individuals presenting with epilepsy of which approximately 70% are medically refractory (Thibert et al., 2012). While absence of *UBE3A* is sufficient to cause epilepsy in AS, a preponderance of AS patients with a large deletion of multiple genes on the maternal chromosome 15q11-13 demonstrate a more significant phenotype. Patients also

commonly exhibit high levels of anxiety and motor-related disturbances such as tremor, which progress with age (Clayton-Smith, 2001; Pelc et al., 2008; Thibert et al., 2013). Both epilepsy and motor dysfunction may be attributed to imbalances in excitation and inhibition. Such imbalances, which are believed to result from diminished synaptic and extrasynaptic $GABA_A$ receptor mediated inhibition, have been documented in the cortex and cerebellum of the AS mouse (Egawa et al., 2012). Moreover, GABAergic dysfunction and the resulting circuit hyperexcitability may contribute to the impaired learning and memory associated with the disorder (Egawa et al., 2012; Judson et al., 2016; Wallace et al., 2012). In the present study, we investigated the potential of ganaxolone, a positive allosteric modulator of $GABA_A$ receptors, to reduce the enhanced seizure susceptibility and rescue the major behavioral and neurological defects in a mouse model of AS. Ganaxolone is an attractive potential treatment agent because it is orally active and has a good safety record (Bialer et al., 2013). Ganaxolone modulates both synaptic and extrasynaptic $GABA_A$ receptors (Martinez Botella et al., 2015). This distinguishes it from benzodiazepines, the positive $GABA_A$ receptor modulators most commonly used clinically, which only act on synaptic $GABA_A$ receptors. Moreover, in contrast to benzodiazepines that have a high propensity for tolerance, studies in animals have indicated that the antiseizure activity of ganaxolone does not diminish with chronic treatment (Reddy and Rogawski, 2000). In addition, in clinical trials ganaxolone has been found to maintain efficacy in some patients for periods of years (Bialer et al., 2013).

Given the lack of tolerance in these prior studies, we surmised that the antiseizure activity of ganaxolone in AS mice would not diminish following 4 week treatment when compared with the effect obtained after 3 days of treatment. However, there is only limited information on the extent to which there is tolerance to the other behavioral actions of ganaxolone, including the

anxiolytic and motor related actions. Therefore, it was of interest to determine if such other effects of ganaxolone would be maintained with 4 week treatment. We initially evaluated the short-term anticonvulsant, anxiolytic, and motor-related effects of ganaxolone in our seizure-prone AS mouse model. Previous studies have demonstrated anticonvulsant and anxiolytic effects of the drug in rodent models within 10 to 30 minutes after injection (Heulens et al., 2012; Kazdoba et al., 2016; Reddy and Rogawski, 2000). These latter effects may in part relate to increased tonic inhibition in the amygdala (Akwa et al., 1999; Romo-Parra et al., 2015). In the AS mice, 3 day ganaxolone treatment improved seizure susceptibility. Although anxiolytic and antiseizure effects have previously been obtained with non-toxic doses of ganaxolone in mice and rats (Gasior et al., 1997; Mareš and Stehlíková, 2010), motor impairment occurs at only modestly greater doses (Hogenkamp et al., 2014) . Therefore, we were concerned that ganaxolone might have untoward actions in AS mice, which have impairments in motor function throughout their lives, manifesting as gait ataxia, poor motor coordination and learning, and defective hind limb clasping (Egawa et al., 2012; Heck et al., 2008; Jiang et al., 1998; Meng et al., 2013; Van Woerden et al., 2007). However, these concerns were found to be unwarranted as ganaxolone did not degrade motor function in AS mice and, indeed, we were able to document improvements in the rotarod and wire hang task.

We also sought to determine if continuous, extended ganaxolone delivery for 4 weeks would provide maintained seizure protection in the AS mouse. Extended ganaxolone administration has been reported for up to 10 days (Reddy and Rogawski, 2000), but to our knowledge, chronic dosing for longer periods has not been studied in rodents. To avoid the stress and anxiety of multiple daily injections, we administered ganaxolone via osmotic mini-pumps implanted subcutaneously, which allow for continuous dosing and a constant plasma

concentrations. After four weeks of continuous administration, we found that ganaxolone treatment resulted in similar anxiolytic, positive motor, and anticonvulsant effects as was obtained with short-term treatment. Thus, in addition to the expected lack of tolerance to the antiseizure efficacy of ganaxolone there was no tolerance to the other therapeutic actions in AS mice. To our knowledge, lack of tolerance to such other therapeutic actions of ganaxolone has not previously been demonstrated.

One of the most surprising effects of ganaxolone is the reversal of motor learning deficits in the AS mice, which we observed with both short- and long-term treatment. AS patients have fine and gross motor impairments that affect many other essential functions such as feeding, movement, and communication (Beckung et al., 2004; Clayton-Smith and Laan, 2003; Lossie et al., 2001). Therefore, if the improvement in motor coordination and motor learning obtained in AS mice translates to humans, ganaxolone could significantly improve quality-of-life in AS patients. Our demonstration of improved motor function in AS mice with ganaxolone is consistent with a previous study showing that gaboxadol enhances motor performance and normalizes Purkinje cell firing in the AS mice (Egawa et al., 2012). In contrast to ganaxolone, which is an allosteric modulator of both synaptic and extrasynaptic $GABA_A$ receptors, gaboxadol is an agonist that acts directly at the GABA recognition site of $GABA_A$ receptors and is highly selective for extrasynaptic receptors. There is no evidence that benzodiazepines, which are selective modulators of synaptic $GABA_A$ receptors, produce motor improvement in AS. Therefore, it is possible that the improved motor performance induced by ganaxolone is a result of its actions on extrasynaptic $GABA_A$ receptors.

An excitatory/inhibitory imbalance has been established in the AS cortex, along with decreased cerebellar tonic inhibition and *Ube3a* loss in cortical GABAergic neurons (Egawa et

al., 2012; Judson et al., 2016; Wallace et al., 2012). These alterations can lead to circuit hyperexcitability and defective sensory integration, detection, and processing, and may be linked to the phenotypic deficits we observe in AS. Previous work has also demonstrated that inhibition of hilar GABAergic interneuron activity impairs spatial learning and memory, and learning ability relies on increased inhibitory synaptic plasticity and GABA release (Andrews-Zwilling et al., 2012; Cui et al., 2008). It has also been suggested that the balance of excitatory and inhibitory neuronal activity in the hippocampus is critical for synaptic plasticity and normal learning (Cui et al., 2008). Therefore, one potential explanation for the action of ganaxolone in AS is that a synthetic neurosteroid analog that functions as a $GABA_A$ positive allosteric modulator may increase the neuronal signal-to-noise ratio, resulting in enhanced information processing and potential learning and memory improvement. Interestingly, we found that ganaxolone rescues both the spatial working memory and hippocampal synaptic plasticity deficits in AS mice, while decreasing LTP in WT treated animals. The contrasting electrophysiological results observed in WT mice were not unexpected, given that progesterone, the precursor of allopregnanolone, decreases LTP in rat CA1 neurons (Foy et al., 2008), and increased tonic inhibition could alter synaptic plasticity. However, the way in which ganaxolone ameliorates learning and memory deficits in the AS mouse remains to be determined.

Extensive experience in human clinical trials has shown ganaxolone to be well tolerated and safe. Moreover, several oral dosage forms (suspension and capsule) are available that allow ganaxolone to be administered conveniently to children and adults. Ganaxolone is not currently approved for any clinical indication, although human studies in various conditions are ongoing. Our present results indicate that ganaxolone might be particularly well suited as a symptomatic treatment for AS, with the potential to not only treat the seizures but also to provide long-lasting

improvement in the diverse neurobehavioral and motor symptoms. Children with AS are at risk of early death due to poorly controlled seizures (Ruggieri and McShane, 1998), and therefore it is of interest to determine if ganaxolone can protect against seizures in AS as we have shown is the case in the mouse model. Consequently, clinical trials are warranted. In addition, our results are consistent with other work (Roden et al., 2010) suggesting that positive modulators of extrasynaptic $GABA_A$ receptors might in general provide unique symptomatic benefits in AS. Investigation of other extrasynaptic $GABA_A$ receptors' active agents including gaboxadol will be of interest.

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References

Akwa, Y., Purdy, R. H., Koob, G. F., Britton, K. T., 1999. The amygdala mediates the anxiolytic-like effect of the neurosteroid allopregnanolone in rat. Behavioural brain research 106, 119-125.

Andrews-Zwilling, Y., Gillespie, A. K., Kravitz, A. V., Nelson, A. B., Devidze, N., Lo, I., Yoon, S. Y., Bien-Ly, N., Ring, K., Zwilling, D., 2012. Hilar GABAergic interneuron activity controls spatial learning and memory retrieval. PloS one 7, e40555.

Beckung, E., Steffenburg, S., Kyllerman, M., 2004. Motor impairments, neurological signs, and developmental level in individuals with Angelman syndrome. Developmental Medicine & Child Neurology 46, 239-243.

Bialer, M., Johannessen, S. I., Levy, R. H., Perucca, E., Tomson, T., White, H. S., 2013. Progress report on new antiepileptic drugs: a summary of the Eleventh Eilat Conference (EILAT XI). Epilepsy research 103, 2-30.

Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., Jackson, M. F., Lambert, J. J., Rosahl, T. W., Wafford, K. A., 2004. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. Proceedings of the National Academy of Sciences of the United States of America 101, 3662-3667.

Carter, R. B., Wood, P. L., Wieland, S., Hawkinson, J. E., Belelli, D., Lambert, J. J., White, H. S., Wolf, H. H., Mirsadeghi, S., Tahir, S. H., 1997. Characterization of the anticonvulsant properties of ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one), a selective, high-affinity, steroid modulator of the γ-aminobutyric acidA receptor. Journal of Pharmacology and Experimental Therapeutics 280, 1284-1295.

Ciarlone, S. L., Grieco, J. C., D'Agostino, D. P., Weeber, E. J., 2016. Ketone ester supplementation attenuates seizure activity, and improves behavior and hippocampal synaptic plasticity in an Angelman syndrome mouse model. Neurobiology of Disease.
Ciarlone, S. L., Weeber, E. J., 2016. Towards targeted therapy for Angelman syndrome. Expert Opinion on Orphan Drugs 4, 317-325.
Clayton-Smith, J., Laan, L., 2003. Angelman syndrome: a review of the clinical and genetic aspects. Journal of Medical Genetics 40, 87-95.
Clayton-Smith, J., 2001. Angelman syndrome: evolution of the phenotype in adolescents and adults. Developmental Medicine & Child Neurology 43, 476-480.
Cui, Y., Costa, R. M., Murphy, G. G., Elgersma, Y., Zhu, Y., Gutmann, D. H., Parada, L. F., Mody, I., Silva, A. J., 2008. Neurofibromin regulation of ERK signaling modulates GABA release and learning. Cell 135, 549-560.
Diamond, D. M., Branch, B. J., Fleshner, M., 1996. The neurosteroid dehydroepiandrosterone sulfate (DHEAS) enhances hippocampal primed burst, but not long-term, potentiation. Neuroscience letters 202, 204-208.
Egawa, K., Kitagawa, K., Inoue, K., Takayama, M., Takayama, C., Saitoh, S., Kishino, T., Kitagawa, M., Fukuda, A., 2012. Decreased tonic inhibition in cerebellar granule cells causes motor dysfunction in a mouse model of Angelman syndrome. Science translational medicine 4, 163ra157-163ra157.
Engel, S. R., Grant, K. A., 2001. Neurosteroids and behavior. International review of neurobiology 46, 321-348.
Flood, J. F., Morley, J. E., Roberts, E., 1992. Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. Proceedings of the National Academy of Sciences 89, 1567-1571.
Foy, M. R., Akopian, G., Thompson, R. F., 2008. Progesterone regulation of synaptic transmission and plasticity in rodent hippocampus. Learning & Memory 15, 820-822.
Gasior, M., Carter, R. B., Goldberg, S. R., Witkin, J. M., 1997. Anticonvulsant and behavioral effects of neuroactive steroids alone and in conjunction with diazepam. Journal of Pharmacology and Experimental Therapeutics 282, 543-553.
Gasior, M., Ungard, J. T., Beekman, M., Carter, R. B., Witkin, J. M., 2000. Acute and chronic effects of the synthetic neuroactive steroid, ganaxolone, against the convulsive and lethal effects of pentylenetetrazol in seizure-kindled mice: comparison with diazepam and valproate. Neuropharmacology 39, 1184-1196.
Heck, D. H., Zhao, Y., Roy, S., LeDoux, M. S., Reiter, L. T., 2008. Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors. Human molecular genetics 17, 2181-2189.
Heulens, I., D'Hulst, C., Van Dam, D., De Deyn, P. P., Kooy, R. F., 2012. Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model. Behavioural brain research 229, 244-249.
Hill-Yardin, E. L., Argyropoulos, A., Hosie, S., Rind, G., Anderson, P., Hannan, A. J., O'Brien, T. J., 2015. Reduced susceptibility to induced seizures in the Neuroligin-3 R451C mouse model of autism. Neuroscience letters 589, 57-61.
Hogenkamp, D. J., Tran, M. B., Yoshimura, R. F., Johnstone, T. B., Kanner, R., Gee, K. W., 2014. Pharmacological profile of a 17β-heteroaryl-substituted neuroactive steroid. Psychopharmacology 231, 3517-3524.
Isaacson, R., Varner, J., Baars, J.-M., De Wied, D., 1995. The effects of pregnenolone sulfate and ethylestrenol on retention of a passive avoidance task. Brain research 689, 79-84.
Ishisaka, M., Tsuruma, K., Shimazawa, M., Shirai, Y., Saito, N., Hara, H., 2013. Increased seizure susceptibility in a mouse with diacylglycerol kinase β deficiency.

Jiang, Y. H., Armstrong, D., Albrecht, U., Atkins, C. M., Noebels, J. L., Eichele, G., Sweatt, J. D., Beaudet, A. L., 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. Neuron 21, 799-811.
Judson, M. C., Wallace, M. L., Sidorov, M. S., Burette, A. C., Gu, B., van Woerden, G. M., King, I. F., Han, J. E., Zylka, M. J., Elgersma, Y., 2016. GABAergic neuron-specific loss of Ube3a causes Angelman syndrome-like EEG abnormalities and enhances seizure susceptibility. Neuron 90, 56-69.
Kazdoba, T. M., Hagerman, R. J., Zolkowska, D., Rogawski, M. A., Crawley, J. N., 2016. Evaluation of the neuroactive steroid ganaxolone on social and repetitive behaviors in the BTBR mouse model of autism. Psychopharmacology 233, 309-323.
Kishino, T., Lalande, M., Wagstaff, J., 1997. UBE3A/E6-AP mutations cause Angelman syndrome. Nature genetics 15, 70-73.
Lossie, A., Whitney, M., Amidon, D., Dong, H., Chen, P., Theriaque, D., Hutson, A., Nicholls, R., Zori, R., Williams, C., 2001. Distinct phenotypes distinguish the molecular classes of Angelman syndrome. Journal of Medical Genetics 38, 834-845.
Mareš, P., Stehlíková, M., 2010. Anticonvulsant doses of ganaxolone do not compromise motor performance in immature rats. Neuroscience letters 469, 396-399.
Martinez Botella, G., Salituro, F. G., Harrison, B. L., Beresis, R. T., Bai, Z., Shen, K., Belfort, G. M., Loya, C. M., Ackley, M. A., Grossman, S. J., 2015. Neuroactive Steroids. 1. Positive allosteric modulators of the (γ-aminobutyric acid) A receptor: Structure–activity relationships of heterocyclic substitution at C-21. Journal of medicinal chemistry 58, 3500-3511.
Matsuura, T., Sutcliffe, J. S., Fang, P., Galjaard, R.-J., Jiang, Y.-h., Benton, C. S., Rommens, J. M., Beaudet, A. L., 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. Nature genetics 15, 74-77.
Meng, L., Person, R. E., Huang, W., Zhu, P. J., Costa-Mattioli, M., Beaudet, A. L., 2013. Truncation of Ube3a-ATS unsilences paternal Ube3a and ameliorates behavioral defects in the Angelman syndrome mouse model. PLoS Genet 9, e1004039.
Meziane, H., Mathis, C., Ungerer, A., Paul, S., 1996. The neurosteroid pregnenolone sulfate reduces learning deficits induced by scopolamine and has promnestic effects in mice performing an appetitive learning task. Psychopharmacology 126, 323-330.
Minassian, B. A., Delorey, T. M., Olsen, R. W., Philippart, M., Bronstein, Y., Zhang, Q., Guerrini, R., van Ness, P., Livet, M. O., Delgado-Escueta, A. V., 1998. Angelman syndrome: correlations between epilepsy phenotypes and genotypes. Annals of neurology 43, 485-493.
Monaghan, E. P., Navalta, L. A., Shum, L., Ashbrook, D. W., Lee, D. A., 1997. Initial human experience with ganaxolone, a neuroactive steroid with antiepileptic activity. Epilepsia 38, 1026-1031.
Nohria, V., Giller, E., 2007. Ganaxolone. Neurotherapeutics 4, 102-105.
Pelc, K., Cheron, G., Dan, B., 2008. Behavior and neuropsychiatric manifestations in Angelman syndrome. Neuropsychiatric disease and treatment 4, 577.
Reddy, D. S., 2010. Neurosteroids: Endogenous role in the human brian and therapeutic potentials. Progress in brain research 186, 113.
Reddy, D. S., Kulkarni, S. K., 2000. Development of neurosteroid-based novel psychotropic drugs. Progress in medicinal chemistry 37, 135-176.
Reddy, D. S., Rogawski, M. A., 2000. Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. Journal of Pharmacology and Experimental Therapeutics 295, 1241-1248.
Reddy, D. S., Rogawski, M. A., 2010a. Ganaxolone suppression of behavioral and electrographic seizures in the mouse amygdala kindling model. Epilepsy research 89, 254-260.
Reddy, D. S., Rogawski, M. A., 2010b. Neurosteroids as endogenous regulators of seizure susceptibility and use in the treatment of epilepsy. Epilepsia 51, 84-84.

Roden, W. H., Peugh, L. D., Jansen, L. A., 2010. Altered GABA A receptor subunit expression and pharmacology in human Angelman syndrome cortex. Neuroscience letters 483, 167-172.
Romo-Parra, H., Blaesse, P., Sosulina, L., Pape, H.-C., 2015. Neurosteroids increase tonic GABAergic inhibition in the lateral section of the central amygdala in mice. Journal of neurophysiology 113, 3421-3431.
Røstergaard, J., Balslev, T., 2001. Efficacy of different antiepileptic drugs in children with Angelman syndrome associated with 15q11–13 deletion: the Danish experience. Developmental Medicine & Child Neurology 43, 718-719.
Ruggieri, M., McShane, M., 1998. Parental view of epilepsy in Angelman syndrome: a questionnaire study. Archives of disease in childhood 79, 423-426.
Thibert, R. L., Larson, A. M., Hsieh, D. T., Raby, A. R., Thiele, E. A., 2013. Neurologic manifestations of Angelman syndrome. Pediatric neurology 48, 271-279.
Thibert, R. L., Pfeifer, H. H., Larson, A. M., Raby, A. R., Reynolds, A. A., Morgan, A. K., Thiele, E. A., 2012. Low glycemic index treatment for seizures in Angelman syndrome. Epilepsia 53, 1498-1502.
Trotter, J., Lee, G. H., Kazdoba, T. M., Crowell, B., Domogauer, J., Mahoney, H. M., Franco, S. J., Müller, U., Weeber, E. J., D'Arcangelo, G., 2013. Dab1 is required for synaptic plasticity and associative learning. The Journal of Neuroscience 33, 15652-15668.
Van Woerden, G. M., Harris, K. D., Hojjati, M. R., Gustin, R. M., Qiu, S., de Avila Freire, R., Jiang, Y.-h., Elgersma, Y., Weeber, E. J., 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. Nature neuroscience 10, 280-282.
Wallace, M. L., Burette, A. C., Weinberg, R. J., Philpot, B. D., 2012. Maternal Loss of Ube3a Produces an Excitatory/Inhibitory Imbalance through Neuron Type-Specific Synaptic Defects. Neuron 74, 793-800.
Williams, C. A., Driscoll, D. J., Dagli, A. I., 2010. Clinical and genetic aspects of Angelman syndrome. Genetics in Medicine 12, 385-395.
Yoo, A., Harris, J., Dubrovsky, B., 1996. Dose–response study of dehydroepiandrosterone sulfate on dentate gyrus long term potentiation. Experimental neurology 137, 151-156.

Figure Legends

Figure 1: Short-term ganaxolone administration significantly improves the anxiety, motor, and audiogenic seizure phenotypes in the AS mouse similar to wild type (WT) controls. (A) WT and AS mice were treated with vehicle (BCD) or ganaxolone (GNX) for 3 days by implanted mini-pump and then tested by EPM. Bars represent the percent total time spent in the open arms of the EPM during a 5 min test period. AS mice spent less time in the open arms than WT mice. GNX did not affect the open arm time of WT mice but GNX did increase the open time of AS mice so that the mean open time value was not significantly different from that in WT mice (WT and WT GNX: n=12; AS and AS GNX: n=13). (B) Mice were tested on an accelerating rotarod on successive days for 4 trials a day for 2 days. AS mice exhibited a significantly reduced average latency to fall. Treatment with GNX did not affect average latency to fall values in WT animals. Average latency to fall of AS animals treated with GNX was not significantly different from that of WT animals (WT and WT GNX: n=12; AS and AS GNX: n=13). (C) Latency to fall in the wire hang test was unaffected by GNX treatment compared to BCD-treated controls. AS mice performed poorly on the wire hang task compared to WT controls. AS mice treated with GNX demonstrated a significant increase in the latency to fall value that was not significantly different from that of WT animals (WT: n=8; WT GNX and AS: n=6; AS GNX: n=4). (D) GNX-treated AS mice exhibited a reduced frequency of audiogenic seizures following 115 dB sound stimulation compared with BCD-treated control mice (AS: n=19; AS GNX: n=16). (E) Latency to seizure following audiogenic stimulation was significantly increased in AS GNX mice compared to BCD-treated controls (AS: n=19; AS GNX: n=16; $*p < 0.05$, $**p<0.01$, $***p < 0.001$).

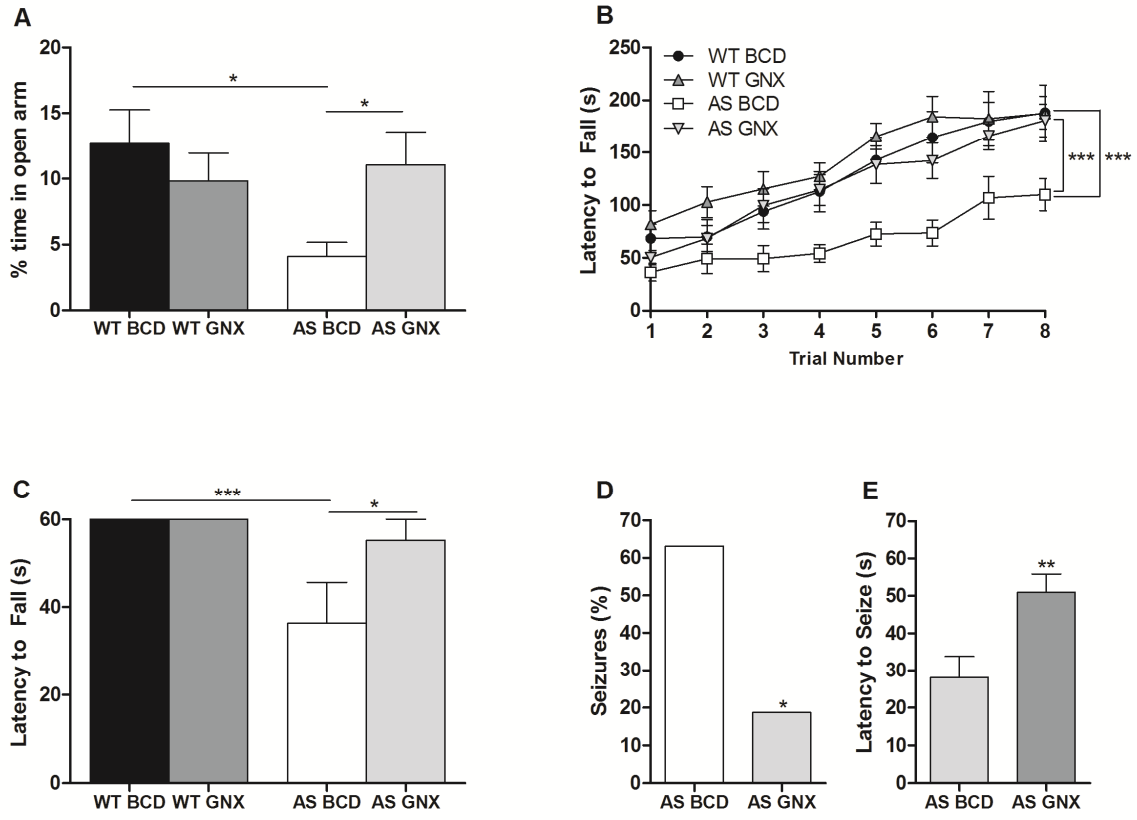
Figure 2: Chronic ganaxolone administration decreases anxiety and improves motor coordination in AS mice without affecting general locomotor activity. (A) Open field: distance traveled. Following 4 weeks of ganaxolone administration, mice underwent open-field testing as control for general locomotor activity. Data represent the overall distance traveled in the open field. There were no significant differences between experimental groups (WT and AS controls: n=12; WT GNX: n=10; AS GNX: n=11). (B) Elevated plus maze: anxiety levels were significantly decreased in AS GNX mice compared to AS controls (WT: n=13; AS: n=9; WT GNX: n=12; AS GNX: n=10). (C) Average latency to fall on the accelerating rotarod was significantly decreased in AS mice compared to WT controls, while AS treated mice demonstrated significant motor improvements compared to AS BCD mice (WT: n=13; AS: n=9; WT GNX: n=12; AS GNX: n=10). (D) Severity of the hind limb clasping score was significantly decreased in AS GNX-treated mice (WT and WT GNX: n=18; AS: n=16; AS GNX: n=17; $*p < 0.05$ and $***p < 0.001$).

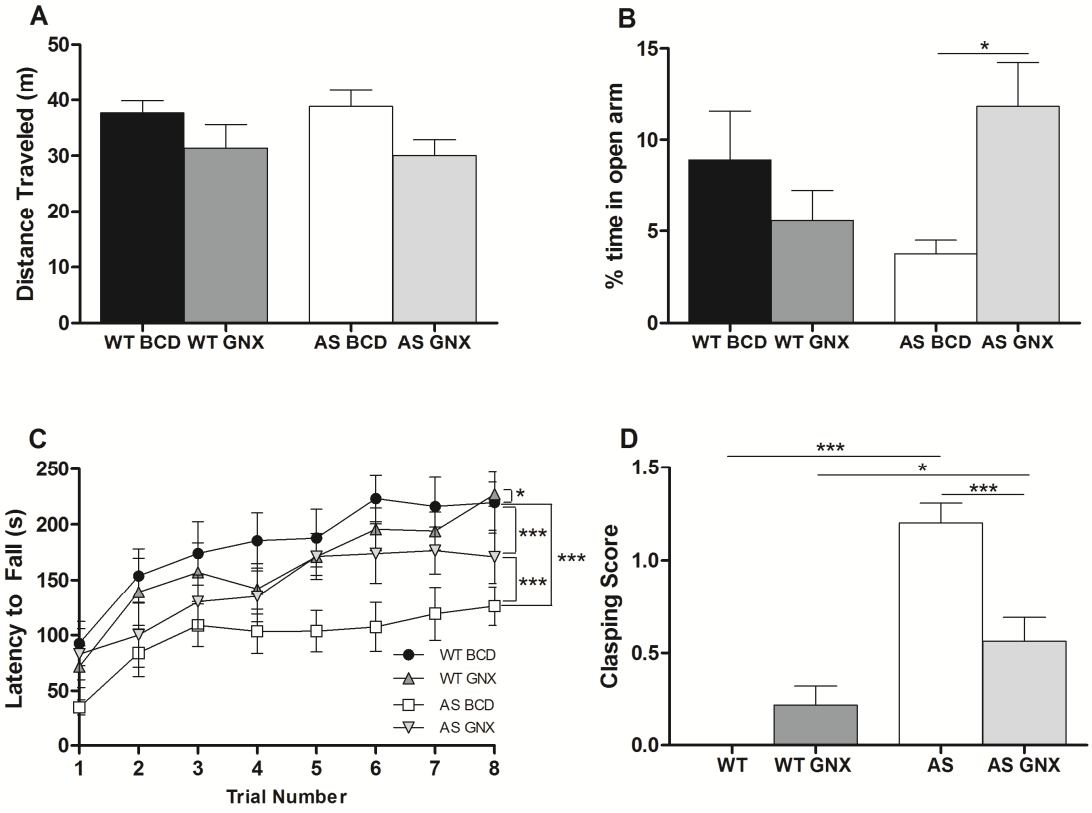
Figure 3. Effects of chronic ganaxolone administration on spontaneous alternation behavior in the Y-maze task. (A) Percentage of spontaneous alternation in the Y-maze. AS mice displayed significant deficits in the Y-maze spontaneous alternation task compared to WT, WT GNX, and AS GNX mice. The dashed line represents the chance level of alternation (random, 50%). (B) The number of total entries in the arms of the Y-maze did not differ significantly between groups (n=8, $*p < 0.05$).

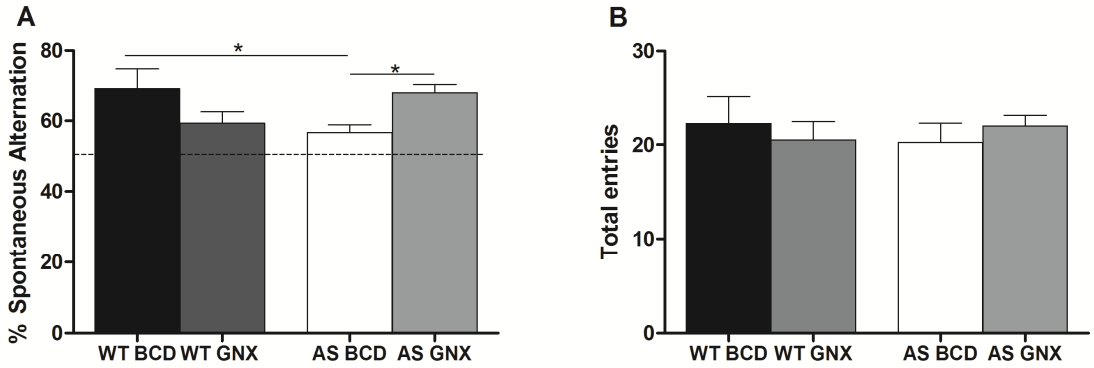
Figure 4. Chronic ganaxolone treatment increases hippocampal LTP induction and maintenance without changes in synaptic transmission. (A) Input-output curves at

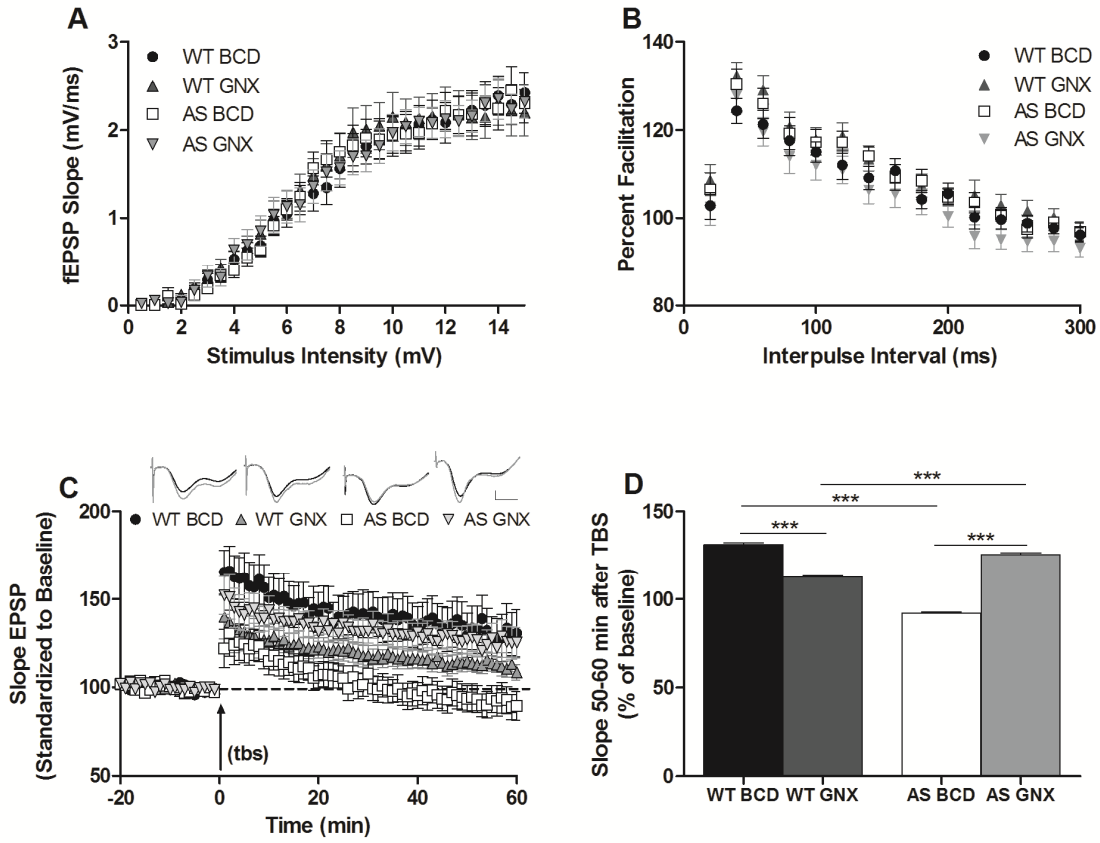
hippocampal SC-CA1 synapses in WT, WT GNX, AS, and AS GNX-treated mice. There were no significant difference between experimental groups. (B) Short-term synaptic plasticity was evaluated by the amount of PPF with IPIs ranging from 20 to 300 ms. There were no significant differences between experimental groups. (C) Long-term potentiation induced by 5 trains of theta-burst stimulation (tbs; arrow). Representative traces are shown for all groups at baseline (black trace) and 50 minutes after tetanic stimulation (grey trace). Scale bar = 1 mV and 5 ms. (D) LTP induction calculated between 50 and 60 min after theta-burst stimulation. Data expressed as mean ± SEM. (WT = 21 slices, n = 4 mice; WT GNX = 22 slices, n = 5 mice; AS = 16 slices, n = 4 mice; AS GNX = 13 slices, n = 3 mice; $p < 0.001$).

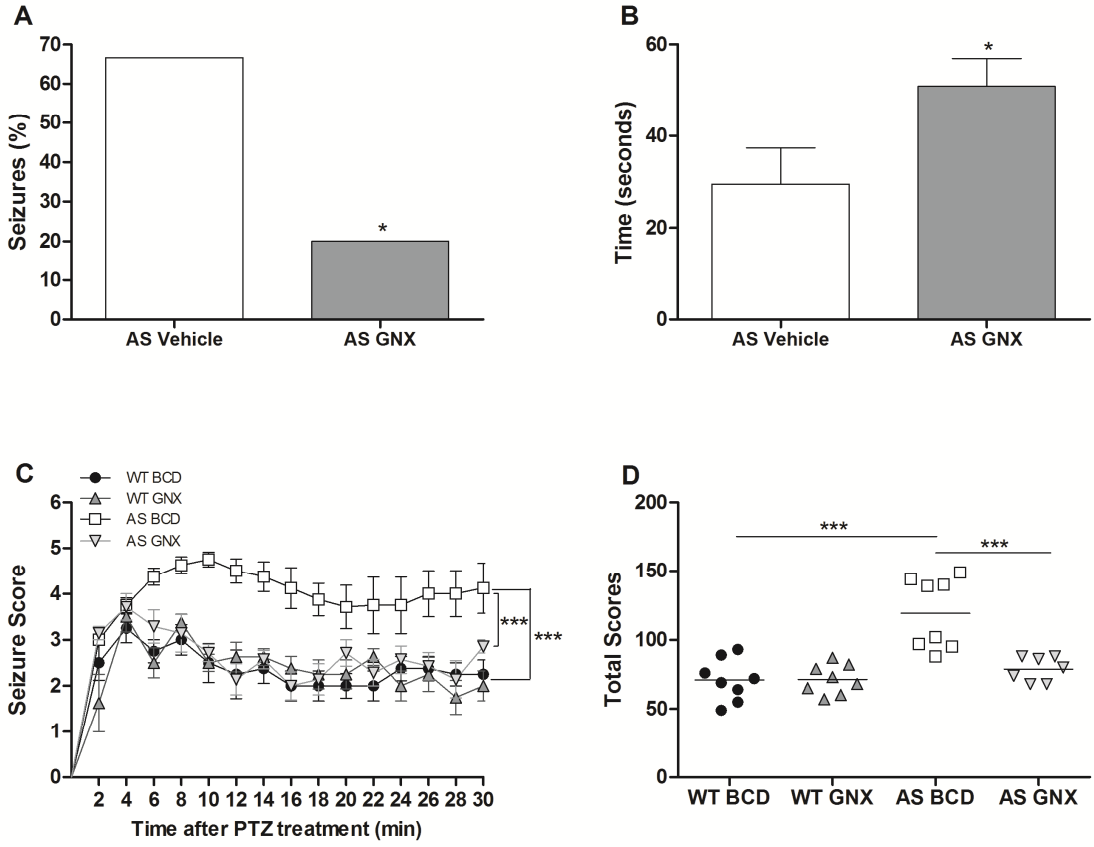
Figure 5. Chronic ganaxolone administration attenuates enhanced seizure susceptibility of AS mice. (A) AS mice treated chronically with GNX for 4 weeks exhibited a reduced frequency of behavioral seizures in response to 115 dB sound stimulation compared with BCD vehicle treated control mice. (B) Latency to seizure following sound stimulation was significantly increased in AS GNX mice compared with BCD vehicle treated controls (AS: n=9; AS GNX: n=10; *$p < 0.05$). (C) AS mice were more susceptible to PTZ-induced seizures than AS GNX and WT control animals. Data points represent mean ± SEM of highest seizure score at successive 2 min intervals following PTZ treatment. (D) Total seizure scores of each animal. The highest total scores were recorded in AS control mice compared to WT controls and AS-treated mice (WT, WT GNX, and AS: n=8; AS GNX: n=7).











- Ganaxolone was anxiolytic, anticonvulsant, and improved motor deficits in AS mice
- Four weeks of ganaxolone treatment recovered spatial working memory and hippocampal LTP deficits
- Tolerance did not develop to the therapeutic effects of ganaxolone
- Modulation of extrasynaptic $GABA_A$ receptors may provide symptomatic benefits in AS