

AWARD NUMBER: W81XWH-15-1-0190

TITLE: Neurosteroids Reverse Tonic Inhibition Deficits in Fragile X Syndrome

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REPORT DATE: October 2017

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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OMB No. 0704-0188

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1. REPORT DATE October 2017			2. REPORT TYPE Final		3. DATES COVERED 15 July 2015 - 14 July 2017	
4. TITLE AND SUBTITLE Neurosteroids Reverse Tonic Inhibition Deficits in Fragile X Syndrome					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER W81XWH-15-1-0190	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr Paul A. Davies E-Mail: paul.davies@tufts.edu					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tufts University School of Medicine 136 Harrison Ave Boston MA 02111-1817					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT Our preliminary studies demonstrate that Fmr1 KO mice at p21 have a decrease in phosphorylation of S443 in the $\alpha 4$ subunit compared to WT and an increase in the phosphorylation of $\beta 3$ subunits at the S408/409 site compared to WT. We have previously showed that phosphorylation of these residues changes the trafficking of the subunits so the changes observed in Fmr1 KO mice would predictably have consequences for trafficking of these essential inhibitory subunits. We noted that there was a significant decrease in tonic inhibition in dentate gyrus granule cells in p21 Fmr1 KO mice compared to WT. A 10 min exposure to neuroactive steroids followed by a 30 min wash induced a >3 fold increase in tonic current in Fmr1 KO animals which was prevented with PKC inhibition. Using a perforated multi-electrode array we have observed in cortical-hippocampal slices seizure like events (SLE) propagating through the dentate gyrus and into the CA3 and CA1 regions of the hippocampus of Fmr1 KO mice but in WT mice SLEs did not propagate through the dentate gyrus. We predict the increase neuronal excitability seen in Fmr1 KO mice is due to the deficits in tonic inhibition.						
15. SUBJECT TERMS GABA receptor, protein kinase C (PKC), Neuroactive steroid, tonic inhibition, dentate gyrus, seizures						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 42	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)	

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1. Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability. In addition, there is increased incidence of anxiety, sleep irregularities, and seizure activity. The underlying cause of FXS is a loss of the fragile X mental retardation protein (FMRP). Studies from both FXS patients and animal models have revealed reduced expression levels of extrasynaptic GABA_AR $\alpha 4$ and δ subunits with a reduced efficacy of tonic inhibition. Neuro-active steroids (NAS) are known allosteric modulators of GABA_AR channel function but recent studies from our laboratory have revealed that NAS also exert persistent effects on the efficacy of tonic inhibition by increasing the PKC-mediated phosphorylation of the $\alpha 4$ subunit which increases the membrane expression and boosts tonic inhibition. We have used a combination of biochemical and electrophysiological methods to assess alterations in GABAergic signaling in the hippocampus of FMRP knock-out mouse (*Fmr1 KO*), a widely validated model of the human syndrome. In addition, we propose an innovative way of reversing the reduced tonic inhibition by boosting GABA_AR trafficking to produce a sustained increase in tonic current with the use of NASs including a novel NAS which has improved bioavailability, and pharmacokinetic properties.

2. Keywords

GABA receptor, protein kinase C (PKC), Neuroactive steroid, tonic inhibition, dentate gyrus, seizures

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: Determine the phosphorylated state and cell surface accumulation of GABA_AR subunits in FXS mice and the role of neurosteroids in altering subunit expression.

1. To analyze the NS-mediated changes in phosphorylation of GABA_ARs .
2. To test the role that NS exposure plays in regulating the cell surface stability of GABA_ARs.

Specific Aim 2. Determining exposure to neurosteroids enhances the efficacy of tonic inhibition in FXS.

1. Measure the efficacy for NS to rescue the deficits of tonic inhibition in *Fmr1 KO* mice.
2. Characterize the changes to phasic inhibition in dentate gyrus from *Fmr1 KO* mice.
3. To examine the deficit of tonic inhibition on circuit excitability in *Fmr1 KO* mice compared to WT and impact of NS treatment.

Specific Aim 3. Ascertaining behavioral phenotypes in *Fmr1 KO* mice will be reversed with NS exposure.

1. To examine audiogenic seizures in *Fmr1 KO* mice following NS treatment.
 2. To compare inappropriate social behavior in *Fmr1 KO* and WT mice.
- Milestone #1: Manuscript on the findings from the study. 18-24 months of study.

What was accomplished under these goals?

We have published our findings on phosphorylation of S408/409 residues on the $\beta 3$ subunit in the *Fmr1 KO* mouse in Vien et al., 2015. The $\beta 3$ subunit, along with $\alpha 4$ subunits, are major subunits contributing to extrasynaptic GABA_ARs. Phosphorylation of these residues decreases the endocytosis of GABA_ARs containing the $\beta 3$ subunit. We assessed the importance of these residues by creating a mouse with the residues mutated to alanines (S408/9A) which also reduces endocytosis. The mouse exhibited autistic like properties such as increased repetitive behavior, decreased social interaction, and altered dendritic spine morphology. Because of the comorbidity of seizures in autism we examined seizure activity in the S408/9A mouse. The mice exhibited increase sensitivity to kainic acid-induced seizures. Similar to our preliminary findings in the *Fmr1 KO* mouse, the S408/9A mouse also had reduced tonic currents and increased phasic currents in dentate gyrus granule cells. Furthermore, we observed a decrease in $\alpha 4$ subunit immunoreactivity in hippocampal sections from S408/9A mouse. These data demonstrate that

changes in the phosphorylation of $\beta 3$ subunits can alter GABA_AR expression and contribute to the pathophysiology of autistic spectrum disorders.

Specific Aim 1. *Determine if the alterations in the phosphorylation state of GABA_AR subunits in *Fmr1* KO mice impacts the cellular distribution within the hippocampus. (PI: Moss).*

***Fmr1* KO mice have age dependent alterations in hippocampal GABA_AR subunit phosphorylation and expression.** We have previously reported a reduction in the GABA_AR-mediated tonic current recorded from hippocampal dentate gyrus neurons. To assess if these modifications reflect alterations in the phosphorylation and/or stability of GABA_ARs, we compared these parameters in hippocampal slices from both WT and *Fmr1* KO genotypes. We examined the phosphorylation of S443 in the $\alpha 4$ subunit, in addition to S408/9 in the $\beta 3$ subunit. These residues are accepted substrates of PKC and their phosphorylation plays key roles in regulating the membrane trafficking of GABA_ARs. In the case of S443 its phosphorylation acts to increase insertion of $\alpha 4$ subunit containing GABA_ARs receptors into the plasma membrane and sustained increase in tonic inhibition. In contrast, phosphorylation of S408/9 acts to reduce receptor endocytosis increasing their accumulation at inhibitory synapses (Abramian et al 2014, Abramian et al 2010, Adams et al 2015).

Hippocampal slices from both genotypes were immunoblotted with an $\alpha 4$ subunit antibody to measure total levels, and a phospho-specific antibody produced against S443 (pS443), which was raised in rabbits against a synthetic peptide derived from the murine $\alpha 4$ subunit in which S443 was phosphorylated (PGSLGSASTRPA). For the $\beta 3$ subunit, samples were blotted with pS408/9 and $\beta 3$ subunit antibodies as detailed previously (Jovanovic et al 2004). The ratios of pS443/ $\alpha 4$ and pS408/9/ $\beta 3$ subunit immunoreactivity were compared between genotypes. We also examined the phosphorylation of S383 in the $\beta 3$ subunit, which is a substrate of CamKII, but not PKC, using the respective phospho-specific antibody pS383 (Saliba et al 2012).

To measure the expression levels of GABA_ARs that mediate phasic inhibition we measured levels of the α 1, α 2 and γ 2 subunit, and for tonic inhibition we employed antibodies against the α 4, α 5 and δ subunits. The levels of the receptor β 1, β 2 and β 3 subunits that are components of subtypes that mediate both forms of GABAergic inhibition were also compared between genotypes.

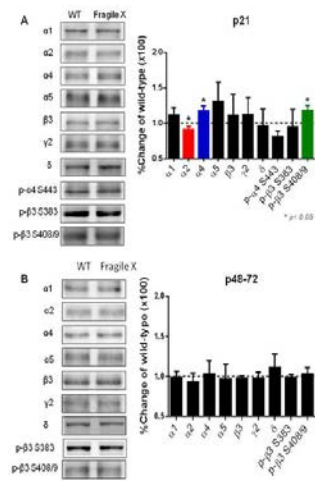


Figure 1. Comparison of GABA_AR expression and phosphorylation in Fmr1 KO mice. 50 μ g of SDS-soluble hippocampal extracts from (A) p21, (B) p48-72 C57/B16 Fmr1 KO mice, or WT controls were subject to immunoblotting with antibodies against the GABA_AR, α 1, α 2, α 4, α 5, β 3, γ 2 and δ . Total subunit expression levels were then normalized to WT. In addition, extracts were immunoblotted with p- α 4 S443, p- β 3 S408/9 and p- β 3 383 antibodies. The ratios of pS409/9/ β 3 and pS383/ β 3 immunoreactivity were compared and normalized to values seen in WT mice. * = significantly different to control $p < 0.05$ (t-test; $n = 3-8$ mice).

Using immunoblotting we compared the expression levels and phosphorylation of GABA_AR subunits in Fmr1 KO mice. We measured these parameters in hippocampal extracts from p21 C57 Fmr1 KO mice, a developmental time point at which these mice exhibit an accepted seizure phenotype (Fig. 1). Immunoblotting revealed that phosphorylation of S443 in the α 4 subunit was significantly decreased in Fmr1 KO mice $80 \pm 9\%$ of control ($n = 4$ for WT and Fmr1 KO $p = 0.001$), while that for total α 4 subunit levels and β 3 S408/9 was increased $118 \pm 4\%$ of control, and $120 \pm 6\%$ of control respectively ($n = 6$ for WT and Fmr1 KO; $p = 0.005$). In contrast, phosphorylation of β 3 S383 was comparable between genotypes. Interestingly, total α 2 was decreased to $91 \pm 5\%$ of WT ($n = 5$ WT, $n = 4$ Fmr1 KO; $P = 0.04$). In contrast to this, the total expression levels of α 1, α 5, β 3, δ and γ 2 subunits were comparable between genotypes. Finally, we examined if these changes in GABA_AR expression level and phosphorylation persist in adult mice. Significantly, in p48-72 C57 Fmr1 KO mice did not exhibit any deficits in

GABA_AR expression levels or phosphorylation (Fig. 1B). This experiment demonstrates that there are alterations in the phosphorylation and expression levels of GABA_ARs in *Fmr1 KO* mice.

Synaptic targeting of $\alpha 4$ subunit containing GABA_ARs in *Fmr1 KO* mice. Because changes in subunit phosphorylation can affect the trafficking of GABA_ARs in the membrane we examined the cellular distribution of the $\alpha 4$ subunit. We stained 40 μm hippocampal slices with antibodies against the $\alpha 4$ subunit together with antibodies against the inhibitory postsynaptic inhibitory protein gephrin. Using confocal microscopy coupled with *Metamorph* analysis we examined the $\alpha 4$ subunit/gephyrin positive puncta in the dentate gyrus between genotypes.

As illustrated in Fig. 2, there appeared to be an increase in $\alpha 4$ subunit immunoreactivity at gephrin positive structures in the brains of *Fmr1 KO* mice suggesting an abnormal redistribution of extrasynaptic $\alpha 4$ subunit to synaptic locations.

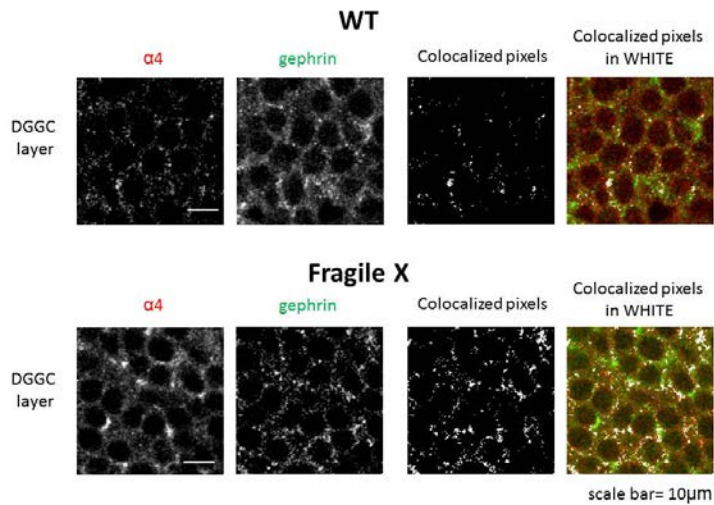


Figure 2. Characterization of $\alpha 4$ -containing inhibitory synapses in *Fmr1 KO*. 40 μm hippocampal slices were stained with antibodies against the GABA_AR $\alpha 4$ subunit and gephrin, a marker of inhibitory synapses. The number of $\alpha 4$ /gephyrin positive puncta was compared within the dentate gyrus between genotypes. In *Fmr1 KO* mice, the number of $\alpha 4$ colocalized with gephrin increased, consistent with an increase in the altered localization of $\alpha 4$ -containing GABA_ARs to inhibitory synapses.

NASs increase the phosphorylation and cell surface stability of GABA_ARs.

We have previously shown that the naturally occurring NAS, THDOC, increased tonic current in part through phosphorylation of S408/409 of the $\beta 3$ subunit ([Abramian et al., 2014](#); [Vien et al., 2015](#)). The

temporal effects of THDOC on GABA_AR phosphorylation were examined in hippocampal slices. S443 phosphorylation was significantly enhanced at 5 min, but returned to basal levels by 20 min. S408/9 phosphorylation did not reach statistical significance until 10 min, but was maintained at 20 min. NS dependent GABA_AR phosphorylation was blocked by GFX (Fig. 3A).

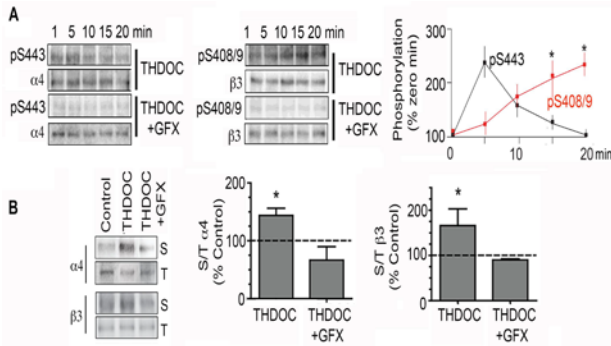


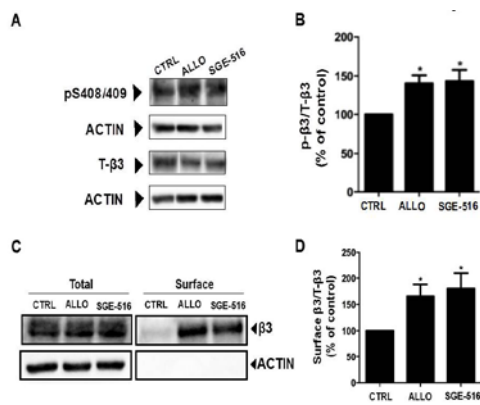
Figure 3. NASs potentiate phosphorylation and cell surface stability of GABA_AR. **A.** Hippocampal slices were treated with, 100 nM THDOC, or 100 nM THDOC with 50 μM GFX for 20 min. SDS soluble lysates were then immunoblotted with pS443, pS408/9, α4 and β3 subunit antibodies. The ratio of pS443/α4 and pS408/9/β3 immunoreactivity were then determined and normalized to values seen at zero time (100%). **B.** Slices were labeled with Sulfo-NHS-SS-biotin. After lysis and purification on

avidin, surface and total fractions were immunoblotted with α4 and β3 subunit antibodies. The ratio of surface:total immunoreactivity (S/T) for each subunit were then normalized to vehicle treated controls (100%; dotted line). * = significantly different to control p<0.05 (t-test, n= 6 mice).

In parallel with potentiating phosphorylation, THDOC increased the cell surface expression levels of both subunits, dependent upon PKC activity (Fig. 3B). Thus, our results suggest that NASs enhance the phosphorylation and cell surface accumulation of GABA_AR containing α4 and β3 subunits.

To further our understanding of the NAS-mediated increase in tonic current we measured the effects of ALLO, and SGE-516 on the phosphorylation and membrane expression of β3 subunits in hippocampal slices. Twenty min exposure to 100nM ALLO, or SGE-516 increased phosphorylation of β3 subunits at S408/409 to 141 ± 10% of control (n=10, p<0.01), and 143 ± 14% of control (n=4, p<0.05) respectively (Fig. 4A&B).

Figure 4. NAS exposure increases phosphorylation and surface expression of $\beta 3$ subunits. A. Exposure to 100 nM of the NASs, ALLO or



SGE-516, for 20 min increases $\beta 3$ S408/409 phosphorylation in acute hippocampal slices. B. The ratio of p- $\beta 3$ /T- $\beta 3$ was measured and values were normalized to those in control (100%). Asterisks represent a significant difference from control (ALLO: $p < 0.01$, Student's *t* test, $n=10$; SGE-516: $p < 0.05$, Student's *t* test, $n=4$). C. Exposure to 100 nM ALLO or SGE-516 for 20 min increases GABA_A- $\beta 3$ -containing receptors at the plasma membrane in acute hippocampal slices. D. The ratio of surface $\beta 3$ /T- $\beta 3$ was measured and values were normalized to cell surface levels in control treated slices (100%). Asterisks represent a significant difference from

control (ALLO: $p < 0.05$, Student's *t* test, $n=8$; SGE-516: $p < 0.05$, Student's *t* test, $n=4$).

In parallel with modulating phosphorylation, exposure to 100 nM ALLO, or SGE-516 increased the cell surface expression levels of receptors containing $\beta 3$ subunits to $166 \pm 22\%$ of control ($n=8$, $p < 0.05$), and $180 \pm 29\%$ of control ($n=4$, $p < 0.05$) respectively (Fig. 4C&D). Therefore, in parallel with inducing sustained effects on GABAergic inhibition ALLO and SGE-516 enhance the cell surface levels and phosphorylation of the GABA_ARs containing $\beta 3$ subunits dependent upon PKC activity.

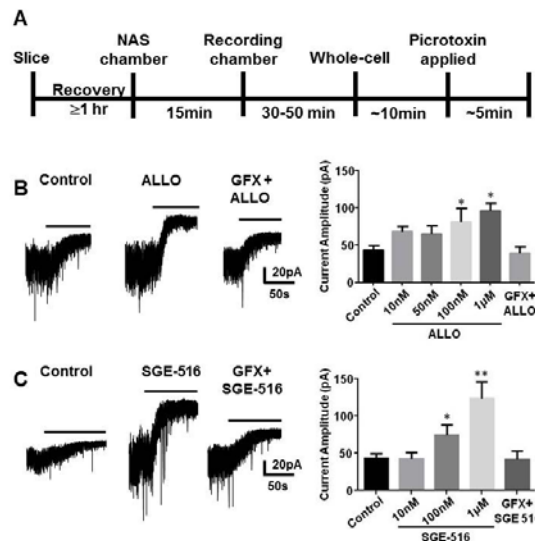
Specific Aim 2. Determining exposure to neurosteroids enhances the efficacy of tonic inhibition in FXS. (PI: Davies)

Neuroactive steroids reverse the deficits in tonic inhibition. We analyzed the sustained effects of ALLO, or the new synthetic NAS SGE-516 on the tonic current in DGGCs in hippocampal slices from 3-5 week old C57 male mice. Hippocampal slices were incubated for 15 min in a chamber containing NASs dissolved in ACSF. Slices were then transferred to the recording chamber of the microscope followed by a wash period between 30 to 60 mins of continuous perfusion of NAS-free ACSF before recordings were started (Fig. 5A).

Slices exposed to ALLO, or SGE-516 demonstrated a concentration-dependent increase in the tonic current measured by the addition of picrotoxin with the maximal effects at 1 μ M. Control, vehicle-

treated slices had a tonic current of 43.6 ± 5.7 pA ($n=12$), whereas the tonic currents for slices treated with SGE-516 ($1 \mu\text{M}$) was 123.0 ± 22.2 pA, ($n=6$, $p= 0.0003$), or ALLO ($1 \mu\text{M}$) was 95.8 ± 10.8 pA ($n=4$, $p= 0.0005$, Fig. 5B&C).

Figure 5. NAS-mediated metabotropic enhancement of tonic inhibitory current in DGGC neurons. A. Scheme demonstrating experimental protocol. Hippocampal slices were allowed to recover for at least



1 hr following slicing. Slices were then incubated for 15 min in a chamber containing NASs dissolved in ACSF. Slices were then transferred to the recording chamber of the microscope followed by a wash period between 30 to 60 min of continuous perfusion of NAS-free ACSF before recordings were started. Recordings were made from DGGCS in hippocampal slices from p21-35 C57 mice in the presence of $1 \mu\text{M}$ GABA followed by $100 \mu\text{M}$ picrotoxin and the difference in holding current was then determined. **Left panel B & C.** Example tonic currents from slices following exposures to vehicle (control) or 100 nM ALLO (B), 100 nM SGE-516 (C) for 15 min. No change in tonic current was observed in slices pre-incubated for 15 min with GFX followed by ALLO,

or SGE-516. Bar above current represents application of picrotoxin ($100 \mu\text{M}$). **Right panel B & C.** Bar graph shows average tonic current was significantly enhanced following exposure to different concentrations of ALLO and SGE-516. In all panels * = significantly different to control ($p < 0.05$; t -test, $n=4-12$ cells).

Synaptic targeting of $\alpha 4$ subunit containing GABA_ARs in *Fmr1* KO mice. We have previously noted that IPSC biophysical properties changed in the *Fmr1* KO mouse, with the IPSC amplitude increasing and the decay prolonging compared to wild-type. The data presented above shows an increase in $\alpha 4$ subunit immunoreactivity at gephyrin positive structures in the hippocampus suggesting the $\alpha 4$ subunit at synapses. We have assessed the effects of the benzodiazepine diazepam (which enhance $\alpha\beta\gamma 2$ GABA_ARs where α can be 1-3 subunits) on sIPSC amplitude, and decay in WT and *Fmr1* KO mice. Additionally, we have examined the $\alpha 4\beta\gamma 2$ specific benzodiazepine Ro15-4513 on sIPSC properties between genotypes.

In wild type mice, diazepam significantly enhanced IPSC amplitude and prolonged decay whereas in *Fmr1 KO* mice only decay was prolonged (Fig 6A&B). Conversely, in the presence of Ro15-4513, IPSC amplitude and decay were unchanged in wild type mice but were significantly enhanced in the *Fmr1 KO* mice (Fig 6C&D).

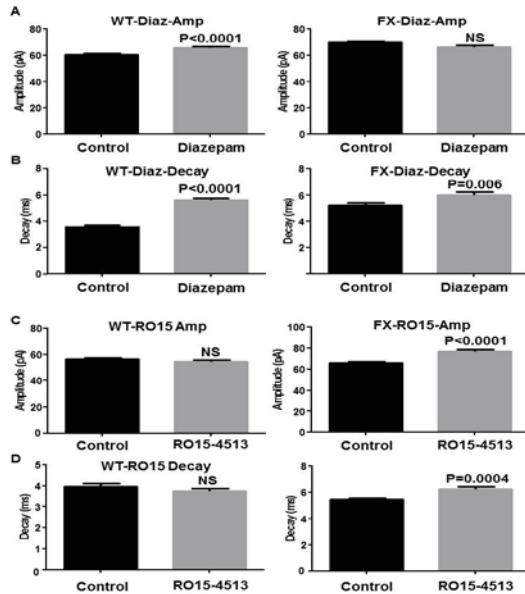


Figure 6. Modulation of DGGC IPSCs by benzodiazepines. Recordings were made from DGGCs in hippocampal slices from p21-35 WT, or *Fmr1 KO* mice. *Fmr1 KO* mice displayed larger sIPSC amplitudes and longer decay times compared to age-matched controls. Bar graphs show the effects of acute (10min) exposures to diazepam (A&B) or Ro15-4513 (C&D) applied to slices from wild-type (left column) and *Fmr1 KO* (right column) mice.

These data suggest the involvement of $\alpha 4$ subunit in synaptic GABA_ARs. This is a new finding in the FXS field and could explain the poor clinical effects of

benzodiazepines because $\alpha 4$ subunits are insensitive to the modulation by most benzodiazepines.

The impact of modified GABAergic inhibition on circuit excitability in *Fmr1 KO* mice. We have examined the circuit excitability of the hippocampus using a perforated multi-electrode array (pMEA) system. A deficit in tonic current observed in *Fmr1 KO* mice is hypothesized to reduce tonic inhibition which would increase the overall circuit excitability by increasing the likelihood of neurons to fire action potentials. It has been proposed that in FXS there is an increase in neuronal excitability and synchrony across various brain structures ([Goncalves et al 2013](#)). Our preliminary data demonstrating a reversal of the tonic current deficit in *Fmr1 KO* with NS treatment would be expected to restore tonic inhibition and reduce the excessive circuit excitability.

Seizure-like events ([Padgett et al](#)) were induced by low Mg^{2+} artificial cerebrospinal fluid (ACSF) to relieve the Mg^{2+} block of NMDA receptors ([Traub et al 1994](#)). We obtained preliminary data with a

pMEA on loan from the supplier. We recorded field potentials from horizontal cortical-hippocampal slices from WT and *Fmr1 KO* mice (Fig.7B).

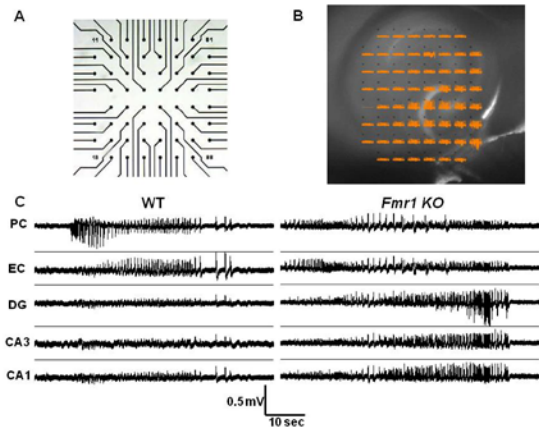


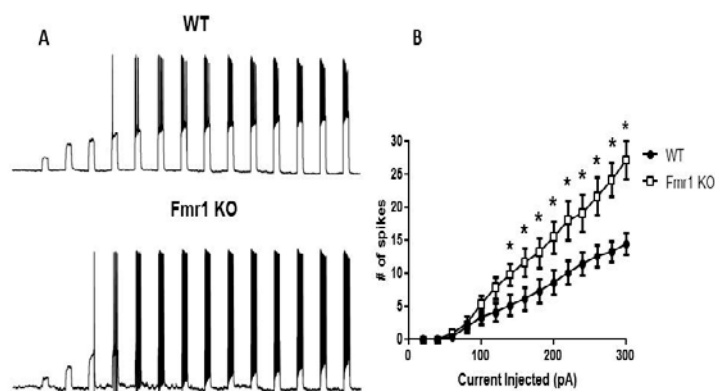
Figure 7. In vitro seizure like events (SLE) in hippocampal slices from WT and *Fmr1 KO* mice in the absence of Mg^{2+} using multi-electrode array (MEA) system. Hippocampal slices from *Fmr1 KO* and WT mice were tested for seizure like events (SLE) in absence of Mg^{2+} using MEA. A. Multi-electrode array chamber B. MEA-layout (iii). Typical slice on MEA-layout (iv) MEA stage & amplifier. C. Traces showing the first SLEs from the dentate gyrus (DG), CA3 neurons (CA3), CA1 neurons (CA1), entorhinal cortex (EC), postrhinal cortex (PC) regions of the hippocampal slices from WT and *Fmr1 KO* mice.

SLEs were induced by perfusing the recording chamber with low Mg^{2+} ACSF. SLEs were characterized by tonic-clonic discharges, with an initial tonic phase followed by a period of clonic after-discharges. Interestingly, we observed a dramatic regional difference in SLE propagation (Fig. 7C). In the WT slices, the first SLE occurred in the entorhinal cortex (EC) and postrhinal cortex (PC) but did not show full propagation through the dentate gyrus (DG). In contrast, the first SLEs in *Fmr1 KO* slices propagated through the DG, indicating an increased potential susceptibility of DG recruitment during onset of epileptiform activity in slices from *Fmr1 KO* mice. The DG is known to be important for epileptogenesis because it forms the so-called dentate-gate that prevents activity spreading from cortex to the hippocampus. Tonic inhibition in the dentate gyrus seems to be critical in preventing excessive neuronal excitability.

Increased excitability of granule cells in *Fmr1 KO* mice. Neuronal excitability is greatly influenced by tonic inhibition. Because of the decrease in DGGC tonic inhibition observed in *Fmr1 KO* mice we next examined input-output curves on DGGCs to measure the intrinsic excitability of the neurons. We delivered current step injections to depolarize the membrane and measured the action potentials elicited from those current injections. There was a clear increase in action potential firing rate at lower current injections in DGGCs from *Fmr1 KO* mice compared to WT DGGCs, with a leftward shift in the relationship between firing rate and injected current (Fig 8).

The ability of NASs to reverse the deficit in tonic current would suggest that NAS exposure could reduce neuronal excitability. Hippocampal slices were exposed to SGE-516 for 15 min followed by a >30 min washout period before the input-output relationship was examined. SGE-516 exposure reduced membrane excitability of DGGCs from *Fmr1 KO* mice, causing a rightward shift in the input-output curve similar to that of vehicle treated WT DGGCs.

Figure 8. DGGCs from *Fmr1 KO* mice are more excitable than from WT mice. *A.* Representative recordings of action potential firing in



*DGGCs from WT and *Fmr1 KO* mice in response to 0.5s current injections from 20 to 300 pA in 20 pA increments. B.* Average input/output curves representing the average number of action potentials from WT (black circles) and *Fmr1 KO* mice (open boxes). Values are means \pm SE; $n = 2$ mice, 6 cells per experimental group. * $P < 0.05$ denotes statistical significance compared with WT littermates using Student's *t*-test.

Specific Aim 3. Ascertaining behavioral phenotypes in *Fmr1 KO* mice will be reversed with NS exposure. (PI: Davies).

Recently, a publication from Sage Therapeutics demonstrated the effectiveness of SGE-516 in protecting *Fmr1 KO* mice against audiogenic seizures (Hammond et al., 2017). Rather than repeat these experiments we examined the EEG activity in *Fmr1 KO*. There are very few published reports on EEG activity from these mice which could provide valuable data on behavior based on electrical activity. In human FXS patients abnormal EEG data in childhood has been suggested as a predictor of anxiety in later life (Sinclair et al., 2017).

Examine the effects of NAS exposure on electroencephalogram activity of *Fmr1 KO* mice. FXS patients have sleep disturbances and seizures in these patients occur more frequently during sleep

([Musumeci et al 1990](#), [Musumeci et al 1999](#)). In the *Fmr1 KO* mouse model of FXS it has been shown that there are abnormal neuronal firing rates, particularly during sleep ([Goncalves et al 2013](#), [Pavlov et al 2009](#)). Electroencephalogram (EEG), and electromyogram (EMG) electrodes were inserted into mice anesthetized with isoflurane. Following acclimation, EEG/EMG activity was continuously monitored for 3 hours.

Preliminary studies demonstrated that EEG activity across all frequencies was increased in *Fmr1 KO* mice compared to WT (Fig. 9). Administration of a single injection of SGE-516 (3 mg/kg i.p.) increased θ and γ activity in WT but decreased the δ activity in *Fmr1 KO* mice.

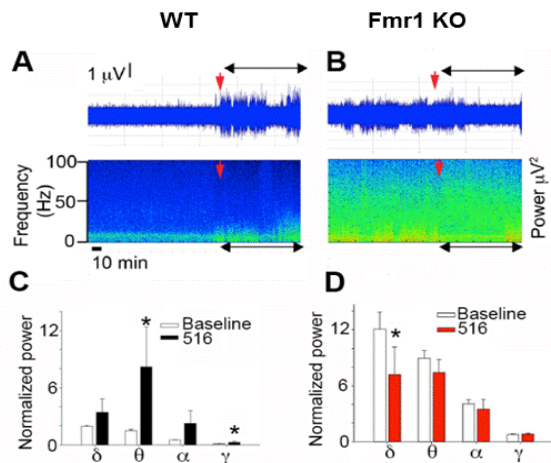


Figure 9. Effects of SGE-516 injection on EEG recordings from WT and *Fmr1 KO* mice EEG recording and corresponding spectrogram for a single WT mouse (A) and *Fmr1 KO* mouse (B). SGE-516 (3mg/Kg i.p.) was injected at 80 min (red arrow). C&D. Cumulative average of specific bands for WT and *Fmr1 KO* mice respectively (n=4). Scales are normalized power (μV^2) for 1 hr before injection and 1 hr after. A 2-way ANOVA was used to calculate significance * $p < 0.05$. All recordings were performed at 1000Hz using a Pinnacle headmount preamplifier.

The synchronized cortical activity has been explained by an increase in neuronal firing rates and predisposes the animal to seizures, hyperactivity, and a reduced computational ability of the cerebral cortex ([Goncalves et al 2013](#)). We believe NAS treatment will normalize EEG activity due to the increased tonic inhibition preventing the increase in neuronal firing.

What opportunities for training and professional development has the project provided?

Dr. Amit Modgil participates in lab meetings, presenting data on a regular basis. Amit will also present his findings to the whole Neuroscience Department once a year in our post-doctoral research seminar

series. He also attends weekly department seminars. In addition the postdoctoral program at Tufts runs a weekly career development seminar series. Speakers in this series are drawn from the local pharmaceutical and biotechnology industry in the local area, which highlights the range of career opportunities for life scientist with doctoral degrees. He also attends didactic and tutorial courses related to animal care and use, laboratory safety, radiation safety and the responsible conduct of research.

Dr Amit Modgil was selected to give an oral presentation at the Gordan Research Seminar for “Fragile X and Autism-Related Disorders”. Dr Modgil also presented a poster at the Gordan Research Conference for “Fragile X and Autism-Related Disorders” in Vermont, June 2016.

How were the results disseminated to communities of interest?

Dr Modgil and Dr Davies presented a poster at the “Neuro Developmental Disorders symposium” at Harvard Medical School, October 2015.

Dr Amit Modgil gave an oral presentation at Gordan Research Seminar for “Fragile X and Autism-Related Disorders”. Dr Modgil and Dr Davies presented posters at the Gordan Research Conference for “Fragile X and Autism-Related Disorders” in Vermont, June 2016.

Findings from the study are being submitted as a manuscript.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

4. Impact

What was the impact on the development of the specific discipline(s) of the project?

The $\alpha 4$ subunits are usually thought to be located extrasynaptically mediating tonic inhibition. In FXS it is believed that the expression of $\alpha 4$ subunit containing GABA_ARs is reduced, thereby decreasing the

contribution of tonic inhibition to the overall inhibition in neurons resulting in excessive excitation. However, our data has suggested that the change in phosphorylation of subunits in *Fmr1 KO* mice leads to a redistribution of subunits which cause a reduction of tonic current and an alteration of phasic inhibition. The relocation of the $\alpha 4$ subunit, in particular, to synaptic sites not only alters phasic current biophysics it also changes the pharmacological response to benzodiazepines. This has not been observed before in FXS models and can help explain the lack of benzodiazepine efficacy in FXS. Our data also point to tonic inhibition contributing significantly to the “dentate gate”, an important characteristic of the dentate gyrus in limiting excessive neuronal activity flowing into the hippocampus and out back into the cortex.

What was the impact on other disciplines?

The breakdown of the dentate gate because of the redistribution of the $\alpha 4$ subunit and reduction of tonic current will impact the knowledge in the epilepsy field. Neuroactive steroids are known to have many impacts on health, including anxiety, depression, and neurogenesis.

What was the impact on technology transfer?

This project could impact the therapeutic targets used by pharmaceutical companies. Most GABAergic compounds to date have been developed as positive allosteric modulators. However, this project indicates that mechanisms which influence trafficking of subunits would be a valid therapeutic target.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Nothing to Report

6. Products

Publications resulting from the work under this award:

Vien TN, Modgil A, Abramian AM, Jurd R, Walker J, Brandon NJ, Terunuma M, Rudolph U, Maguire J, **Davies PA**, Moss SJ (2015). Compromising the phosphodependent regulation of the GABAAR $\beta 3$ subunit reproduces the core phenotypes of autism spectrum disorders. *Proc Natl Acad Sci U S A.*, 112:14805-14810. Published; acknowledged federal support.

Modgil A, Parakala ML, Ackley MA, James J. Doherty JJ, Stephen J. Moss SJ, **Davies PA** (2017). Endogenous and synthetic neuroactive steroids evoke sustained increases in the efficacy of GABAergic inhibition via a protein kinase C-dependent mechanism. *Neuropharmacology*, **113**:314-322.; acknowledged federal support.

Other publications during reporting period:

Kelley MR, Deeb TZ, Brandon NJ, Dunlop J, **Davies PA**, Moss SJ (2016). Compromising KCC2 transporter activity enhances the development of continuous seizure activity. *Neuropharmacology*, **108**:103-110.

Nakamura Y, Morrow DH, Modgil A, Huyghe D, Deeb TZ, Lumb MJ, **Davies PA**, Moss SJ. Proteomic Characterization of Inhibitory Synapses Using a Novel pHluorin-tagged γ -Aminobutyric Acid Receptor, Type A (GABAA), $\alpha 2$ Subunit Knock-in Mouse. *J Biol Chem.* 2016;291:12394-407. Published; acknowledged federal support.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name: Paul A. Davies
Project Role: co-PI, Initiating PI
Researcher Identification (ORCID ID): [0000-0002-3973-3143](https://orcid.org/0000-0002-3973-3143)
Nearest person month worked: 3 calendar months
Contribution to Project: Has designed experiments and performs analysis and interpretation of the results. He also supervises other members of the research team and is responsible for writing reports and producing manuscripts.

Funding Support: NIH-NIMH R01 MH097446

Name: Stephen J. Moss
Project Role: co-PI
Researcher Identification:
Nearest person month worked: 0.5 calendar months
Contribution to Project: Supervising the experiments designed to assay the effects of neurosteroids on GABA_AR phosphorylation and cell surface accumulation.

Funding Support: NIH-NIDA DA037170-01, NIH-NIMH R01 MH097446, NIH-NINDS R01 NS081986, NIH-NINDS, 1R01NS087662, AstraZeneca; IMED/GABA_BR drug development.

Name: Amit Modgil
Project Role: Postdoctoral Researcher
Researcher Identification:
Nearest person month worked: 6 calendar months
Contribution to Project: Has performed the electrophysiology experiments, making hippocampal slices and examining the effects of their exposure to neurosteroids.

Funding Support: Funded from R01 MH097446

Name: Manasa L. Parakala

Project Role: Graduate student

Researcher Identification:

Nearest person month worked: 6 calendar months

Contribution to Project: Has performed the biochemistry experiments examining GABA_AR phosphorylation and cell surface accumulation.

Funding Support: Funded from R01 MH097446

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: SAGE Therapeutics

Location of Organization: Cambridge, MA

Partner's contribution to the project:

Collaboration. SAGE Therapeutics share the necessary proprietary compounds and data necessary to support the success of this project

8. Special Reporting Requirements

Collaborative Award

9. Appendices

Vien TN, Modgil A, Abramian AM, Jurd R, Walker J, Brandon NJ, Terunuma M, Rudolph U, Maguire J, Davies PA, Moss SJ. Compromising the phosphodependent regulation of the GABAAR β 3 subunit reproduces the core phenotypes of autism spectrum disorders. *Proc Natl Acad Sci U S A*. 2015;112:14805-14810.

Modgil A, Parakala ML, Ackley MA, James J. Doherty JJ, Stephen J. Moss SJ, Davies PA (2017). Endogenous and synthetic neuroactive steroids evoke sustained increases in the efficacy of GABAergic inhibition via a protein kinase C-dependent mechanism. *Neuropharmacology*, 113:314-322.



Endogenous and synthetic neuroactive steroids evoke sustained increases in the efficacy of GABAergic inhibition via a protein kinase C-dependent mechanism



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ARTICLE INFO

Article history:

Received 10 May 2016

Received in revised form

3 October 2016

Accepted 9 October 2016

Available online 12 October 2016

Keywords:

GABA receptor

Protein kinase C (PKC)

Neuroactive steroid

Tonic inhibition

Dentate gyrus

ABSTRACT

The neuroactive steroid (NAS) tetrahydrodeoxycorticosterone (THDOC) increases protein kinase C (PKC) mediated phosphorylation of extrasynaptic GABA_A receptor (GABA_AR) subunits leading to increased surface expression of $\alpha 4/\beta 3$ subunit-containing extrasynaptic GABA_ARs, leading to a sustained increase in GABA_AR tonic current density. Whether other naturally occurring and synthetic NASs share both an allosteric and metabotropic action on GABA_ARs is unknown. Here, we examine the allosteric and metabotropic properties of allopregnanolone (ALLO), and synthetic NASs SGE-516 and ganaxolone. ALLO, SGE-516, and ganaxolone all allosterically enhanced prototypical synaptic and extrasynaptic recombinant GABA_ARs. In dentate gyrus granule cells (DGGCs) all three NASs, when applied acutely, allosterically enhanced tonic and phasic GABAergic currents. In separate experiments, slices were exposed to NASs for 15 min, and then transferred to a steroid naïve recording chamber followed by ≥ 30 min wash before tonic currents were measured. A sustained increase in tonic current was observed following exposure to ALLO, or SGE-516 and was prevented by inhibiting PKC with GF 109203X. No increase in tonic current was observed with exposure to ganaxolone. In agreement with the observations of an increased tonic current, the NASs ALLO and SGE-516 increased the phosphorylation and surface expression of the $\beta 3$ subunit-containing GABA_ARs. Our studies demonstrate that neuroactive steroids have differential abilities to induce sustained increases in the efficacy of tonic inhibition by promoting GABA_AR phosphorylation and membrane trafficking dependent on PKC activity.

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1. Introduction

γ -Aminobutyric acid type A receptors (GABA_ARs) mediate both phasic and tonic inhibitory neurotransmission in the CNS and are the sites of action of benzodiazepines, barbiturates, general anesthetics and neuroactive steroids (NAS). GABA_ARs containing the $\alpha 4$ subunit are located primarily extrasynaptically in the dentate gyrus of the hippocampus, neocortex, striatum and the thalamus where they are persistently activated by low concentrations of GABA. They have distinct pharmacological properties that distinguish them from synaptic GABA_ARs (Glykys and Mody, 2007). Extrasynaptic

GABA_ARs are responsible for mediating the tonic inhibition that determines the gain and offset of the neuronal output, thus regulating the excitability of neurons and the activity of neuronal circuits (Belelli et al., 2009; Semyanov et al., 2004). In addition to regulating neuronal activity, $\alpha 4/\delta$ subunit-containing GABA_ARs are implicated in a multitude of neurological disorders including epilepsy, neurodevelopment disorders and anxiety disorders (Belelli et al., 2009; Brickley and Mody, 2012; D'Hulst and Kooy, 2007).

The endogenous NASs ALLO, and THDOC, and the synthetic NAS ganaxolone, are potent positive allosteric modulators (PAMs) of GABA_ARs. Consistent with their role as GABA_AR PAMs, NASs have dose dependent anxiolytic, anti-convulsant, hypnotic and sedative actions (Mitchell et al., 2008; Paul and Purdy, 1992). The ability of NASs to allosterically potentiate both phasic and tonic inhibition makes them attractive anticonvulsants however, despite these

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favorable properties the therapeutic potential of NASs is very limited because of their low bioavailability, and rapid clearance preventing the concentration in the brain to reach therapeutic levels (Rupperecht, 2014). Recently, Botella et al (Botella et al., 2015), developed a series of synthetic NASs with modification that highly improves their pharmacokinetic properties yet retain similar pharmacological profile as ALLO. Among those compounds, SGE-516 was developed with improved bioavailability, and pharmacokinetic properties.

In addition to their actions as PAMs, recent studies have demonstrated that THDOC has a metabotropic mechanism of action on GABA_ARs. THDOC increase the PKC-dependent phosphorylation of S443 in $\alpha 4$ subunits and S408/9 in $\beta 3$ subunits. Upon THDOC exposure, enhanced phosphorylation at S443 and S408/9 leads to a sustained increase in GABA_AR tonic current density due to increased receptor insertion into the plasma membrane and decreased receptor endocytosis (Abramian et al., 2010, 2014; Adams et al., 2015). In this study we sought to examine if the PKC-dependent metabotropic pathway previously identified with THDOC exposure is stimulated by another endogenous NAS, ALLO, the first generation synthetic NAS, ganaxolone, and a more recently developed synthetic NAS, SGE-516.

2. Methods

2.1. Recombinant GABA_A pharmacology

Cellular electrophysiology was used to measure the pharmacological properties of ALLO, ganaxolone and SGE-516 in heterologous cell systems. Compounds were tested for their ability to affect GABA mediated currents at a submaximal agonist dose (GABA EC₂₀ = 2 μ M).

LTK cells were stably transfected with the $\alpha 1\beta 2\gamma 2$ subunits of the GABA receptor and CHO cells are transiently transfected with the $\alpha 4\beta 3\delta$ subunits via the Lipofectamine method. Cells were passaged at a confluence of about 50–80% and then seeded onto 35 mm sterile culture dishes containing 2 ml culture complete medium without antibiotics or antimycotics. Cells were cultivated at a density that enabled the recording of single cells without visible connections to other cells.

Whole-cell currents were measured with HEKA EPC-10 amplifiers using PatchMaster software. Bath solution for all experiments contained (in mM): NaCl 137, KCl 4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, D-Glucose 10, pH 7.4 with NaOH. Intracellular (pipette) solution contained (in mM): KCl 130, MgCl₂ 1, Mg-ATP 5, HEPES 10, EGTA 5, pH 7.2 with KOH. During experiments, cells and solutions were maintained at room temperature (19 °C - 30 °C). For manual patch-clamp recordings, cell culture dishes were placed on the dish holder of the microscope and continuously perfused (1 ml/min) with bath solution. After formation of a Gigaohm seal between the patch electrode and the cell (pipette resistance range: 2.5 M Ω - 6.0 M Ω ; seal resistance range: >1 G Ω) the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior (whole-cell patch configuration).

Cells were voltage-clamped at a holding potential of -80 mV. GABA_A receptors were activated by 2 μ M GABA and compounds were sequentially applied at increasing concentrations for 30s prior to a 2s application of GABA. GABA and compounds were applied to cells via the Dynaflo perfusion system (Celletricon, Sweden). Test compounds were dissolved in DMSO to form 10 mM stock solution and serially diluted to 0.01, 0.1, 1, and 10 μ M in bath solution. There was no effect on GABA currents when DMSO was applied to cells at its maximal concentration in solution (0.1%). All concentrations of test compound were tested on each cell. The relative percentage potentiation was defined as the peak amplitude

in response to GABA EC₂₀ in the presence of test compound divided by the peak amplitude in response to GABA EC₂₀ alone, multiplied by 100.

2.2. Hippocampal slice preparation

Brain slices were prepared from 3- to 5-week-old male C57 mice. Mice were anesthetized with isoflurane, decapitated, and brains were rapidly removed and submerged in ice-cold cutting solution containing (mM): 126 NaCl, 2.5 KCl, 0.5 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 1.5 sodium pyruvate, and 3 kynurenic acid. Coronal 310 μ m thick slices were cut with the vibratome VT1000S (Leica Microsystems, St Louis, MO, USA). The slices were then transferred into incubation chamber filled with prewarmed (31–32 °C) oxygenated artificial cerebro-spinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 1.5 sodium pyruvate, 1 glutamine, 3 kynurenic acid and 0.005 GABA bubbled with 95% O₂ -5% CO₂. Slices were allowed to recover at 32 °C for at least 1hr before recording. Exogenous GABA was added in an attempt to standardize ambient GABA in the slice and provide an agonist source for newly inserted extrasynaptic GABA_ARs.

2.3. Electrophysiology recordings

After recovery, a single slice was transferred to a submerged, dual perfusion recording chamber (Warner Instruments, Hamden, CT, USA) on the stage of an upright microscope (Nikon FN-1) with a 40 \times water immersion objective equipped with DIC/IR optics. Slices were maintained at 32 °C and gravity-superfused with ACSF solution throughout experimentation and perfused at rate of 2 ml/min with oxygenated (O₂/CO₂ 95/5%) ACSF.

Whole-cell currents were recorded from the dentate gyrus granule cells (DGGCs) in 310- μ m-thick coronal hippocampal slices. Patch pipettes (5–7 M Ω) were pulled from borosilicate glass (World Precision Instruments) and filled with intracellular solution of the composition (in mM) as follows: 140 CsCl, 1 MgCl₂, 0.1 EGTA, 10 HEPES, 2 Mg-ATP, 4 NaCl and 0.3 Na-GTP (pH = 7.2 with CsOH). A 5 min period for stabilization after obtaining the whole-cell recording conformation (holding potential of -60 mV) was allowed before currents were recorded using an Axopatch 200B amplifier (Molecular Devices), low-pass filtered at 2 kHz, digitized at 20 kHz (Digidata 1440A; Molecular Devices), and stored for off-line analysis.

2.4. Electrophysiology analysis

For tonic current measurements, an all-points histogram was plotted for a 10 s period before and during 100 μ M picrotoxin application, once the response reached a plateau level. Recordings with unstable baselines were discarded. Fitting the histogram with a Gaussian distribution gave the mean baseline current amplitude and the difference between the amplitudes before and during picrotoxin was considered to be the tonic current. The negative section of the all-points histogram which corresponds to the inward IPSCs was not fitted with a Gaussian distribution (Kretschmannova et al., 2013; Nusser and Mody, 2002). Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by >20%.

Spontaneous inhibitory post-synaptic currents (sIPSCs) were analyzed using the mini-analysis software (version 5.6.4; Synaptosoft, Decatur, GA). Minimum threshold detection was set to 3 times the value of baseline noise signal. To assess sIPSC kinetics, the

recording trace was visually inspected and only events with a stable baseline, sharp rising phase, and single peak were used to negate artifacts due to event summation. Only recordings with a minimum of 200 events fitting these criteria were analyzed. sIPSCs amplitude, and frequency from each experimental condition was pooled and expressed as mean \pm SEM. To measure sIPSC decay we averaged 100 consecutive events and fitted the decay to a double exponential and took the weighted decay constant (τ_w). Statistical analysis was performed by using Student *t*-test (paired and unpaired where appropriate), where $p < 0.05$ is considered significant.

2.5. Metabolic labeling and biotinylation

Hippocampi were dissected out of acute slices from 8 to 12 week old C57/Bl6 mice and lysed with phosphate buffer including: 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na₃VO₄, 10 mM pyrophosphate, 0.1% SDS and 1% Triton after drug treatment. The $\beta 3$ subunit was isolated using immunoprecipitation with $\beta 3$ antibodies, after correction for protein content and the specific activity of labeling. Results were attained by SDS/PAGE followed by autoradiography (Abramian et al., 2010). For biotinylation experiments hippocampi were dissected out of acute slices from 8 to 12 week old C57/Bl6 mice and incubated in artificial cerebrospinal fluid (ACSF) described above at 30 °C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/mL NHS-SS-biotin (Pierce). Excess biotin was removed by a 50 mM glycine quenching buffer, followed by washing of slices three times in ice-cold ACSF. The tissue was snap frozen on dry ice for 5 min, thawed at 4 °C and lysed. The lysates were solubilized with 2% Triton at 4 °C on a rotating wheel for 1 h. The insoluble material was removed by centrifugation, and 350–500 μ g of protein lysate were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 °C. Bound material was eluted with sample buffer and subjected to SDS/PAGE and then immunoblotted with the indicated antibodies. Blots were then quantified using the CCD-based ChemiDoc XRS + system. Antibodies against the $\beta 3$ subunit and the phospho- $\beta 3$ antibody were generated and verified by the laboratory of S.J.M. (Vien et al., 2015).

3. Results

3.1. Comparing the effects of endogenous and synthetic neurosteroids on the activity of recombinant GABA_ARs

ALLO and ganaxolone are known PAMs of both synaptic and extrasynaptic GABA_AR-mediated currents. The ability of SGE-516 to act as a PAM was compared to ALLO and ganaxolone using the whole-cell recordings of recombinant human GABA_A receptors expressed in mammalian cells. The $\alpha 1\beta 2\gamma 2$ or $\alpha 4\beta 3\delta$ subunit combinations were chosen as representatives of typical synaptic and extrasynaptic GABA_A receptors respectively. Similar to previous reports (Botella et al., 2015), ALLO, ganaxolone and SGE-516 allosterically potentiated currents induced by EC₂₀ concentration of GABA in a concentration-dependent manner in both synaptic- and

extrasynaptic-type GABA_ARs. ALLO potentiated $\alpha 1\beta 2\gamma 2$ receptors with an EC₅₀ of 115 nM and E_{max} of 229% and potentiated $\alpha 4\beta 3\delta$ receptors with an EC₅₀ of 57 nM and E_{max} of 426%. SGE-516 potentiated $\alpha 1\beta 2\gamma 2$ receptors with an EC₅₀ of 61 nM and E_{max} of 219%. Likewise, SGE-516 potentiated $\alpha 4\beta 3\delta$ receptors with an EC₅₀ of 193 nM and E_{max} of 400%. Ganaxolone potentiated $\alpha 1\beta 2\gamma 2$ receptors with an EC₅₀ of 256 nM and E_{max} of 307% and potentiated $\alpha 4\beta 3\delta$ receptors with an EC₅₀ of 94 nM and E_{max} of 225% (Table 1). To confirm the presence of the $\gamma 2$ subunit in $\alpha 1\beta 2\gamma 2$ receptors, we also checked the effects of diazepam, a well-known benzodiazepine PAM specifically for synaptic GABA_ARs (data not shown). It was observed that diazepam potentiates the GABA evoked currents in $\alpha 1\beta 2\gamma 2$ receptors with an EC₅₀ of 69 nM and E_{max} of 184%. In contrast, it did not alter GABA evoked currents in $\alpha 4\beta 3\delta$ receptors. The results indicate that like ALLO, and ganaxolone, SGE-516 is a potent and efficacious PAM for both synaptic and extrasynaptic type GABA_ARs.

3.2. Acute exposure to NASs allosterically potentiates tonic current in the dentate gyrus granule cells

The results from the above experiments with synaptic-, and extrasynaptic-like GABA_ARs expressed in HEK293 cells demonstrated the ability of ALLO, SGE-516, and ganaxolone to potentiate sub-maximal GABA-mediated currents. We next examined the ability of acute application of these NASs to allosterically modulate phasic and tonic currents in dentate gyrus granule cells (DGGCs) in hippocampal slices from 3 to 5 weeks old male C57/Bl6 mice. As would be expected, both ALLO, and SGE-516 modulated the tonic holding current in DGGCs in hippocampal slices (Fig. 1). At 100 nM, ALLO modulated the tonic current from 31.8 \pm 5.9 pA, to 58.3 \pm 13.0 pA, ($n = 5$) and 100 nM SGE-516 modulated the tonic current from 34.2 \pm 11.1 pA, to 49.2 \pm 8.9 ($n = 7$). The only significant modulation was observed with 100 nM ganaxolone which modulated DGGC tonic current from 42.1 \pm 17.5 to 78.8 \pm 23.2 pA ($n = 6$; $p = 0.004$ paired *t*-test).

3.3. Comparing the acute effects of NASs on phasic currents in DGGCs

We also compared the properties of inhibitory synaptic currents in DGGCs before and during exposure to NASs (Fig. 2). It was observed that there was no significant difference in the mean sIPSC amplitude before and during exposure with 100 nM ALLO ($p = 0.85$, $n = 5$), 100 nM SGE-516 ($p = 0.46$, $n = 7$) and 100 nM ganaxolone ($p = 0.07$, $n = 6$) (Table 2). However, the mean sIPSC decay time significantly increased in the presence of ALLO, SGE-516, and ganaxolone ($p = 0.03$, $p = 0.01$, and $p = 0.04$ respectively, Table 2).

3.4. Exposure to NASs metabotropically enhances tonic current in DGGCs

Recently we have shown that, in addition to the allosteric modulation of GABA_A receptors, THDOC, exert sustained effects on

Table 1
Properties of neuroactive steroids on recombinant GABA_ARs.

Compound	$\alpha 1\beta 2\gamma 2$		$\alpha 4\beta 3\delta$	
	EC ₅₀ (pEC ₅₀ \pm S.E.M.)	E _{max} \pm S.E.M. (%)	EC ₅₀ (pEC ₅₀ \pm S.E.M.)	E _{max} \pm S.E.M. (%)
ALLO	115 (6.9 \pm 0.2)	229 \pm 19	57 (7.2 \pm 0.3)	426 \pm 42
SGE-516	61 (7.2 \pm 0.3)	219 \pm 21	193 (6.7 \pm 0.1)	505 \pm 19
Ganaxolone	256 (6.6 \pm 0.2)	400 \pm 27	94 (7.0 \pm 0.1)	225 \pm 8

EC₅₀ values are given in nM with -pEC₅₀ \pm S.E.M., $n = 3$ for all.

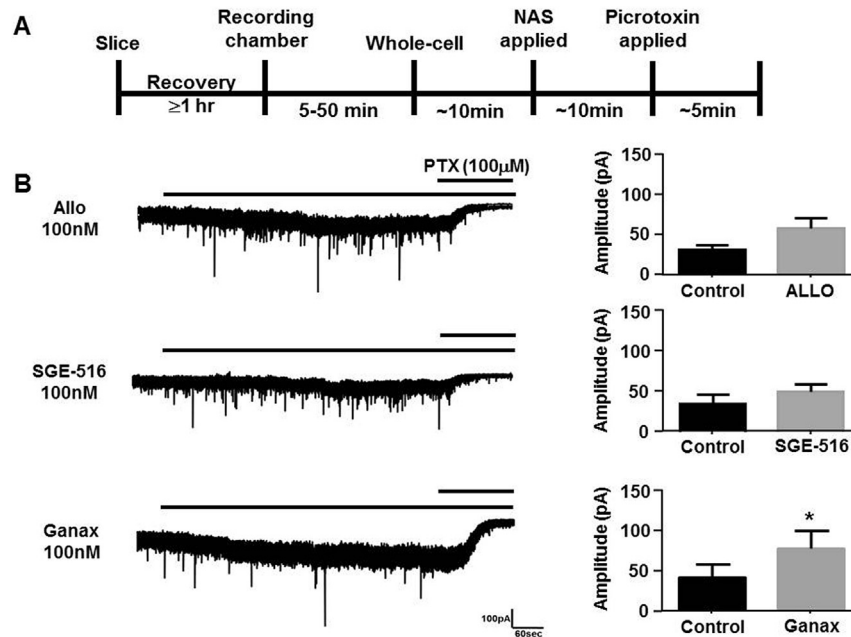


Fig. 1. NASs allosterically modulate DGGC tonic currents. **A.** Scheme demonstrating experimental protocol. Hippocampal slices from p21–35 (C57/Bl6) mice were allowed to recover for at least 1 h following slicing. Slices were transferred to the recording chamber of the microscope. After achieving the whole-cell configuration approximately 10 min was allowed for membrane currents to stabilize. Hippocampal slices were acutely exposed to 100 nM ALLO, SGE-516, or ganaxolone (Ganax) for 10 min followed by 100 μ M picrotoxin (PTX). **B.** Example of current recordings from DGGCs showing modulation of tonic currents by acutely applied NASs (left panel). Bars above current recordings represent application of NAS and picrotoxin. Bar graphs summarizing the average tonic current (mean \pm SEM) before and during acute exposure with ALLO, SGE-516, or ganaxolone (right panel). * = significantly different to control ($p = 0.004$; t -test), $n = 4$ –12 cells.

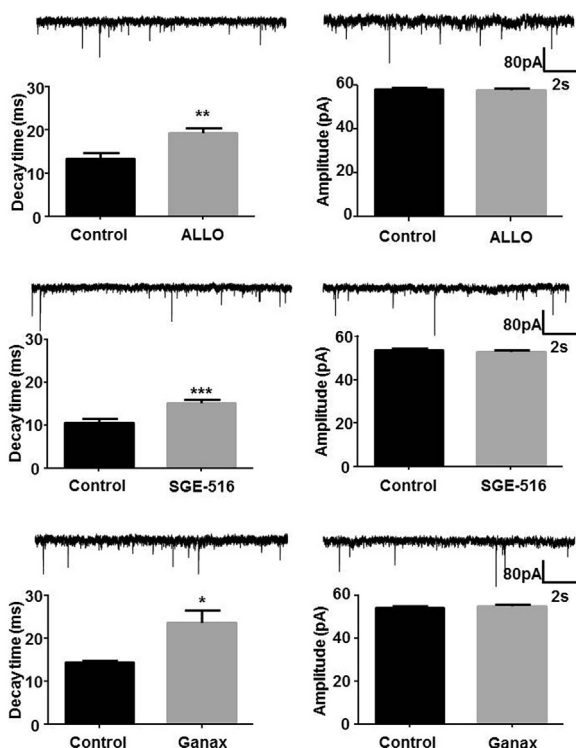


Fig. 2. Allosteric modulation of phasic currents by acutely applied NASs. Recordings of sIPSCs from DGGCs before and during acute exposure to 100 nM ALLO, 100 nM SGE-516, or 100 nM ganaxolone for 10 min. Example of current recordings from DGGCs showing phasic currents before and during acutely applied NASs (left panel). Bar graphs summarizing the effects of acute exposure of ALLO, SGE-516, or ganaxolone on the amplitude and decay of sIPSCs (right panel). *** $p = 0.01$, ** $p = 0.03$, * $p = 0.04$, paired t -test, $n = 5$ –7 cells.

GABAergic tonic current by enhancing the PKC-dependent phosphorylation of the $\alpha 4$ and $\beta 3$ subunits, leading to enhanced insertion and stability of GABA_ARs into the membrane and a long lasting increase in tonic current (Abramian et al., 2010, 2014).

Here, we analyzed the sustained effects of ALLO, or the new synthetic NAS SGE-516 on the tonic current in DGGCs in hippocampal slices from 3 to 5 week old C57 male mice. Hippocampal slices were incubated for 15 min in a chamber containing NASs dissolved in ACSF. Slices were then transferred to the recording chamber of the microscope followed by a wash period between 30 and 60 min of continuous perfusion of NAS-free ACSF before recordings were started (Fig. 3A).

Slices exposed to ALLO, or SGE-516 demonstrated a concentration-dependent increase in the tonic current measured by the addition of picrotoxin with the maximal effects at 1 μ M. Control, vehicle-treated slices had a tonic current of 43.9 ± 5.7 pA ($n = 12$), whereas the tonic currents for slices treated with SGE-516 (1 μ M) was 123.0 ± 22.2 pA, ($n = 6$, $p = 0.0003$), or ALLO (1 μ M) was 95.8 ± 10.8 pA ($n = 4$, $p = 0.0005$, Fig. 3B&C).

In addition, we also examined if the synthetic NAS, ganaxolone, also had a metabotropic effect following 15 min incubation. In contrast to the naturally occurring NASs THDOC, and ALLO, and the synthetic NAS, SGE-516, ganaxolone (1 μ M) did not significantly alter the magnitude of tonic current in DGGCs (57.4 ± 6.3 pA, $n = 7$, $p = 0.14$, Fig. 3D).

To assess if the effects of NASs are dependent upon PKC, hippocampal slices were treated with the established PKC inhibitor GF 109203X (GFX 50 μ M) for 15 min followed by co-exposure of to ALLO, or SGE-516 and GFX for 15 min. When tonic current was measured following ≥ 30 min washout, there was no significant difference to the tonic current measured in ALLO/GFX, or SGE-516/GFX treated slices with vehicle treated slices (Fig. 3).

We measured the tonic current by blocking extrasynaptic GABA_ARs with picrotoxin. Because picrotoxin also inhibits glycine

Table 2
Allosteric modulation of DGGC sIPSCs evoked by 100 nM ALLO, SGE-516, and ganaxolone.

sIPSC	Control	ALLO	Control	SGE-516	Control	Ganax
Amplitude (pA)	59.2 ± 0.7	59.1 ± 0.6	53.7 ± 0.7	52.9 ± 0.7	54.2 ± 0.6	55.1 ± 0.7
Frequency (Hz)	4.5 ± 0.9	3.4 ± 1.0	4.1 ± 0.4	4.6 ± 0.6	6.4 ± 1.3	5.6 ± 1.7
Decay (ms)	13.4 ± 1.3	19.2 ± 1.3 ^b	10.5 ± 1.1	15.1 ± 0.8 ^a	14.4 ± 0.5	23.6 ± 2.9 ^c

Data is mean SEM, n = 5 to 7 neurons.

^a p = 0.01.

^b p = 0.03.

^c p = 0.04.

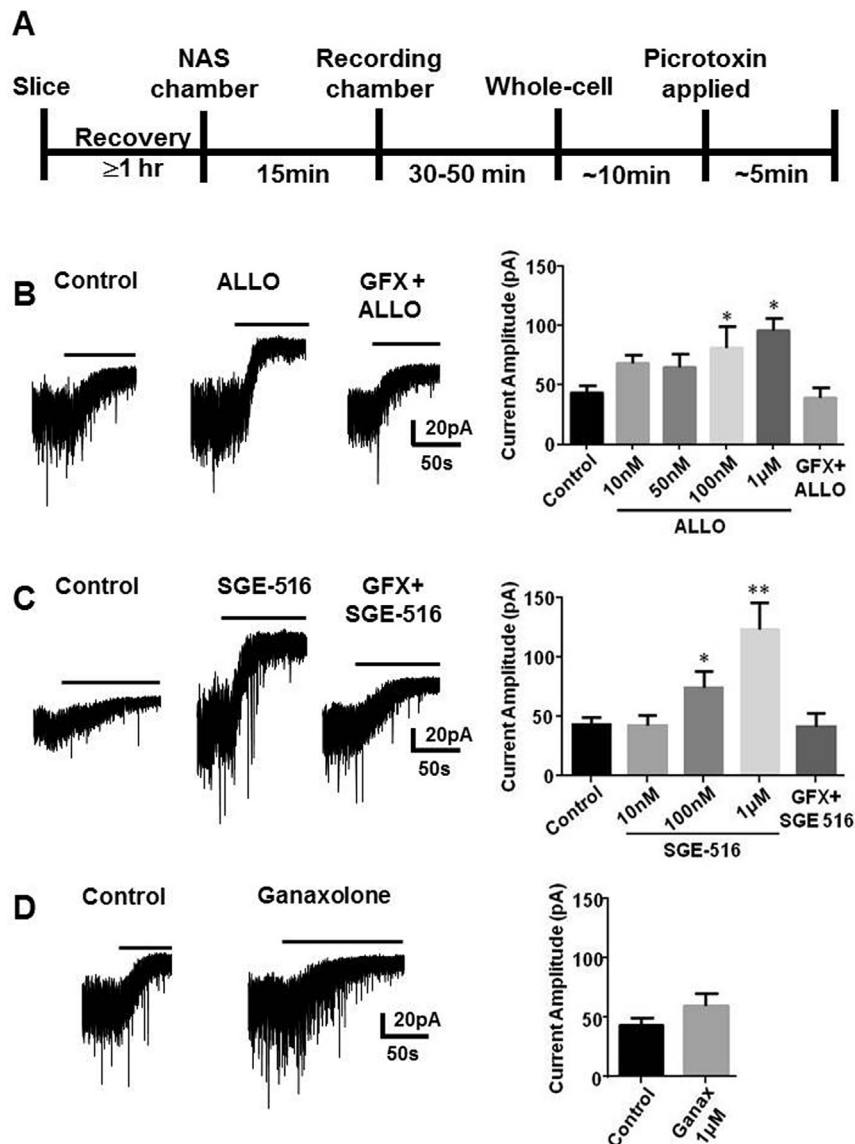


Fig. 3. NAS-mediated metabotropic enhancement of tonic inhibitory current in DGGC neurons. A. Scheme demonstrating experimental protocol. Hippocampal slices were allowed to recover for at least 1 h following slicing. Slices were then incubated for 15 min in a chamber containing NASs dissolved in ACSF. Slices were then transferred to the recording chamber of the microscope followed by a wash period between 30 and 60 min of continuous perfusion of NAS-free ACSF before recordings were started. Recordings were made from DGGCs in hippocampal slices from p21–35 C57 mice in the presence of 5 µM GABA followed by 100 µM picrotoxin and the difference in holding current was then determined. **Left panel B, C, D.** Example tonic currents from slices following exposures to vehicle (control) or 100 nM ALLO (B), 100 nM SGE-516 (C), or 1 µM ganaxolone (D) for 15 min. No change in tonic current was observed in slices pre-incubated for 15 min with GFX followed by ALLO, or SGE-516. Bar above current represents application of picrotoxin (100 µM). **Right panel B, C, D.** Bar graph shows average tonic current was significantly enhanced following exposure to different concentrations of ALLO and SGE-516. No significant change in tonic current was observed following exposure to 1 µM ganaxolone for 15 min. In all panels * = significantly different to control (p < 0.05; un-paired t-test, n = 4–12 cells).

receptors we examined if glycine receptors contributed to tonic current by using the specific glycine receptor inhibitor, strychnine. There was no difference in tonic current measured under control

conditions in the absence or presence of strychnine (100 nM). Similarly, there was no difference in the increase in tonic current following a 15 min exposure to 100 nM ALLO in the absence or

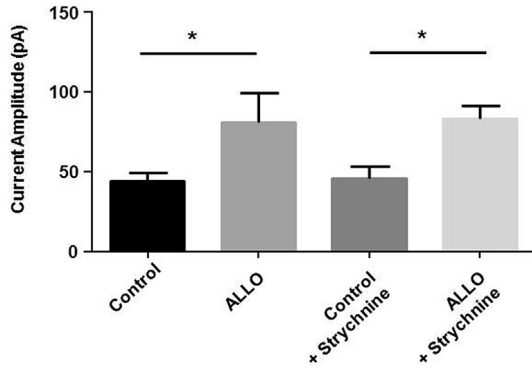


Fig. 4. Glycine receptors do not contribute to tonic current in DGCCs. Hippocampal slices were incubated for 15 min with 100 nM ALLO or vehicle dissolved in ACSF then transferred to the recording chamber and washed for 30–60 min with NAS-free ACSF before recordings were started. Tonic current was measured by applying 100 μ M picrotoxin in the absence or presence of the glycine receptor, strychnine (100 nM). Exposure to ALLO caused a significant increase in tonic current. Addition of strychnine did not alter the tonic current measured with picrotoxin. * $p = 0.01$, unpaired t -test, $n = 4$ –12 neurons.

presence of strychnine (Fig. 4). These results suggest that glycine receptors have an undetected contribution to tonic current in DGCCs and that the metabotropic increase in tonic current by NAS exposure do not involve glycine receptors.

Collectively, these results suggest that the exposure of hippocampal slices to SGE-516 and ALLO has a strong sustained metabotropic effects on tonic current. In contrast, ganaxolone produce major effects via allosteric mechanism as compared to metabotropic effects.

3.5. The metabotropic effects of NASs on phasic current in DGCCs

In parallel with our measurements on tonic current we assessed the effects of prior exposure of NASs on sIPSC properties. The amplitude of sIPSCs was significantly increased after incubation with 100 nM SGE-516 compared to vehicle control (control 39 ± 0.9 pA, $n = 12$; SGE-516 58 ± 1.9 pA, $n = 6$, $p = 0.0001$). This increase in sIPSC amplitude following exposure to SGE-516 was significantly reduced following co-exposure with SGE-516 and GFX (Fig. 5C). No change in sIPSC amplitude was observed following exposure with 100 nM ALLO or 1 μ M ganaxolone (Fig. 5B&D). In

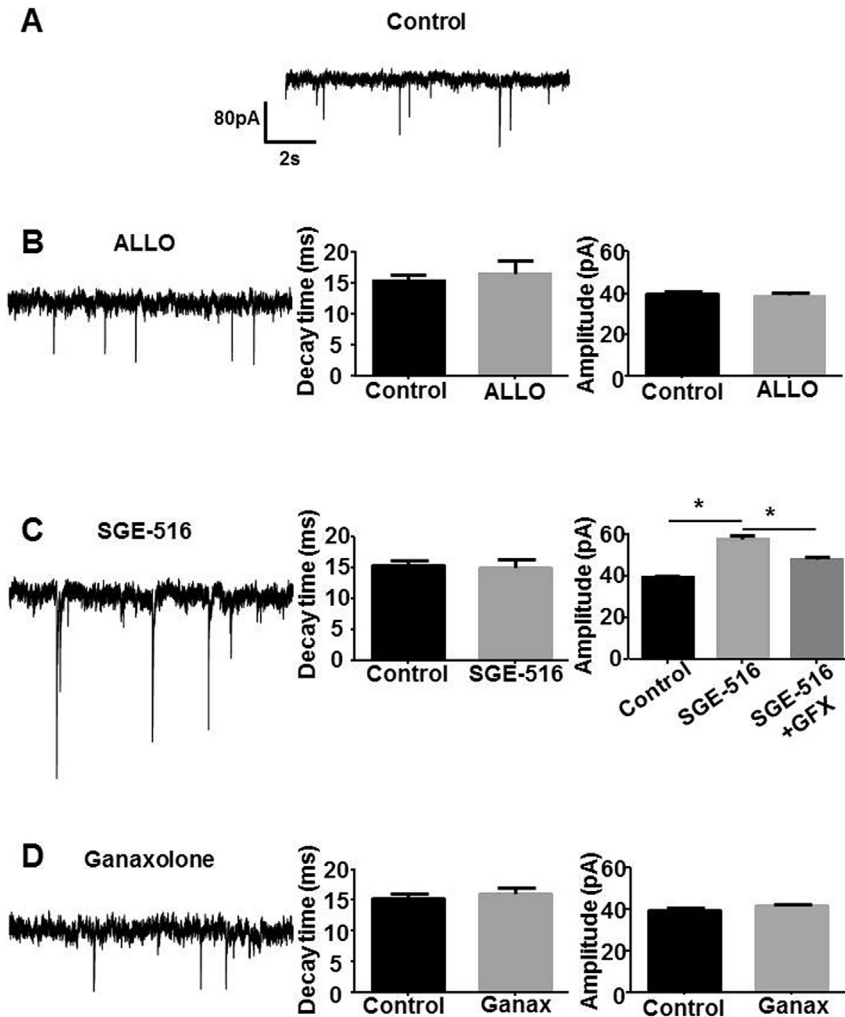


Fig. 5. sIPSC amplitude and decay was largely unchanged following exposure to NASs. A. IPSC recordings were made from DGCCs in hippocampal slices from p21–35 C57 mice. The slices were exposed to vehicle (control, $n = 6$ neurons from 3 mice), B. 100 nM ALLO ($n = 4$ neurons from 2 mice), C. 100 nM SGE-516 ($n = 5$ neurons from 2 mice), or D. 1 μ M ganaxolone ($n = 5$ neurons from 2 mice) for 15 min and then washed for >30 min prior to measurement of sIPSCs. Bar graphs shows average sIPSC decay and amplitude. Only sIPSC amplitude was significantly enhanced following exposure to 100 nM SGE-516 but GFX ($n = 5$ neurons from 2 mice) significantly reduced SGE-516 enhancement. * = significantly different to control ($p < 0.05$; unpaired t -test).

contrast to the changes observed with the allosteric modulation of IPSC decay time, no metabotropic-mediated changes in sIPSC decay time was observed following exposure to ALLO, SGE-516, or ganaxolone (Fig. 5).

3.6. SGE-516 and ALLO increase the phosphorylation and cell surface stability of GABA_ARs

We have previously shown that the naturally occurring NAS, THDOC, increased tonic current in part through phosphorylation of S408/409 of the $\beta 3$ subunit (Abramian et al., 2014; Vien et al., 2015). To further our understanding of the NAS-mediated increase in tonic current we measured the effects of ALLO, SGE-516, on the phosphorylation and membrane expression of $\beta 3$ subunits in hippocampal slices. Twenty min exposure to 100 nM ALLO, or SGE-516 increased phosphorylation of $\beta 3$ subunits at S408/409 to $141 \pm 10\%$ of control ($n = 10$, $p < 0.01$), and $143 \pm 14\%$ of control ($n = 4$, $p < 0.05$) respectively (Fig. 6A&B).

In parallel with modulating phosphorylation, exposure to 100 nM ALLO, or SGE-516 increased the cell surface expression levels of receptors containing $\beta 3$ subunits to $166 \pm 22\%$ of control ($n = 8$, $p < 0.05$), and $180 \pm 29\%$ of control ($n = 4$, $p < 0.05$) respectively (Fig. 6C&D). Therefore, in parallel with inducing sustained effects on GABAergic inhibition ALLO and SGE-516 enhance the cell surface levels and phosphorylation of the GABA_ARs containing $\beta 3$ subunits dependent upon PKC activity.

4. Discussion

We have previously shown that the endogenous NAS, THDOC, exerts a sustained increase on GABAergic tonic current in cultured neurons. The THDOC-mediated increase in $\alpha 4/\beta 3$ subunit-mediated current is independent of the allosteric potentiation typically described of NASs. Rather, we described a metabotropic signaling mechanism by which THDOC induced a PKC-mediated

phosphorylation of $\alpha 4$ and $\beta 3$ subunits with a subsequent increase in the insertion of GABA_ARs, and a reduction in their endocytosis (Abramian et al., 2010, 2014; Adams et al., 2015). Here we have assessed this putative regulatory mechanism for other endogenous and synthetic NASs in the dentate gyrus.

To do so, hippocampal slices were exposed to NAS for 15 min followed by ≥ 30 min wash in a separate recording chamber before the start of the experiment. Both ALLO and SGE-516 significantly enhanced the tonic current compared to vehicle treated slices via a metabotropic mechanism. This is in contrast with the non-significant allosteric enhancement of tonic current we observed with ALLO and SGE-516. However, we also examined the first generation synthetic NAS, ganaxolone, and although it significantly produced an allosteric potentiated tonic current it failed to metabotropically modulate tonic current. This result demonstrates that the metabotropic pathway that increases the PKC-mediated phosphorylation of $\alpha 4$ and $\beta 3$ subunits can be stimulated by both endogenous and synthetic NASs but is not universally stimulated by all steroids.

Ganaxolone, ALLO, and SGE-516 all allosterically prolonged the decay of sIPSCs, and allosterically potentiated recombinant GABA_ARs demonstrating their recognized role as GABA_ARs PAMs. However, in our metabotropic studies only SGE-516 significantly altered sIPSC properties, with an increase in sIPSC amplitude. Our previous data has demonstrated the link between PKC-mediated phosphorylation of $\beta 3$ subunits with a subsequent reduction in GABA_AR endocytosis and an increase in IPSC amplitude (Jovanovic et al., 2004; Kittler et al., 2005; Vien et al., 2015).

In DGGCs, from mice that are 3 weeks and older the predominant extrasynaptic combination is thought to be $\alpha 4\beta n\delta$ or even just $\alpha 4\beta n$ (Bencsits et al., 1999; Chandra et al., 2006; Mortensen and Smart, 2006). Extrasynaptic GABA_ARs in hippocampal CA1 pyramidal neurons have the $\alpha 5$ subunit whereas, extrasynaptic GABA_ARs in hippocampal interneurons are comprised of $\alpha 1\beta n\delta$ subunits (Caraiscos et al., 2004; Glykys et al., 2007). We have

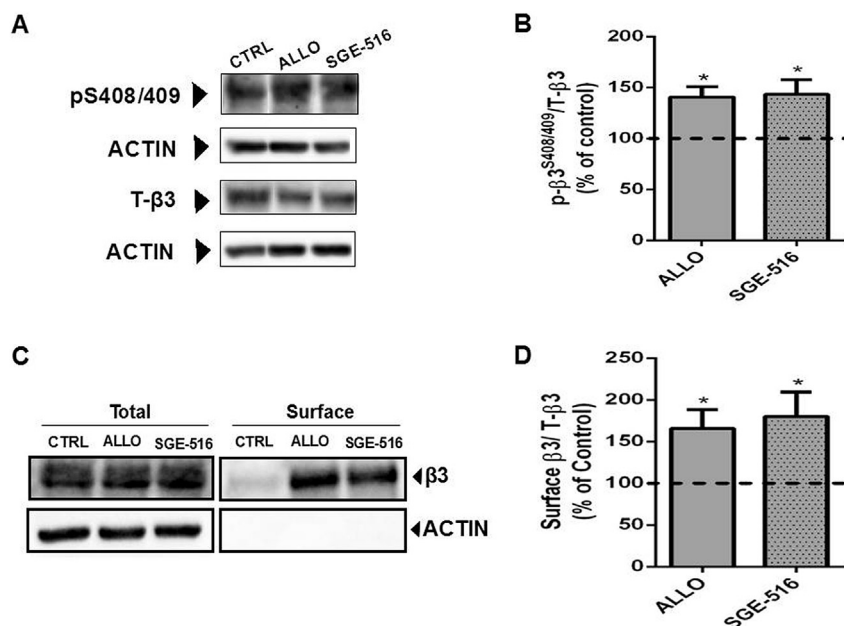


Fig. 6. NAS exposure increases phosphorylation and surface expression of $\beta 3$ subunits. **A.** Exposure to 100 nM of the NASs, ALLO or SGE-516, for 20 min increases $\beta 3$ S408/409 phosphorylation in acute hippocampal slices. **B.** The ratio of p- $\beta 3$ /T- $\beta 3$ was measured and values were normalized to those in control (100%). Asterisks represent a significant difference from control (ALLO: $p < 0.01$, Student's t -test, $n = 10$ slices, from 10 mice; SGE-516: $p < 0.05$, Student's t -test, $n = 4$ slices, from 4 mice). **C.** Exposure to 100 nM ALLO or SGE-516 for 20 min increases GABA_A- $\beta 3$ -containing receptors at the plasma membrane in acute hippocampal slices. **D.** The ratio of surface $\beta 3$ /T- $\beta 3$ was measured and values were normalized to cell surface levels in control treated slices (100%). Asterisks represent a significant difference from control (ALLO: $p < 0.05$, Student's t -test, $n = 8$ slices; SGE-516: $p < 0.05$, Student's t -test, $n = 4$ slices).

previously shown that with NAS exposure, $\alpha 5$ and $\alpha 1$ subunits are not phosphorylated nor is there an increase in $\alpha 5$ and $\alpha 1$ subunit trafficking to the membrane surface (Abramian et al., 2014). We have only observed the NAS-evoked PKC-dependent increase in tonic current through the phosphorylation of $\alpha 4$ and $\beta 3$ subunits and this phosphorylation occurs independently of the presence of δ subunits (Abramian et al., 2014), suggesting a brain region selective change in extrasynaptic GABA_AR trafficking. Extrasynaptic GABA_ARs in dentate gyrus granule cells have been suggested to be a mix of $\alpha 4\beta 2\delta$ and $\alpha 4\beta 3\delta$ subunits (Herd et al., 2008). GABA_ARs comprised of $\alpha 4\beta 2\delta$ have been shown to undergo increased endocytosis upon PKC phosphorylation of $\beta 2$ S410 (Bright and Smart, 2013). A hypothetical consequence of NAS-mediated increase in PKC phosphorylation of GABA_A subunits would be the predominance of extrasynaptic GABA_ARs comprised of $\alpha 4\beta 3\delta$ subunits.

Reduced expression of extrasynaptic GABA_ARs has been demonstrated in human and rodent models of postpartum depression, schizophrenia, epilepsies, Angelman, Rett, and Fragile X syndromes (Curia et al., 2009; D'Hulst and Kooy, 2007; Gantois et al., 2006; Maguire and Mody, 2008; Maldonado-Aviles et al., 2009). We have previously described a decrease in phosphorylation of residues S408/409 of the $\beta 3$ subunit following status epilepticus leading to an increase in GABA_AR endocytosis and increased excitation (Terunuma et al., 2008). Thus, by increasing the phosphorylation of the $\beta 3$ subunit by exposure to NAS the expression and trafficking of these extrasynaptic GABA_AR subunits could be manipulated and may provide novel therapeutic areas for these difficult to treat disorders.

The NAS-mediated increase in phosphorylation and surface levels of extrasynaptic GABA_ARs leading to an increase in tonic current is a further mechanism by which NASs can increase the inhibitory tone in the brain in addition to the more commonly described allosteric effect. While the allosteric modulation only exists while the NAS is present, the PKC-mediated metabotropic enhancement can cause a prolonged increase in inhibitory tone. We have noted before that other positive allosteric modulators such as the intravenous general anesthetic, propofol, do not have the ability to metabotropically enhance extrasynaptic GABA_ARs (Abramian et al., 2014). Here, we have demonstrated that not all steroidal modulators (ganaxolone) have the ability to increase tonic current through this metabotropic mechanism either. However, the metabotropic pathway seems to be activated by various NAS including endogenous THDOC, and ALLO and the synthetic NAS, SGE-516. The activation of this pathway increases phosphorylation of the $\beta 3$ subunit, leading to increased surface expression of the receptor and an increase in tonic current. All of which leads to an interesting area of potential therapeutic targets aimed at modulating the trafficking of a particularly important subset of GABA_ARs.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. SJM serves as a consultant for SAGE therapeutics and AstraZeneca, relationships that are regulated by Tufts University and do not impact on this study. JJD and MAA are employees of SAGE therapeutics.

Author contributions

AM performed and analyzed the patch clamp experiments and drafted the paper, MLP performed phosphorylation and biotinylation experiments and drafted the paper, MAA conceived and coordinated the study, JJD conceived and coordinated the study,

SJM conceived and coordinated the study, and wrote the paper PAD conceived and coordinated the study and wrote the paper.

Acknowledgements

This work was supported by grants from the National Institutes of Health (NIH)-National Institute of Alcoholism and Alcohol Abuse grant AA017938 (P.A.D), NIH-National Institute of Mental Health grant, MH097446, and DOD, AR140209 (PAD & SJM), NIH-National Institute of Neurological Disorders and Stroke grant NS051195, NS056359, NS081735, NS080064, NS087662 (SJM), the Simons Foundation #206026 (S.J.M.).

References

- Abramian, A.M., Comenencia-Ortiz, E., Modgil, A., Vien, T.N., Nakamura, Y., Moore, Y.E., Maguire, J.L., Terunuma, M., Davies, P.A., Moss, S.J., 2014. Neurosteroids promote phosphorylation and membrane insertion of extrasynaptic GABA_A receptors. *Proc. Natl. Acad. Sci. U. S. A.* 111, 7132–7137.
- Abramian, A.M., Comenencia-Ortiz, E., Vithlani, M., Tretter, E.V., Sieghart, W., Davies, P.A., Moss, S.J., 2010. Protein kinase C phosphorylation regulates membrane insertion of GABA_A receptor subtypes that mediate tonic inhibition. *J. Biol. Chem.* 285, 41795–41805.
- Adams, J.M., Thomas, P., Smart, T.G., 2015. Modulation of neurosteroid potentiation by protein kinases at synaptic- and extrasynaptic-type GABA_A receptors. *Neuropharmacology* 88, 63–73.
- Belelli, D., Harrison, N.L., Maguire, J., Macdonald, R.L., Walker, M.C., Cope, D.W., 2009. Extrasynaptic GABA_A receptors: form, pharmacology, and function. *J. Neurosci.* 29, 12757–12763.
- Bencsits, E., Ebert, V., Tretter, V., Sieghart, W., 1999. A significant part of native gamma-aminobutyric acid receptors containing alpha4 subunits do not contain gamma or delta subunits. *J. Biol. Chem.* 274, 19613–19616.
- Botella, G.M., Salituro, F.G., Harrison, B.L., Beresis, R.T., Bai, Z., Shen, K.S., Belfort, G.M., Loya, C.M., Ackley, M.A., Grossman, S.J., Hoffmann, E., Jia, S.L., Wang, J.M., Doherty, J.J., Robichaud, A.J., 2015. Neuroactive steroids. 1. Positive allosteric modulators of the (gamma-aminobutyric acid)(A) receptor: structure-activity relationships of heterocyclic substitution at C-21. *J. Med. Chem.* 58, 3500–3511.
- Brickley, S.G., Mody, I., 2012. Extrasynaptic GABA(A) receptors: their function in the CNS and implications for disease. *Neuron* 73, 23–34.
- Bright, D.P., Smart, T.G., 2013. Protein kinase C regulates tonic GABA(A) receptor-mediated inhibition in the hippocampus and thalamus. *Eur. J. Neurosci.* 38, 3408–3423.
- 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., MacDonald, J.F., Orser, B.A., 2004. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3662–3667.
- Chandra, D., Jia, F., Liang, J., Peng, Z., Suryanarayanan, A., Werner, D.F., Spigelman, I., Houser, C.R., Olsen, R.W., Harrison, N.L., Homanics, G.E., 2006. GABA_A receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc. Natl. Acad. Sci. U. S. A.* 103, 15230–15235.
- Curia, G., Papouin, T., Seguela, P., Avoli, M., 2009. Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome. *Cereb. Cortex* 19, 1515–1520.
- D'Hulst, C., Kooy, R.F., 2007. The GABA_A receptor: a novel target for treatment of fragile X? *Trends Neurosci.* 30, 425–431.
- Gantois, I., Vandesompele, J., Speleman, F., Reyniers, E., D'Hooge, R., Severijnen, L.A., Willemsen, R., Tassone, E., Kooy, R.F., 2006. Expression profiling suggests underexpression of the GABA(A) receptor subunit delta in the fragile X knockout mouse model. *Neurobiol. Dis.* 21, 346–357.
- Glykys, J., Mody, I., 2007. Activation of GABA_A receptors: views from outside the synaptic cleft. *Neuron* 56, 763–770.
- Glykys, J., Peng, Z., Chandra, D., Homanics, G.E., Houser, C.R., Mody, I., 2007. A new naturally occurring GABA(A) receptor subunit partnership with high sensitivity to ethanol. *Nat. Neurosci.* 10, 40–48.
- Herd, M.B., Haythornthwaite, A.R., Rosahl, T.W., Wafford, K.A., Homanics, G.E., Lambert, J.J., Belelli, D., 2008. The expression of GABA_A beta subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. *J. Physiol.* 586, 989–1004.
- Jovanovic, J.N., Thomas, P., Kittler, J.T., Smart, T.G., Moss, S.J., 2004. Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. *J. Neurosci.* 24, 522–530.
- Kittler, J.T., Chen, G., Honing, S., Bogdanov, Y., McAinsh, K., Arancibia-Carcamo, I.L., Jovanovic, J.N., Pangalos, M.N., Haucke, V., Yan, Z., Moss, S.J., 2005. Phospho-dependent binding of the clathrin AP2 adaptor complex to GABA_A receptors regulates the efficacy of inhibitory synaptic transmission. *Proc. Natl. Acad. Sci. U. S. A.* 102, 14871–14876.

- Kretschmannova, K., Hines, R.M., Revilla-Sanchez, R., Terunuma, M., Tretter, V., Jurd, R., Kelz, M.B., Moss, S.J., Davies, P.A., 2013. Enhanced tonic inhibition influences the hypnotic and amnesic actions of the intravenous anesthetics etomidate and propofol. *J. Neurosci.* 33, 7264–7273.
- Maguire, J., Mody, I., 2008. GABA(A)R plasticity during pregnancy: relevance to postpartum depression. *Neuron* 59, 207–213.
- Maldonado-Aviles, J.G., Curley, A.A., Hashimoto, T., Morrow, A.L., Ramsey, A.J., O'Donnell, P., Volk, D.W., Lewis, D.A., 2009. Altered markers of tonic inhibition in the dorsolateral prefrontal cortex of subjects with schizophrenia. *Am. J. Psychiatry* 166, 450–459.
- Mitchell, E.A., Herd, M.B., Gunn, B.G., Lambert, J.J., Belelli, D., 2008. Neurosteroid modulation of GABAA receptors: molecular determinants and significance in health and disease. *Neurochem. Int.* 52, 588–595.
- Mortensen, M., Smart, T.G., 2006. Extrasynaptic alphabeta subunit GABAA receptors on rat hippocampal pyramidal neurons. *J. Physiol.* 577, 841–856.
- Nusser, Z., Mody, I., 2002. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J. Neurophysiol.* 87, 2624–2628.
- Paul, S.M., Purdy, R.H., 1992. Neuroactive steroids. *FASEB J.* 6, 2311–2322.
- Rupprecht, R., 2014. New perspectives in neurosteroid action: open questions for future research. *Front. Cell Neurosci.* 8, 268.
- Semyanov, A., Walker, M.C., Kullmann, D.M., Silver, R.A., 2004. Tonic active GABA A receptors: modulating gain and maintaining the tone. *Trends Neurosci.* 27, 262–269.
- Terunuma, M., Xu, J., Vithlani, M., Sieghart, W., Kittler, J., Pangalos, M., Haydon, P.G., Coulter, D.A., Moss, S.J., 2008. Deficits in phosphorylation of GABA(A) receptors by intimately associated protein kinase C activity underlie compromised synaptic inhibition during status epilepticus. *J. Neurosci.* 28, 376–384.
- Vien, T.N., Modgil, A., Abramian, A.M., Jurd, R., Walker, J., Brandon, N.J., Terunuma, M., Rudolph, U., Maguire, J., Davies, P.A., Moss, S.J., 2015. Compromising the phosphodependent regulation of the GABAAR beta3 subunit reproduces the core phenotypes of autism spectrum disorders. *Proc. Natl. Acad. Sci. U. S. A.* 112, 14805–14810.

Compromising the phosphodependent regulation of the GABA_AR β3 subunit reproduces the core phenotypes of autism spectrum disorders

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Edited by Richard L. Huganir, The Johns Hopkins University School of Medicine, Baltimore, MD, and approved October 22, 2015 (received for review July 24, 2015)

Alterations in the efficacy of neuronal inhibition mediated by GABA_A receptors (GABA_ARs) containing β3 subunits are continually implicated in autism spectrum disorders (ASDs). In vitro, the plasma membrane stability of GABA_ARs is potentiated via phosphorylation of serine residues 408 and 409 (S408/9) in the β3 subunit, an effect that is mimicked by their mutation to alanines. To assess if modifications in β3 subunit expression contribute to ASDs, we have created a mouse in which S408/9 have been mutated to alanines (S408/9A). S408/9A homozygotes exhibited increased phasic, but decreased tonic, inhibition, events that correlated with alterations in the membrane stability and synaptic accumulation of the receptor subtypes that mediate these distinct forms of inhibition. S408/9A mice exhibited alterations in dendritic spine structure, increased repetitive behavior, and decreased social interaction, hallmarks of ASDs. ASDs are frequently comorbid with epilepsy, and consistent with this comorbidity, S408/9A mice exhibited a marked increase in sensitivity to seizures induced by the convulsant kainic acid. To assess the relevance of our studies using S408/9A mice for the pathophysiology of ASDs, we measured S408/9 phosphorylation in *Fmr1* KO mice, a model of fragile X syndrome, the most common monogenetic cause of ASDs. Phosphorylation of S408/9 was selectively and significantly enhanced in *Fmr1* KO mice. Collectively, our results suggest that alterations in phosphorylation and/or activity of β3-containing GABA_ARs may directly contribute to the pathophysiology of ASDs.

GABA receptor | phosphorylation | autism spectrum disorder | tonic inhibition | phasic inhibition

GABA_A receptors (GABA_ARs) are Cl⁻ selective ligand-gated ion channels that mediate phasic and tonic inhibition in the adult brain. Consistent with their roles in limiting neuronal excitability, benzodiazepines, barbiturates, general anesthetics, and neurosteroids exert their anxiolytic, anticonvulsant, hypnotic, and sedative effects via potentiating GABA_AR activity (1). GABA_ARs are heteropentamers constructed from α1–6, β1–3, γ1–3, δ, ε, θ, and π subunits. Phasic inhibition is principally mediated by receptors assembled from α1–3, β1–3, and γ2 subunits, whereas those receptors that mediate tonic inhibition contain α4–6, β1–3, and δ subunits (2). Studies using KO mice have shown that the β3 subunit is an essential component of receptor subtypes that mediate phasic and tonic inhibition (3). Together with the *Fmr1* gene (Fragile X mental retardation), mutations to the 15q11–13 locus, where the GABA_AR β3 gene resides, are the leading monogenetic causes of autism spectrum disorders (ASDs) (4). Moreover, β3 subunit mutations have been described in seizure disorders, and alterations in subunit expression levels have also been reported in ASDs (3, 5).

In vitro studies have revealed that the β3 subunit plays a critical role in regulating the plasma membrane accumulation and synaptic targeting of GABA_ARs via phosphorylation of the

intracellular serine residues 408 and 409 (S408/9) (6, 7). S408/9 are substrates of cAMP-dependent PKA, PKC, Ca²⁺-calmodulin type 2-dependent protein kinases (Cam KIIs), and cGMP-dependent protein kinase, and they are principally dephosphorylated by protein phosphatase 2A (8). S408/9 are the principal mediators of high-affinity binding to the clathrin adaptor molecule AP2 within the β3 subunit, and thereby facilitate GABA_AR endocytosis (9). Phosphorylation of S408/9 reduces the affinity of the β3 subunit for AP2 by 100-fold, and mutation of S408/9 to alanine residues (S408/9A) has been shown to mimic the effects of phosphorylation on AP2 binding to the β3 subunit (9, 10). Accordingly, overexpression of the mutant β3 S408/9A subunit in cultured hippocampal neurons leads to an increase in the number and size of inhibitory synapses (7).

Studies in animal models of ASDs have reported modifications in the expression levels of some GABA_AR mRNAs and proteins (11, 12). However, the mechanisms underlying these alterations in subunit expression and if they contribute to ASDs remain to be addressed. Therefore, in this study, we have analyzed the role that modified β3 subunit phosphorylation may play in the pathophysiology of ASDs. To test this role, we created a

Significance

Alterations in the efficacy of neuronal inhibition mediated by GABA_A receptors (GABA_ARs) containing β3 subunits are continually implicated in autism spectrum disorders (ASDs). In vitro, the plasma membrane stability of GABA_ARs is potentiated via phosphorylation of serine residues 408 and 409 (S408/9) in the β3 subunit, an effect that is mimicked by their mutation to alanines. Here, we created a mouse in which S408/9 have been mutated to alanines (S408/9A). S408/9A mice exhibited altered dendritic spine structure, increased repetitive behavior, decreased social interaction, and an epileptic phenotype. Thus, mutation of S408/9 reproduces the core deficits seen in humans with ASDs. Collectively, our results suggest that alterations in phosphorylation and/or activity of β3-containing GABA_ARs may directly contribute to the pathophysiology of ASDs.

Author contributions: T.N.V., A.M., A.M.A., R.J., J.W., M.T., U.R., and J.M. designed research; T.N.V., A.M., A.M.A., R.J., J.W., N.J.B., M.T., U.R., and J.M. performed research; A.M.A., M.T., and U.R. contributed new reagents/analytic tools; T.N.V., A.M., A.M.A., R.J., J.W., N.J.B., M.T., U.R., J.M., and P.A.D. analyzed data; and T.N.V., N.J.B., U.R., J.M., P.A.D., and S.J.M. wrote the paper.

Conflict of interest statement: S.J.M. serves as a consultant for AstraZeneca and SAGE Therapeutics, relationships that are regulated by Tufts University and do not have an impact on this study.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1514657112/-DCSupplemental.

mouse in which the principal sites of phosphodependent regulation within the receptor $\beta 3$ subunit, S408/9, have been mutated to S408/9A, a mutation that mimics the effects of their phosphorylation. S408/9A mice exhibited increased phasic but decreased tonic inhibition events, which correlated with alterations in the membrane stability of the receptor subtypes that mediate these distinct forms of inhibition. S408/9A mice exhibited alterations in dendritic spine structure, increased repetitive-like behavior, and decreased social interaction, which are hallmarks of ASDs. Therefore, our results provide evidence that alterations in the activity of GABA_ARs containing $\beta 3$ subunits directly contribute to ASDs.

Results

Creation of a S408/9A Knock-In Mouse. Alterations in the efficacy of GABAergic inhibition mediated by $\beta 3$ -containing GABA_ARs are strongly linked to ASDs. Phosphorylation of GABA_ARs regulates their exocytosis and endocytosis, and thereby their residence time on the neuronal plasma membrane and accumulation at inhibitory synapses (8). Central to these regulatory processes is the phosphorylation of S408/9 in the $\beta 3$ subunit, which reduces the affinity of GABA_ARs for the clathrin adaptor protein AP2, as measured by using synthetic peptides corresponding to the $\beta 3$ subunit and purified AP2 (9, 13). The significance of these findings has recently been questioned by studies that suggest that the arginine residues flanking S408/9 are the principal determinants of AP2 binding in the $\beta 3$ subunit (14). To examine the significance of S408/9 further, we expressed the intracellular domain of the $\beta 3$ subunit as a GST fusion protein (GST $\beta 3$) or a fusion protein in which the respective residues were mutated to alanines (GST $\beta 3$ S408/9A) in *Escherichia coli*. The respective fusion proteins were then phosphorylated in vitro using purified PKC to final stoichiometries of ~ 0.35 and ~ 0.03 mol/mol, respectively, and then exposed to the $\mu 2$ subunit of AP2 (15) (Fig. S1A). Phosphorylation of GST $\beta 3$ significantly reduced $\mu 2$ binding, whereas phosphorylation of GST $\beta 3$ S408/9A was without effect. Likewise GST $\beta 3$ S408/9A bound significantly lower levels

of $\mu 2$ compared with GST $\beta 3$. Collectively, these results suggest a key role for S408/9 and their phosphorylation in determining the affinity of GABA_ARs for AP2 (Fig. S1A and B).

To assess the significance of $\beta 3$ subunit phosphorylation in determining the efficacy of GABAergic inhibition, we created a knock-in mouse in which S408/9 were mutated to S408/9A using homologous recombination in ES cells (Fig. S1C and D). Mutation of the respective codons in exon 9 of the $\beta 3$ subunit was confirmed by DNA sequencing (Fig. S1C and D), and the respective mice were backcrossed on the C57BL/6J background in excess of 10 generations. S408/9A homozygotes were viable and bred normally and did not exhibit any overt phenotypes. Likewise, there did not appear to be any gross anatomical changes in the structure of the hippocampus (Fig. S1E). In hippocampal slices from WT mice, treatment with the PKC activator phorbol 12,13-dibutyrate produced a large increase in pS408/9 immunoreactivity, an effect not replicated in S408/9A mice (Fig. 1A). To control for possible global changes in GABA_AR phosphorylation mice, we analyzed phosphorylation of Y367 in the $\gamma 2$ subunit, an accepted substrate of Src family kinases (16). Treatment of slices with vanadate, an inhibitor of tyrosine phosphatases, induced similar increases in Y367 phosphorylation in WT and S408/9A mice (Fig. 1A).

Characterization of GABA_AR expression levels and synaptic targeting in S408/9A mice. Next, we examined the effects of the S408/9A mutation on the cell surface accumulation of the GABA_ARs using biotinylation. The S408/9A mutation increased the cell surface expression levels of the $\beta 3$ subunit to $145 \pm 8\%$ of WT ($P < 0.05$, $n = 4$ mice of each genotype; Fig. 1B) without modifying total subunit expression levels ($P > 0.05$, $n = 4$ mice of each genotype; Fig. 1C). In the dentate gyrus, phasic inhibition is mediated by GABA_AR assembled from $\alpha 1/2$, $\beta 2$, $\beta 3$, and $\gamma 2$ subunits, whereas subtypes containing $\alpha 4-6$, $\beta 2$, $\beta 3$, and δ subunits are responsible for tonic current. Therefore, we assessed the effects of the S408/9 mutation on the plasma membrane accumulation of the receptor $\alpha 2$ and $\alpha 4$ subunits. The plasma membrane levels of the $\alpha 2$ subunit were increased in S408/9A mice to $119.0 \pm 5.2\%$ of control ($P < 0.05$, $n = 5-6$ mice of each genotype; Fig. 1B). In

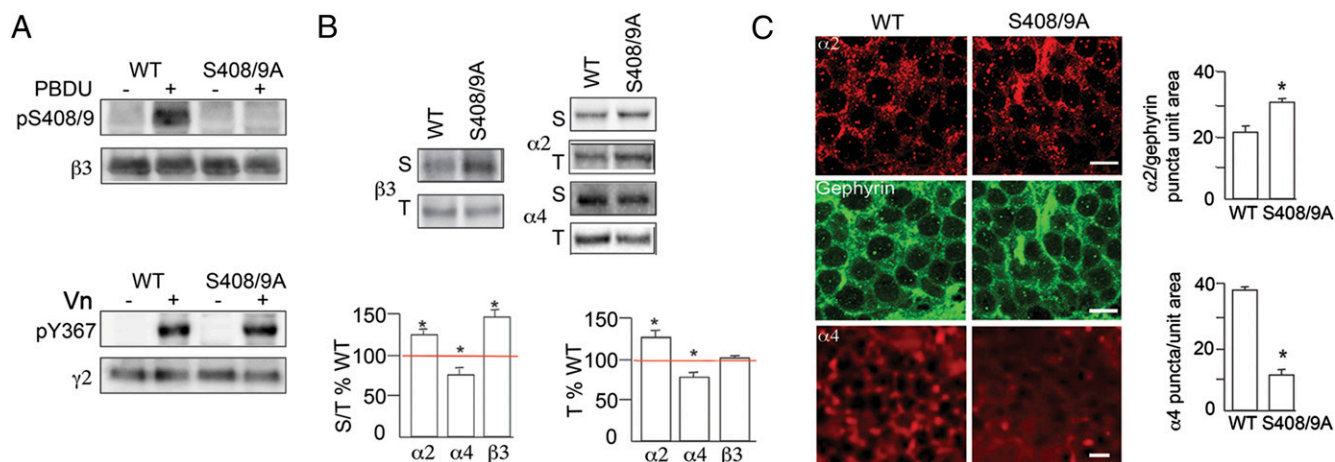


Fig. 1. GABA_AR expression in S408/9A mice. (A) Treatment of hippocampal slices from WT mice with 100 nM phorbol 12,13-dibutyrate (PDBU) increased pS408/9 immunoreactivity consistent with published studies demonstrating that both residues are substrates of PKC (8). In contrast, pS408/9 immunoreactivity was not detected in S408/9A mice. (B) Biotinylation revealed that the surface levels of the $\beta 3$ subunits, along with the synaptic $\alpha 2$ subunits, were increased in the S408/9A mice, whereas surface accumulation of GABA_ARs containing $\alpha 4$ subunits was decreased. Hippocampal slices were subjected to biotinylation, followed by immunoblotting with anti- $\alpha 2$, anti- $\alpha 4$, and anti- $\beta 3$ subunit antibodies. The ratio of surface/total (S/T%WT) was measured in S408/9A mice and WT control mice, and values were then normalized to WT controls (100%, red line). Total expression levels were also compared between genotypes (T%WT). *Significantly different from control ($P < 0.05$, t test; $n = 5-6$ mice of each genotype). (C) Forty-micron hippocampal slices were stained with antibodies against the $\alpha 2$ and $\alpha 4$ subunits and gephyrin fluorescent-conjugated secondary antibodies, followed by confocal microscopy. The number of $\alpha 2$ /gephyrin-positive puncta and $\alpha 4$ puncta was then compared within the dentate gyrus between genotypes. Preventing phosphorylation of the $\beta 3$ subunit increases the number of inhibitory synapses in the dentate gyrus of the hippocampus but reduces $\alpha 4$ puncta immunoreactivity. *Significantly different from control ($P < 0.001$, t test; $n = 3$ mice of each genotype). (Scale bars: 10 μ m.)

contrast, the $\alpha 4$ subunit was decreased to $81.0 \pm 4.5\%$ of levels seen in WT ($P < 0.05$, $n = 6$ mice of each genotype; Fig. 1B). These changes in cell surface accumulation were mirrored by parallel changes in increased total expression levels of the $\alpha 2$ subunits and decreased total expression levels of the $\alpha 4$ subunits to $120.7 \pm 4.3\%$ and $85.3 \pm 7.0\%$, respectively, compared with WT controls ($P < 0.05$, $n = 4-8$ mice of each genotype; Fig. 1B).

To characterize the subcellular distribution of GABA_ARs in S408/9A mice, hippocampal sections were subjected to immunohistochemistry with antibodies against GABA_AR $\alpha 2$ or $\alpha 4$ subunits. In some experiments, sections were also stained with antibodies against the inhibitory scaffold protein gephyrin. Sections were then visualized using confocal microscopy, and subunit expression levels were quantified within the dentate gyrus. The number of inhibitory synapses defined as $\alpha 2$ /gephyrin puncta were increased in S408/9A mice compared with WT littermates (23.4 ± 2.5 vs. 32.0 ± 0.6 for WT and S408/9A mice, respectively; $P < 0.05$, $n = 3$ mice of each genotype; Fig. 1C). In contrast, the number of puncta of $\alpha 4$ subunit immunoreactivity was decreased (13.1 ± 1.2 vs. 37.4 ± 0.8 puncta per $2,500 \mu\text{m}^2$ for S408/9A and WT mice, respectively; $P < 0.001$; Fig. 1C). Additionally, in contrast to the effects seen with GABA_ARs, the levels of gephyrin, postsynaptic density protein-95, and the AMPA receptor subunit GluA1 were unaffected by the S408/9A mutation (Fig. S2).

Collectively, these results reveal that S408/9A mice have deficits in the cell surface accumulation of GABA_ARs that mediate tonic inhibition but an increased level of those GABA_ARs that mediate phasic inhibition.

S408/9A exhibited an increase in phasic inhibition and decreased tonic current. To examine the functional significance of the alterations in GABA_AR expression seen in S408/9A mice, we used patch-clamp recording to analyze GABAergic inhibition in dentate gyrus granule cells (DGGCs). In DGGCs from S408/9A mice, tonic current was significantly decreased (24.7 ± 4.1 vs. 11.9 ± 2.2 pA for WT and S408/9A mice, respectively; $P < 0.005$, $n = 10-11$ cells, three mice of each genotype; Fig. 2A). In contrast, the amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) was significantly increased in S408/9A mice compared with WT controls (28.4 ± 2.4 vs. 42.5 ± 3.3 pA for WT and S408/9A mice, respectively; $P < 0.005$, $n = 17-18$ cells, three mice of each genotype; Fig. 2B). However, the frequency (4.5 ± 0.2 vs. 4.4 ± 0.1 Hz for WT and S408/9A mice, respectively) and decay time (44.7 ± 12.8 vs. 24.8 ± 2.5 ms for WT and S408/9A mice, respectively; $P > 0.05$, $n = 17-18$ cells, three mice of each genotype) of sIPSCs was comparable between genotypes.

We also assessed the impact of the modifications in GABAergic inhibition on neuronal excitability. First, we compared the properties of excitatory postsynaptic currents (EPSCs) in DGGCs. In S408/9A mice, EPSC amplitude was significantly increased (14.6 ± 1.05 vs. 18 ± 1.02 pA for WT and S408/9A mice, respectively; $P < 0.05$; Fig. 2C) with no difference in frequency (1.4 ± 0.3 vs. 1.3 ± 0.2 Hz for WT and S408/9A mice, respectively). Moreover, their decay was also prolonged (2.1 ± 0.1 vs. 2.4 ± 0.2 ms for WT and S408/9A mice, respectively; $P < 0.05$, $n = 14-16$ cells, three mice of each genotype). However, net excitability of DGGCs and their resting membrane potentials were not modified by the S408/9A mutation ($n = 19-20$ cells, three mice of each genotype; Fig. 2D and Table S1).

Therefore, S408/9A mice have reduced tonic but enhanced phasic inhibition, but these changes do not lead to any gross changes in neuronal excitability, presumably because S408/9A mice also exhibit elevations in EPSC amplitude.

S408/9A Mice Exhibit Deficits in Social Interaction and Increased Repetitive Behavior. ASDs have a common core of behavioral deficits, including reduced social interaction and increased repetitive behavior. Therefore, we assessed if any of these parameters are altered in S408/9A mice. First, we assessed if the

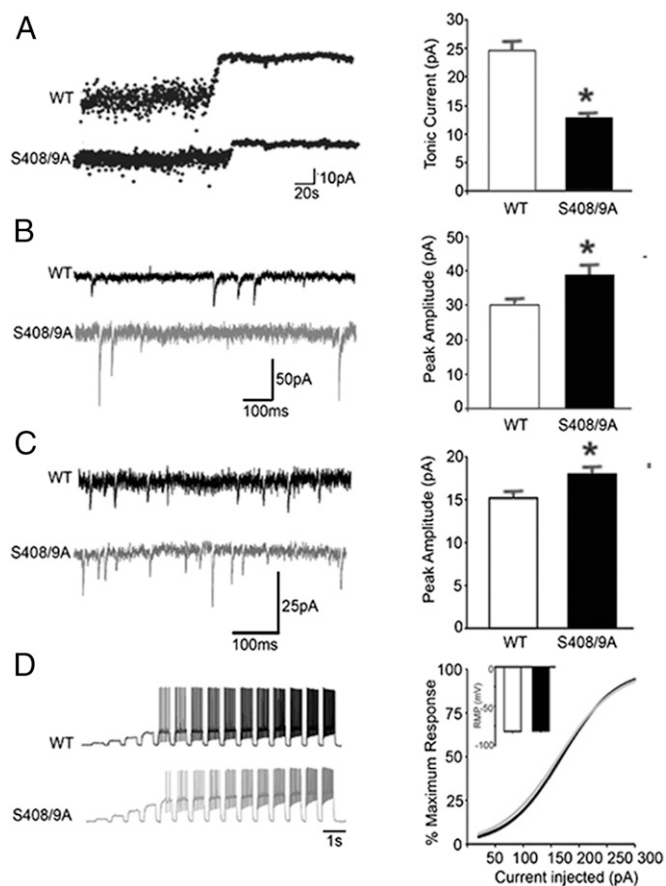


Fig. 2. Characterization of phasic and tonic inhibition in S408/9A mice. (A) Tonic conduction is decreased in S408/9A mice. Tonic current in DGGCs was compared between genotypes in the presence and absence of gabazine. The tonic current amplitude of S408/9A mice was significantly smaller than tonic current amplitude of WT mice. *Significantly different from control ($P < 0.01$, t test; $n = 10-11$ cells, three mice of each genotype). (B) sIPSCs were recorded from DGGCs of WT mice (black) and S408/9A mice (gray). *Significantly different from WT control ($P < 0.001$, t test; $n = 17-18$ cells, three mice of each genotype). (C) Examples of EPSCs recorded from DGGCs of WT mice (black) and S408/9A mice (gray). *Significantly different from control ($P < 0.05$, t test; $n = 14-16$ cells, three mice of each genotype). (D) Representative recordings of action potential firing in DGGCs from WT mice (black) and S408/9A mice (gray) in response to 0.5-s current injections from 20 to 300 pA in 20-pA increments. Average input/output curves from DGGCs from WT mice (black) and S408/9A mice (gray) are shown as a Boltzmann function generated by the averages of the fitted parameters. (Inset) No difference in the resting membrane potential (RMP) was observed between genotypes. Values are mean \pm SEM ($n = 19-21$ cells, three mice of each genotype).

respective mutation modifies motor coordination as measured using the rotarod, a critical control for data interpretation in rodent behavioral experiments. The latency to fall in the rotarod test was not different at 16–32 rpm in S408/9A mice compared with WT mice (Fig. S3A). Likewise, there were no differences between genotypes for the total distance traveled or dwell time in the center of the open field (Fig. S3B and C). We also compared anxiety-like behavior using the light/dark test. WT and S408/9A mice spent equivalent time in the light chamber ($46.9 \pm 2.2\%$ vs. $41.8 \pm 2.5\%$ for WT and S408/9A mice, respectively; $P > 0.05$, $n = 12$ mice of each genotype; Fig. S3D).

Social behavior was measured using the two-choice and three-chamber social interaction test. In the two-choice assay, WT controls spent more time in the chamber containing the novel mouse than in the chamber containing the empty cage, whereas S408/9A mice had no preference for the novel mouse over the

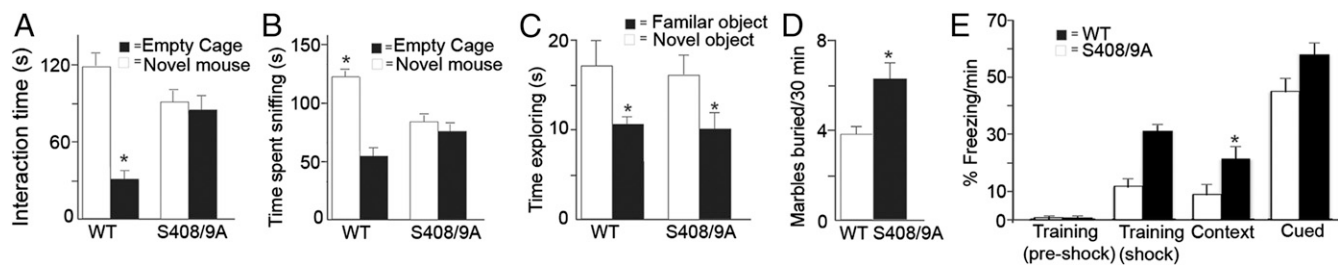


Fig. 3. Characterization of social interaction and repetitive-like behavior in S408/9A mice. (A) Preference for an empty cage or novel mouse was determined in a two-choice social interaction setup. *Significantly different from control ($P < 0.01$, ANOVA with Tukey's post hoc comparison; $n = 12$ mice of each genotype). (B) Three-chamber social interaction assay was used to measure the preference for a novel mouse or an empty cage. The amount of time mice spent in a predefined sniffing zone around the empty cage or novel mouse was digitally tracked. *Significantly different from control ($P < 0.001$, ANOVA with Tukey's post hoc comparison; $n = 12$ –16 mice of each genotype). (C) Preference for a familiar object or a novel object was determined for the genotypes. *Significantly different from control ($P < 0.05$, ANOVA with Tukey's post hoc comparison; $n = 10$ –12 mice of each genotype). (D) Repetitive-like behavior was assessed using the marble burying assay. ($P < 0.01$, t test; $n = 18$ –19 mice of each genotype). (E) Learning and memory were assessed using the fear-conditioning assay. The percentage of freezing per minute was assessed during training and for context-dependent and cued-dependent learning. *Significantly different from control ($P < 0.05$, t test; $n = 12$ –13 mice of each genotype).

empty cage (time spent interacting with empty cage: 49.8 ± 7.4 s (WT), 86.6 ± 17.6 s (S408/9A); time spent interacting with novel mouse: 119.4 ± 19.0 s (WT), 92.7 ± 17.7 s (S408/9A); $n = 12$ mice of each genotype, $P < 0.01$; Fig. 3A). Consistent with the profound deficits in social interaction observed in the two-choice assay, S408/9A mice exhibited no preference for the novel mouse in the three-chamber social interaction assay. WT controls spent more time in the novel mouse sniffing zone than in the empty cage sniffing zone (time spent interacting with empty cage: 55.4 ± 5.8 s (WT), 71.9 ± 8.4 s (S408/9A); time spent interacting with novel mouse: 123.8 ± 5.1 s (WT), 85.7 ± 6.9 s (S408/9A); $n = 12$ –16 mice of each genotype, $P < 0.01$; Fig. 3B). In contrast, S408/9A mice showed no preference for the novel mouse over an empty cage, consistent with ASD-like deficits in social interaction. As a control for our measurements for social preference, we examined the ability of S408/9A mice to discriminate between a novel object and a familiar object. Both WT controls and S408/9A mice spent more time interacting with the novel object ($66 \pm 0.9\%$ vs. $64 \pm 7.7\%$ of total time for WT and S408/9A mice, respectively; $P < 0.05$, $n = 10$ –11 mice of each genotype; Fig. 3C).

To measure repetitive-like behavior in S408/9A mice, we used the marble burying assay. S408/9A mice displayed an increase in repetitive-like behavior in the marble burying test (6.0 ± 0.6 marbles in 30 min compared with 3.8 ± 0.5 marbles in WT controls; $P < 0.001$, $n = 18$ –19 mice of each genotype; Fig. 3D). Therefore, S408/9A mice exhibited an increase in repetitive-like behavior and deficits in social interaction, which are behavioral characteristics commonly observed in ASDs.

Finally, we assessed if the S408/9A mutation has an impact on cognition by comparing contextual and cued fear conditioning between genotypes. S408/9A mice exhibited enhanced context-dependent learning (training: $11.8 \pm 3.0\%$ freezing per minute (WT), $32.1 \pm 4.3\%$ freezing per minute (S408/9A); $P > 0.05$; context: $8.9 \pm 3.4\%$ freezing per minute (WT), $21.4 \pm 4.2\%$ freezing per minute (S408/9A); $P < 0.05$; Fig. 3E) compared with WT controls but no statistical differences in cue-dependent learning (cued: $45.1 \pm 6.6\%$ freezing per minute (WT), $60.8 \pm 6.4\%$ freezing per minute (S408/9A); $P > 0.05$; Fig. 3E).

S408/9A Mice Have Increased Seizure Susceptibility. ASDs are frequently comorbid with epilepsy. Therefore, we analyzed the impact of the S408/9A mutation on seizure susceptibility. Mice were implanted with EEG electrodes, and seizures were induced with 20 mg/kg of kainic acid and monitored to ensure seizures reached stage 3–4 on the Racine scale (forelimb clonus and rearing with forelimb clonus). Representative EEG traces during epileptiform events captured 30 min after kainic acid injection illustrate a

marked increase in epileptiform activity in S408/9A mice over WT controls (Fig. 4A). Strikingly, S408/9A mice exhibited a faster onset to the first epileptiform event compared with WT mice (4.6 ± 0.7 vs. 11.2 ± 1.1 min of latency for S408/9A and WT mice, respectively; $P < 0.001$, $n = 8$ –10 mice of each genotype; Fig. 4B). Additionally, S408/9A mice entered status epilepticus at an earlier time point than WT controls (41.8 ± 4.3 vs. 61.5 ± 8.6 min for S408/9A and WT mice, respectively; $P < 0.05$, $n = 8$ –10 mice of each genotype; Fig. 4B). Furthermore, S408/9A mice spent a greater percentage of the total time experiencing epileptiform activity compared with WT controls ($82.5 \pm 2.1\%$ vs. $65.4 \pm 6.4\%$ for S408/9A and WT mice, respectively; $P < 0.05$, $n = 8$ –10 mice of each genotype; Fig. 4B). Therefore, the S408/9A mutation significantly increases seizure susceptibility.

S408/9A Mice Exhibited Increased Hippocampal Dendritic Spine Density.

Alterations in spine structure are a common feature in ASDs (17). Therefore, we assessed if the alterations in GABAergic inhibition have an impact on dendritic structure. Brains from both genotypes were subjected to Golgi staining and visualized using stereology. We quantified the density of spines along 30- μ m-long sections of

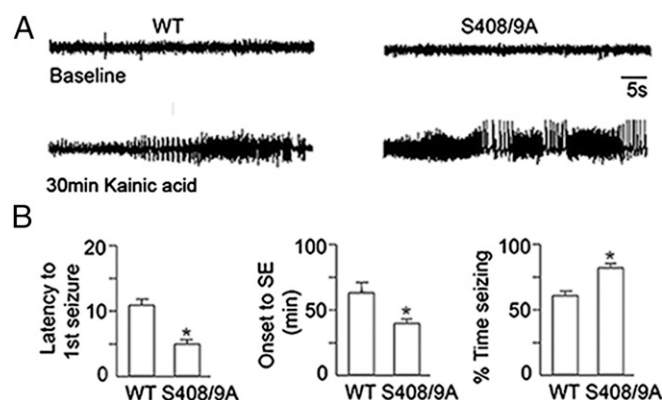


Fig. 4. Characterization of seizure susceptibility in S408/9A mice. (A) Representative EEG traces for WT and S408/9A mice at baseline and 30 min after kainic acid injection. (B) Latency to the first epileptiform activity, onset of status epilepticus (SE), and total time experiencing epileptiform activity were digitally tracked and measured. S408/9A mice had a shorter latency to the first epileptiform activity, faster onset of status epilepticus, and spent a great percentage of time experiencing epileptiform activity compared with WT controls. *Significantly different from control ($P < 0.05$, t test; $n = 8$ –10 mice of each genotype).

dendrites in the molecular layer above the dentate gyrus of the hippocampus. In S408/9A mice, a pronounced increase in spine density was seen (28.6 ± 0.7 vs. 34.8 ± 1.0 per 30- μm dendrite for WT and S408/9A mice, respectively; $P < 0.0001$, $n = 12$ neurons in three independent experiments; Fig. 5A). Similarly, the spines in S408/9A mice appeared to be more filopodia-like in structure compared with their equivalents in WT mice, and some exhibited pronounced branching. Therefore, S408/9A mice displayed similar abnormalities in spine structure to those abnormalities reported in *Fmr1* KO mice and patients with ASDs.

S408/9A Phosphorylation Is Increased in *Fmr1* KO (Fragile X Syndrome) Mice. To assess the relevance of our studies further, we measured S408/9 phosphorylation in *Fmr1* KO mice, a model of Fragile X syndrome (FXS). S408/9 phosphorylation was significantly increased to $285.6 \pm 24.3\%$ in *Fmr1* KO mice compared with WT controls ($P < 0.005$; $n = 3$ mice of each genotype; Fig. 5B). In contrast to S408/9, phosphorylation of S383 was similar in *Fmr1* KO mice compared with WT controls ($P > 0.05$, $n = 3$ mice of each genotype; Fig. 5B).

Discussion

Deficits in the efficacy of neuronal inhibition mediated by GABA_ARs containing the $\beta 3$ subunit are widely believed to contribute to the pathophysiology of ASDs. Accordingly, modifications in GABA_AR $\beta 3$ subunit gene structure and/or protein expression levels, together with both deletions and duplications of the 15q11–13 locus, are leading causes of ASDs (5, 18).

In vitro studies have shown that phosphorylation of S408/9 regulates the cell surface stability and synaptic accumulation of $\beta 3$ -containing GABA_ARs (7), and, here, we have analyzed the significance of this putative regulatory process for the pathophysiology of ASDs. To do so, we created a S408/9A knock-in mouse using homologous recombination. S408/9A mice were viable and did not exhibit any overt phenotypes or gross alterations in the structure of the hippocampus. Consistent with the lowered affinity for AP2 in the S408/9A mutation in vitro, the cell surface expression levels of the $\beta 3$ subunit in the hippocampus were increased in S408/9A mice compared with WT controls. Within the dentate gyrus, the $\beta 3$ subunit assembles with the $\alpha 2/\gamma 2$ or $\alpha 4/\delta$ subunits to form GABA_AR subtypes that mediate phasic and tonic inhibition, respectively (2). Strikingly, in the dentate gyrus of S408/9A mice, there was a significant increase in the expression levels of the $\alpha 2$ subunit and the number of inhibitory synapses. In contrast, deficits in the expression levels of the $\alpha 4$ subunit were seen. Thus, in addition to membrane trafficking, S408/9 and/or their phosphorylation may play a role in regulating the assembly of individual GABA_AR subtypes. Significantly, our findings of alterations in $\alpha 4$ and $\beta 3$ subunit expression

are consistent with GABAergic dysfunction in *Mecp2* and *Fmr1* KO mice, which are accepted models of ASDs (11, 19, 20).

Consistent with this result, the amplitude of sIPSCs was increased and their decay was slowed in DGGCs from S408/9A mice. In contrast, a reciprocal decrease in the cell surface accumulation of the $\alpha 4$ subunit was seen in S408/9A mice, which paralleled a reduction in tonic current. The gross excitability of DGGCs as measured by current injection was not altered between strains, reflecting the increase in EPSC amplitude in S408/9A mice. Therefore, preventing the phosphodependent modulation of the GABA_ARs by mutating S408/9 in the $\beta 3$ subunit to alanines alters the equilibrium between phasic and tonic inhibition in the dentate gyrus.

Previous in vitro studies have suggested that for synaptic GABA_ARs containing $\alpha 1/2\beta 3\gamma 2$ subunits, mutation of S408/9A does not compromise receptor assembly, or transport to the plasma membrane, but selectively reduces their endocytosis (9). Therefore, the increased synaptic accumulation of the $\alpha 2$ subunit in S408/9A mice presumably results from their enhanced residence time on the plasma membrane. In contrast to synaptic receptors, the mechanisms that control the membrane trafficking of receptors containing $\alpha 4$ subunits are less well defined. However, it is evident from our results that phosphorylation of S408/9 may play opposing roles in regulating the assembly and membrane trafficking of $\alpha 4$ subunit containing GABA_ARs to those roles established for subtypes that mediate phasic inhibition. Clearly, further studies are required to identify the role that S408/9 and their phosphorylation plays in regulating the differential membrane trafficking of GABA_AR subtypes that mediate phasic and tonic inhibition.

In parallel with these modifications in GABAergic inhibition, the number of dendritic spines was strikingly increased in the mutant mice. Significantly, overexpressing S408/9A cDNA in cultured hippocampal neurons leads to similar changes in spine maturity to those changes seen in S408/9A mice (7). It is emerging that tonic inhibition plays a central role in reducing neuronal excitability, particularly at depolarizing membrane potentials (21). Given the emerging role for tonic inhibition in regulating neuronal output, the deficits in the efficacy of this process in S408/9A mice may lead to prolonged neuronal depolarization and enhanced intracellular Ca^{2+} signaling, which would be predicted to have significant effects on spine architecture.

In addition to modifications in dendritic structure, ASDs have a common core of behavioral symptoms, including increased repetitive behavior and anxiety, together with deficits in social interaction (17). Therefore, we compared these parameters between genotypes. Compared with WT controls, S408/9A mice exhibited decreased social interaction as measured in both the two-choice and three-chamber assays; however, S408/9A mice and WT controls

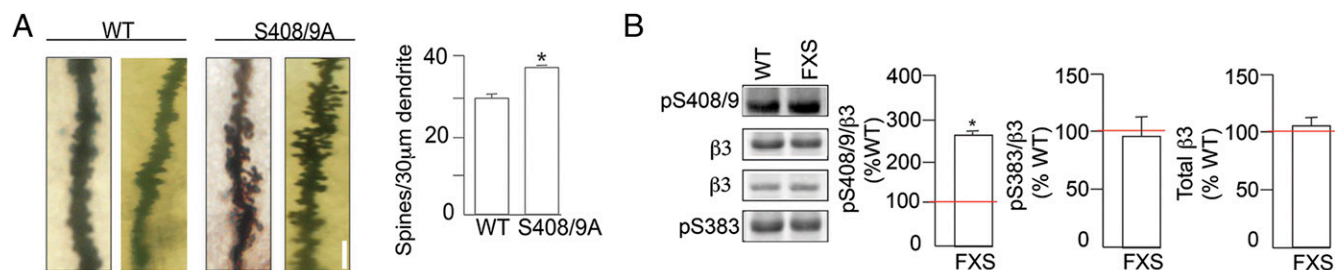


Fig. 5. Characterization of dendritic spine density in DGGCs from S408/9A mice and $\beta 3$ phosphorylation in *Fmr1* KO (FXS) mice. (A) Representative dendrites from DGGCs of two different mice. (Scale bar: 2 μm .) The number of spines per 30 μm of dendrite was then determined and compared between genotypes. *Significantly different from control ($n = 12$ neurons from three independent experiments, three mice of each genotype; $P < 0.0001$, t test). (B) Hippocampal lysates from WT and *Fmr1* KO mice on a FVB/N (Friend leukemia virus B strain) background were subjected to SDS/PAGE and immunoblotted with pS408/9, pS383, and $\beta 3$ subunit antibodies. The pS408/9/ $\beta 3$ or pS383/ $\beta 3$ ratios in *Fmr1* KO mice (FXS) were normalized to WT controls (100%; red line). *Significantly different from control ($P < 0.01$, t test; $n = 3$ mice of each genotype).

shared a similar preference for a novel object over a familiar object. S408/9A mice also exhibited an increase in repetitive-like behavior as measured using the marble burying assay. Significantly, the mutation did not alter locomotor activity, behavior in the open field, or behavior in the light/dark test. S408/9A mice did not appear to exhibit any modifications in anxiety. ASDs are often comorbid with epilepsy, and consistent with this comorbidity, S408/9A mice exhibit increased sensitivity to kainate-induced seizures. Thus, although the changes in phasic and tonic inhibition seen in S408/9A mice do not appear to lead to changes in net neuronal excitability, they are important determinants of epileptogenesis. Finally, we noted that in the *Fmr1* KO mouse model of FXS, specific alterations in S408/9 phosphorylation were seen. The mechanism linking deficits in FMRP expression and enhanced S408/9 phosphorylation requires further experimentation. However, it is interesting to note that FMRP is part of a signaling complex that contains protein phosphatase 2A (PP2A), the principle phosphatase that dephosphorylates S408/9 (10, 22, 23). Thus, deficits in FMRP expression levels may affect the stability and/or subcellular targeting of PP2A, leading to reduced rates of S408/9 dephosphorylation.

In conclusion, our results suggest that alterations in the phosphorylation status of GABA_A β 3 resulting in compromised GABAergic inhibition are central to the pathophysiology of FXS. Therefore, restoring the efficacy of tonic inhibition may be a useful therapeutic strategy to alleviate the burdens of ASDs.

- Rudolph U, Möhler H (2006) GABA-based therapeutic approaches: GABAA receptor subtype functions. *Curr Opin Pharmacol* 6(1):18–23.
- Brickley SG, Mody I (2012) Extrasynaptic GABA(A) receptors: Their function in the CNS and implications for disease. *Neuron* 73(1):23–34.
- DeLorey TM, et al. (1998) Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. *J Neurosci* 18(20):8505–8514.
- Abrahams BS, Geschwind DH (2008) Advances in autism genetics: On the threshold of a new neurobiology. *Nat Rev Genet* 9(5):341–355.
- Delahanty RJ, et al. (2011) Maternal transmission of a rare GABRB3 signal peptide variant is associated with autism. *Mol Psychiatry* 16(1):86–96.
- Brandon NJ, et al. (1999) Synaptic targeting and regulation of GABA(A) receptors. *Biochem Soc Trans* 27(4):527–530.
- Jacob TC, et al. (2009) GABA(A) receptor membrane trafficking regulates spine maturity. *Proc Natl Acad Sci USA* 106(30):12500–12505.
- Nakamura Y, Darnieder LM, Deeb TZ, Moss SJ (2015) Regulation of GABAARs by phosphorylation. *Adv Pharmacol* 72:97–146.
- Kittler JT, et al. (2005) Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. *Proc Natl Acad Sci USA* 102(41):14871–14876.
- Terunuma M, et al. (2008) Deficits in phosphorylation of GABA(A) receptors by intimately associated protein kinase C activity underlie compromised synaptic inhibition during status epilepticus. *J Neurosci* 28(2):376–384.
- Martin BS, Corbin JG, Huntsman MM (2014) Deficient tonic GABAergic conductance and synaptic balance in the fragile X syndrome amygdala. *J Neurophysiol* 112(4):890–902.
- DeLorey TM (2005) GABRB3 gene deficient mice: A potential model of autism spectrum disorder. *Int Rev Neurobiol* 71:359–382.
- Jacob TC, Moss SJ, Jurd R (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci* 9(5):331–343.
- Smith KR, et al. (2012) Stabilization of GABA(A) receptors at endocytic zones is mediated by an AP2 binding motif within the GABA(A) receptor β 3 subunit. *J Neurosci* 32(7):2485–2498.
- Moss SJ, Doherty CA, Haganir RL (1992) Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular

Materials and Methods

All protocols were approved by Tufts University's Institutional Animal Care and Use Committee and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (24). More detailed information on materials and methods is provided in *SI Materials and Methods*.

Creation of the S408/9A Mice. The S408/9A mice were created using homologous recombination as detailed previously (25).

Biochemical Measurements. Antibodies used in this study have been described previously, as have the methods for immunoblotting and immunoprecipitation (22, 26).

Electrophysiology and EEG Recordings. Phasic and tonic inhibition was measured using the patch-clamp technique (27). The Pinnacle Technology system and LabChart (ADInstruments) were used for EEG recordings and analysis as previously described (28).

Behavior. Measurements using the rotarod and activity in the open field and anxiety were assessed as described previously (26).

ACKNOWLEDGMENTS. We thank Jay Boltax and Hew Mun Lau (McLean Hospital) for technical assistance in generating the mutant allele in ES cells. This work was supported by Grant 206026 from the Simons Foundation (to S.J.M.); NIH–National Institute of Neurological Disorders and Stroke Grants NS051195, NS056359, NS081735, R21NS080064, and NS087662 (to S.J.M.); NIH–National Institute of Mental Health Grant MH097446 (to P.A.D. and S.J.M.); and US Department of Defense Grant AR140209 (to P.A.D. and S.J.M.). J.M. is supported by Grant NS073574, and U.R. is supported by Grant R01MH080006.

- domains of the beta 1, gamma 2S, and gamma 2L subunits of the gamma-aminobutyric acid type A receptor. *J Biol Chem* 267(20):14470–14476.
- Jurd R, Tretter V, Walker J, Brandon NJ, Moss SJ (2010) Fyn kinase contributes to tyrosine phosphorylation of the GABA(A) receptor gamma2 subunit. *Mol Cell Neurosci* 44(2):129–134.
- Hutsler JJ, Zhang H (2010) Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain Res* 1309:83–94.
- Kang JQ, Barnes G (2013) A common susceptibility factor of both autism and epilepsy: Functional deficiency of GABA A receptors. *J Autism Dev Disord* 43(1):68–79.
- Olmos-Serrano JL, et al. (2010) Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. *J Neurosci* 30(29):9929–9938.
- Chao HT, et al. (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. *Nature* 468(7321):263–269.
- Włodarczyk AI, et al. (2013) Tonic GABAA conductance decreases membrane time constant and increases EPSP-spike precision in hippocampal pyramidal neurons. *Front Neural Circuits* 7:205.
- Jovanovic JN, Thomas P, Kittler JT, Smart TG, Moss SJ (2004) Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. *J Neurosci* 24(2):522–530.
- Narayanan U, et al. (2007) FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A. *J Neurosci* 27(52):14349–14357.
- Committee on Care and Use of Laboratory Animals (1996) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.
- Terunuma M, et al. (2014) Postsynaptic GABAB receptor activity regulates excitatory neuronal architecture and spatial memory. *J Neurosci* 34(3):804–816.
- Tretter V, et al. (2009) Deficits in spatial memory correlate with modified gamma-aminobutyric acid type A receptor tyrosine phosphorylation in the hippocampus. *Proc Natl Acad Sci USA* 106(47):20039–20044.
- Kretschmannova K, et al. (2013) Enhanced tonic inhibition influences the hypnotic and amnestic actions of the intravenous anesthetics etomidate and propofol. *J Neurosci* 33(17):7264–7273.
- Lee V, Maguire J (2013) Impact of inhibitory constraint of interneurons on neuronal excitability. *J Neurophysiol* 110(11):2520–2535.

Supporting Information

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SI Materials and Methods

Mice. Ten- to 12-wk-old male S408/9A mice and the corresponding WT littermates were housed in a 12-h light/dark cycle with standard rodent food and water ad libitum. For behavioral testing, animals were acclimatized to the testing room for 1 h before the start of all procedures.

Gabrb3 (Gene That Encodes for the $\beta 3$ GABA_AR Subunit Protein). S408/9A mice were generated by gene targeting in murine ES cells using standard techniques. Briefly, a targeting vector was constructed containing 7.4 kb of homologous genomic sequence (from 3 kb upstream of the 5' end of exon 9 to 4.4 kb downstream of the 5' end of exon 9). The genomic DNA was subcloned from a substrain 129S5 BAC, obtained from the Wellcome Trust Sanger Institute. In exon 9 (the final exon), the codons for S408/9 of the GABA_AR $\beta 3$ subunit were mutated to alanine codons (S408/9A). A loxP-flanked neomycin expression cassette (loxP-FRT-PGKneobpA-FRT-SA-4xSv40pA-SD-loxP) was inserted 0.3 kb upstream of the 5' end of exon 9. An HSV-TK (herpes simplex virus-thymidine kinase) cassette for negative selection was positioned 3' of the homology. The NotI-linearized targeting vector was electroporated into TC-1 ES cells (derived from substrain 129S6, a gift from Phil Leder, Department of Genetics, Harvard Medical School, Belmont, MA). Correctly targeted clones were identified by PCR and sequenced to verify the double-point mutation and the loxP sites, and they were injected into C57BL/6J mouse blastocysts. Chimeric male mice were bred with WT C57BL/6J females, and the entire neomycin expression cassette was excised by Cre/loxP-mediated recombination. Mice were backcrossed to C57BL/6J for a minimum of 10 generations. Experimental animals were derived from heterozygous breedings. The proposed allele symbol for the $\beta 3$ -S408/9A allele in the MGI database is *Gabrb3*^{tm2Uru}.

Antibodies. Polyclonal rabbit antibodies against $\alpha 2$, $\alpha 4$, $\beta 2$, and δ were gifts from Verena Tretter and Werner Sieghart (both from Medical University Vienna, Vienna). Anti- $\beta 3$ and anti-phospho- $\beta 3$ (phospho-S408/9 and phospho-S383) antibodies were generated and verified by the S.J.M. laboratory (9, 10, 12). The following antibodies were purchased from commercial vendors: monoclonal anti- $\alpha 1$ (clone N95/35; NeuroMab), monoclonal anti-gephyrin (catalog no. 147011; Synaptic Systems), and monoclonal anti-actin (catalog no. A2228; Sigma-Aldrich).

Western Blot. Standard Western blotting protocols were used as previously described (10). Briefly, hippocampi were rapidly dissected, flash-frozen, and lysed in lysis buffer composed of the following: 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 2 mM Na₃VO₄, 10 mM pyrophosphate, 0.1% SDS, and 50 mM NaF. Total protein concentration was established, and 40 μ g of hippocampal lysate was subjected to SDS/PAGE, transferred to nitrocellulose membranes, and blocked with 5% (wt/vol) BSA in Tris-buffered saline-Tween 20 for 1 h. Membranes were immunoblotted with the indicated primary antibodies, and following extensive rinsing, they were probed with HRP-conjugated secondary antibodies and detected with enhanced chemiluminescence. Blots were imaged, and data were normalized to actin and quantified with the CCD-based LAS 3000 system (FujiFilm).

Surface Biotinylation. Three hundred fifty-micron-thick hippocampal slices from 10- to 12-wk-old male WT control (C57BL/6)

mice or age-matched male S408/9A mice were prepared on a vibratome (Leica VT1000S) in cold, oxygenated artificial cerebral spinal fluid (ACSF) composed of 124 mM NaCl, 3 mM KCl, 25 mM NaHCO₃, 2 mM MgSO₄, 2 mM CaCl₂, 1.1 mM NaH₂PO₄, and 10 mM glucose (pH 7.4). After sectioning, slices were allowed a 1-h recovery period in oxygenated, ice-cold ACSF. Following the recovery period, slices were incubated for 30 min at 4 °C with 1 mg/mL NHS-SS-biotin (Pierce). Following three rinses in ice-cold ACSF to remove excess biotin, hippocampal samples were lysed. After adjusting for protein concentration, hippocampal lysates were incubated with streptavidin beads (Pierce) overnight at 4 °C. Bound substances were subjected to SDS/PAGE, followed by immunoblotting with the indicated antibodies. Immunoblots were quantified using the CCD-based LAS 3000 system. For phospho-specific antibodies, the ratio of phospho- $\beta 3$ /total- $\beta 3$ was calculated and S408/9A values were normalized to WT control (100%).

EEG Recordings. EEGs were performed as previously described (28). Age-matched male littermates, aged 10–12 wk, were used for recording EEGs in awake, behaving animals. Mice were deeply anesthetized and implanted with prefabricated head mounts containing six-pin connectors with two electromyogram reference electrodes (Pinnacle Technology) 2.0 mm posterior to the bregma, along the midsagittal suture, and 2 mm below the dura. Following a 1-wk recovery period, EEG activity was monitored using the Pinnacle Technology turnkey system with a 100 \times amplifier and were high-pass-filtered at 1.0 Hz (PowerLabs; ADInstruments). A 30-min baseline was obtained before an i.p. injection of 20 mg·kg⁻¹ kainic acid (Tocris), followed by continuous EEG monitoring for an additional 120 min. Epileptiform activity was defined as electrographic events with amplitudes greater than twofold the standard deviation of the averaged baseline that last a minimum of 10 s and are separated from another event by greater than 10 s. Epileptiform activity was additionally confirmed by an increase in the power and frequency of high-amplitude events. Latency to the first seizure was defined as the time from injection of kainic acid until the first electrographic seizure. Duration of epileptiform activity was calculated from the cumulative time in minutes of epileptiform activity divided by 120 min. The onset of status epilepticus was defined as epileptiform activity lasting longer than 5 min with no silent period greater than 10 s. Duration of epileptiform activity, latency to the first epileptiform event, and latency to onset of status epilepticus were quantified using LabChart, version 7 (ADInstruments).

Immunohistochemistry. Ten- to 12-wk-old male mice were perfused intracardially with 4% (wt/vol) paraformaldehyde, brains were dissected and immersed in 30% (wt/vol) sucrose for 72 h, and 40- μ m-thick sections were prepared from fresh-frozen tissue using a microtome. Free-floating sections were processed for immunohistochemistry. Tissue was extensively rinsed in BupH-PBS (Thermo Fisher Scientific) and blocked for 1 h in 10% (wt/vol) BSA with 0.3% Triton X-100 in BupH-PBS. Tissue was incubated overnight at room temperature with the indicated antibodies, extensively rinsed in BupH-PBS, and incubated for 2 h in Alexa Fluor 488 and/or Alexa Fluor 568 secondary antibodies (Life Technologies, Thermo Fisher Scientific). After extensive rinsing with BupH-PBS, tissues were mounted, dried, and coverslipped with ProLong Gold anti-fade reagent with DAPI (Invitrogen). Images were obtained with a Nikon Ti microscope and analyzed with

ImageJ (NIH) software. Controls consisted of incubating slices in the absence of primary antibody.

Electrophysiology. The brains of male mice were rapidly dissected and placed in oxygenated, ice-cold ACSF solution containing 225 mM sucrose, 2.95 mM KCl, 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , 0.5 mM CaCl_2 , 10 mM MgSO_4 , 10 mM D-glucose, and 3 mM kynurenic acid and were bubbled with 95% O_2 /5% CO_2 (330–340 mosmol $^{-1}$). Coronal (350- μm -thick) slices were cut using a vibratome. The slices were then incubated at -33°C in ACSF containing 126 mM NaCl, 2.5 mM KCl, 26 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 2 mM CaCl_2 , 10 mM D-glucose, and 2 mM MgCl_2 saturated with 95% O_2 /5% CO_2 (pH 7.4, 300–310 mosmol $^{-1}$) for a minimum of 1 h before experimentation. Hippocampal slices were viewed on a fixed-stage upright microscope (Nikon FN-1) with a 40 \times water immersion objective equipped with differential interference contrast/infrared (DIC/IR) optics. Slices were maintained at 32°C and gravity-superfused with ACSF solution throughout experimentation. For current-clamp recordings in S408/9A mice, slices were incubated in ACSF in the absence of kynurenic acid. Input/output curves were produced as previously described from DGGCs (28). Briefly, visually identified DGGCs were injected with 0.5-s square wave current injections from 20 to 300 pA in 20-pA increments.

For voltage-clamp recordings, the internal pipette solution contained 140 mM CsCl, 1 mM MgCl_2 , 0.1 mM EGTA, 10 mM Hepes, 2 mM Mg-ATP, 4 mM NaCl, and 0.3 mM Na-GTP (pH 7.25, 280–290 mosM). Series resistance and whole-cell capacitance were continually monitored throughout the experiment. After these parameters had stabilized for a minimum of 5 min, synaptic currents were recorded using an Axopatch 200B amplifier (Molecular Devices), filtered at 2 kHz, sampled at 20 kHz, digitized (Digidata 1320A; Molecular Devices), and stored for off-line analysis (Minianalysis; Synsoft, Inc.). Access resistance of 10–20 $\text{M}\Omega$ ($\sim 80\%$ compensation) was monitored using a voltage step of -5 mV, and data from cells were discarded when $>20\%$ change occurred.

sIPSCs were analyzed using pClamp 9.2 (Clampfit; Axon Instruments) and MiniAnalysis software using pooled population data expressed as the mean \pm SEM. The decay phase was fitted with a monoexponential function (τ decay), and rise time was analyzed by comparing the mean with 10–90% rise time. Tonic current was calculated as the difference between the holding current before and after application of the indicated GABA $_A$ R antagonist (≥ 200 μM SR95531 or 100 μM picrotoxin). The mean holding current of a 10-ms epoch was sampled every 100 ms for a single data point, and a Gaussian fit was applied to the resulting all-points histogram. Epochs containing synaptic events or an unstable baseline were excluded from the analysis. Input/output curves were fitted with a Boltzmann function: $f(W) = \text{Max}/(1 + \exp[(I - I_{50})/k])$, where Max = maximum response, k = slope, and I_{50} = amplitude of current that produces 50% of the maximum response.

Fear Conditioning. Fear conditioning was carried out in a single chamber with foot-shock bars. For conditioning and context testing, the chamber walls were covered with patterned panels. The conditioning procedures were a 180-s baseline period, followed by two trials separated by 120-s intervals. For each trial, a 15-s 70-dB tone was played, which terminated with the application of a 1.5-mA foot shock during the last 1 s. Twenty-four hours later, context-dependent memory was assessed by placing the mice in the chambers containing the patterned panels (conditioning environment). The amount of time the mice spent freezing during the trials was digitally tracked and analyzed. Two hours after context testing, cue-dependent memory was assessed by placing the mouse in a chamber without the patterned panels and measuring the amount of time it spent freezing during a 120-s

baseline period, followed by two 70-dB tones played for 15 s separated from one another by 60-s intervals.

Open-Field Test. Following a thorough cleaning of the arena with 70% (vol/vol) ethanol, individual mice were placed in the center of a 40 \times 40-cm arena and movement was digitally tracked (EthoVision XT 7.0; Noldus Technology). Time spent in a predefined center arena and total distance traveled were calculated.

Light/Dark Box Assay. Following a thorough cleaning with 70% (vol/vol) ethanol of the two equally sized chambers (each chamber is 21 \times 21 cm), individual mice were placed in the dark chamber and allowed to roam freely between the dark and light chambers. Movement and time spent in the different chambers were digitally tracked and calculated (EthoVision XT 7.0).

Marble Burying. Mice were transferred into a new cage with 6-inch deep bedding, and 20 glass marbles were placed evenly spaced apart in a 4 \times 5 pattern on its surface. Mice were individually placed in the cage, and the number of marbles buried to two-thirds of their depth after 30 min was counted.

Two-Choice Social Assay. In the two-choice assay, the subject mouse is placed at the bottom of a T-shaped container made of Plexiglas with two perpendicular arms (the long arm is 63.5 \times 10 cm, and each short arm is 55 \times 10 cm, surrounded by 32-cm high walls). All chambers and wire cups were cleaned with 70% (vol/vol) ethanol between each subject mice. The first session is a habituation period, where the subject mouse was able to roam freely across the three arms. This session was followed by a testing session, where a novel object (empty cup) was placed in the left arm or a novel mouse (stranger mouse under the wire cup) was placed in the right arm. The subject mouse was able to move freely across all three arms, and the cumulative time spent in the left arm or right arm was digitally tracked and quantified (EthoVision XT 7.1; Noldus Technology).

Social Novelty. Briefly, on the first day of training, mice were exposed to the open-field arena (40 \times 40 cm) for 10 min. On the second day of training, mice were placed in an empty open-field arena for 5 min, followed by 5 min of exposure to two empty cages. On the third day, testing for social novelty was carried out by placing the subject mouse in an empty open-field arena for 5 min, followed by another 5 min when an empty cage (familiar object) or a novel object (empty salt shaker) was placed in opposite corners of the open-field arena. The amount of time the mice spent exploring in a predefined zone around the objects was digitally tracked (EthoVision XT 7.0).

Three-Chamber Social Test. Briefly, we constructed a three-chamber apparatus with Plexiglas containing three equally sized chambers (30 \times 30 cm); two doors that can be remotely opened separate the middle chamber from the left and right chambers. A wire cup (4-inch diameter; item no. 010591315704, organizeit.com) was used as the empty cage or housed a stranger mouse (novel mouse), and the sniff zone 5 cm from the center of the wire cup was predefined. All chambers and wire cups were cleaned with 70% (vol/vol) ethanol between subject mice. A 10-min habituation period where the subject mouse was placed in the center chamber with the doors to the other chambers closed was followed by another 10-min session where the subject mouse was able to roam freely across all three chambers. These sessions were followed by a third 10-min session where a novel object (empty cup) was placed in the left chamber or a novel mouse (stranger mouse under the wire cup) was placed in the right chamber. The subject mouse was freely able to move across all three chambers, and the amount of time it spent in the predefined sniff zone (5-cm radius proximal to each cage) was

digitally monitored (EthoVision XT 7.1). A fourth 10-min session was used to test for social novelty, where a second stranger mouse was placed in the previously empty cage. The subject mouse was then able to roam freely across all three chambers, with the right chamber holding the previously encountered mouse (familiar mouse) and the left chamber holding the novel mouse (stranger mouse). Cumulative time spent in the left or right sniffing zone was digitally tracked and quantified. (EthoVision XT 7.1).

Golgi Staining. For Golgi staining, we used the protocol recommended by the manufacturer of the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Inc.). Briefly, mice were deeply anesthetized, and brains were rapidly extracted and rinsed in Milli-Q water generated at Tufts University within the S.J.M. laboratory. Brains were immersed in the provided solutions for 14 d at room temperature in the dark. Following Golgi-Cox immersion, 200- μ m-

thick coronal sections were prepared on a vibratome. Sections were then developed according to the manufacturer's protocol and dehydrated in 50%, 75%, 95%, and absolute ethanol; cleared in xylene; and coverslipped with Permount (catalog no. SP15; Thermo Fisher Scientific). Dendritic spine analysis was performed on dendritic spines located immediately adjacent to the DGGC layer in the molecular layer of the hippocampus using images collected with a 60 \times objective (Nikon). Spine density in 30- μ m-long sections was calculated.

Statistical Analysis. All data are presented as the mean \pm SEM and were analyzed with the Student's *t* test, one-way ANOVA with Turkey's multiple comparison test, and two-way ANOVA with Bonferroni's post hoc comparison. Unless otherwise indicated, $P < 0.05$ was considered statistically significant. All analyses were done using Prism 6 (GraphPad).

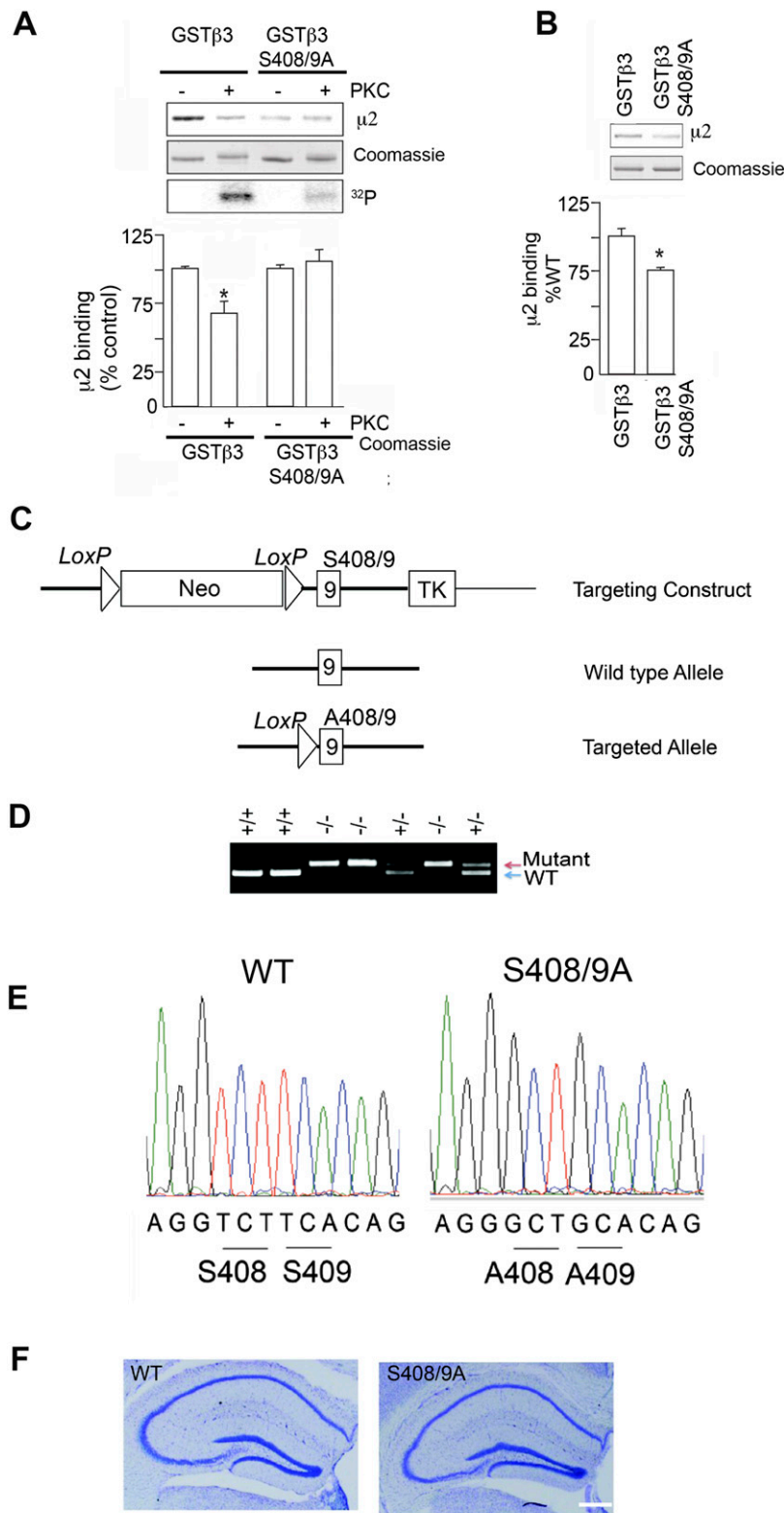


Fig. S1. Characterization of β3 phosphorylation and generation of S408/9A mice. (A) GST fusion proteins encoding the major intracellular domain of the β3 subunit (GST-β3) and a mutant fusion protein in which residues S408/9 were mutated to alanines (GSTβ3S408/9A) were purified from *E. coli*. Fusion proteins were subjected to *in vitro* phosphorylation using purified PKC. GSTβ3 was phosphorylated to 0.35 mol/mol, and GSTβ3S408/9A was purified to 0.03 mol/mol. Phosphorylated fusion proteins were then exposed to brain lysates and immunoblotted with μ2 antibodies. Coomassie staining of the respective samples and an autoradiogram of samples exposed to ³²P-γ-ATP and PKC are shown. The level of μ2 binding was normalized to fusion proteins that had been incubated with heat-inactivated PKC. (B) AP2 binding to GSTβ3 and GSTβ3S408/9A was directly compared as outlined above. (C) Schematic diagram of the targeting

Legend continued on following page

construct for generating $\beta 3$ S408/9A knock-in mice. The $\beta 3$ S408/9A mice were generated by homologous recombination in ES cells. A targeting plasmid was constructed that contained a floxed neomycin (Neo) selection cassette in intron 8 of $\beta 3$ genomic DNA and a flanking thymidine kinase-negative selection marker (TK). (D) Following Cre-mediated excision of the selection cassette in vivo, the presence of the mutant codons in exon 9 was confirmed by DNA sequencing. (E) PCR product of WT and $\beta 3$ S408/9A DNA samples detecting the presence or absence of the remaining loxP site. (F) No gross morphological changes were observed in the hippocampus between WT and S408/9A mice as determined by Nissl staining. (Scale bar: 100 μm .)

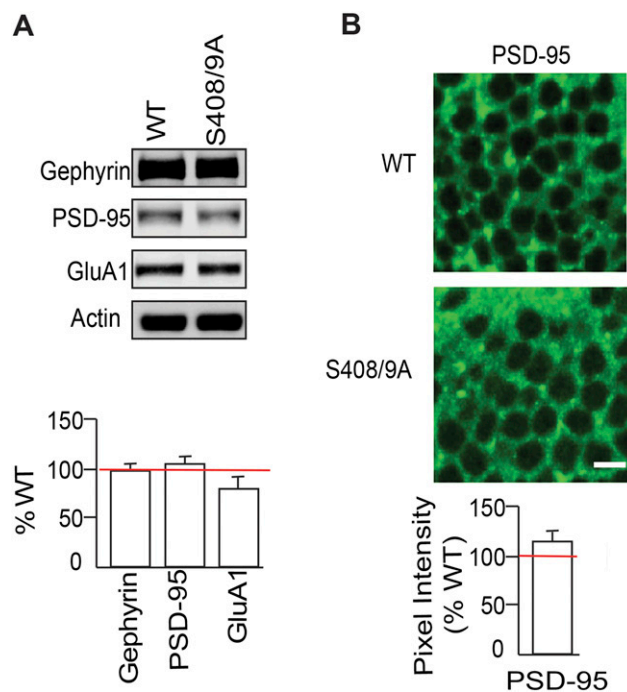


Fig. S2. Analyzing glutamate receptor expression in S408/9A mice. (A) Postsynaptic protein expression is unaltered in S408/9A mice. To determine whether the observed changes in inhibitory synapses were associated with alterations in postsynaptic proteins, the total expression of the inhibitory synaptic marker gephyrin, excitatory synaptic marker PSD-95, and AMPA receptor GluA1 were measured. Hippocampal lysates from S408/9A and WT control mice were subjected to SDS/PAGE and immunoblotted with gephyrin, PSD-95, and GluA1 antibodies. Protein expression in S408/9A mice was normalized to WT controls (100%; red line). No significant differences in postsynaptic protein expression were observed ($P > 0.05$, t test; $n = 8$ mice of each genotype). (B) Forty-micron hippocampal slices were stained with antibodies against the postsynaptic protein PSD-95, followed by confocal microscopy. The pixel intensity of PSD-95 staining was then compared within the dentate gyrus between genotypes. Preventing phosphorylation of the $\beta 3$ subunit does not alter PSD-95 immunoreactivity ($P > 0.05$, t test; $n = 8$ mice of each genotype). (Scale bar: 10 μm .)

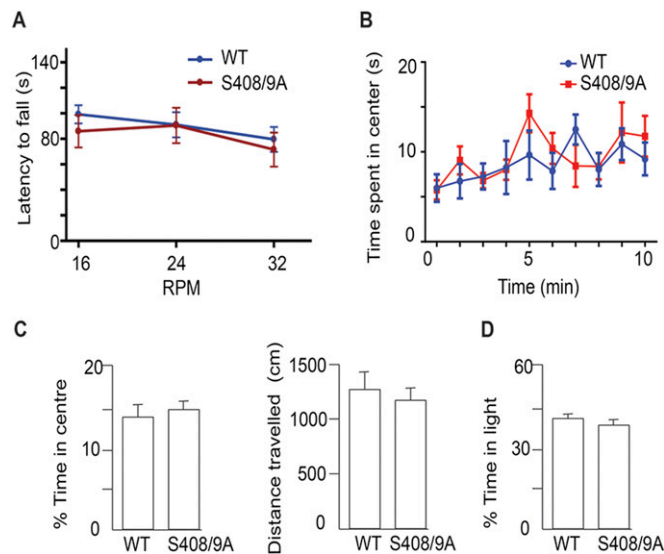


Fig. 53. Characterization of motor activity and anxiety-like behavior in S408/9A mice. (A) Locomotor activity of WT and S408/9A mice was examined using the rotarod test, and the latency to fall was determined. There were no significant differences in motor activity ($P > 0.05$, t test; $n = 10$ –11 mice of each genotype). RPM, revolutions per minute. (B) Time spent in the center of the arena in an open field was compared for WT and S408/9A mice over a 10-min time course. (C) In the open-field assay, there were no significant differences in the percentage of time spent in the middle of the open-field arena or in the distance traveled between genotypes ($P > 0.05$, t test; $n = 12$ mice of each genotype). (D) Percentage of time spent in the light arena of the light/dark box assay was determined for WT and S408/9A mice. There were no significant differences in the percentage of time spent in the light arena between genotypes ($P > 0.05$, t test; $n = 12$ mice of each genotype).

Table S1. Comparing the excitability of DGGCs between genotypes

No. of action potentials fired in DGGCs	WT	S408/9A
Current injection amplitude, pA		
20	0.4 ± 0.4	0.0 ± 0.0
40	0.6 ± 0.5	1.0 ± 0.8
60	4.3 ± 1.8	6.9 ± 2.5
80	10.9 ± 2.9	17.1 ± 4.2
100	21.5 ± 3.5	24.5 ± 4.4
120	30.9 ± 3.6	34.0 ± 4.7
140	39.1 ± 3.6	42.6 ± 5.1
160	46.8 ± 3.8	49.7 ± 5.4
180	55.6 ± 3.8	57.9 ± 5.6
200	63.5 ± 4.3	65.0 ± 5.8
220	71.5 ± 4.6	72.2 ± 5.9
240	80.7 ± 4.7	77.5 ± 6.1
260	87.8 ± 4.9	83.2 ± 6.1
280	94.4 ± 5.0	89.5 ± 6.5
300	100.0 ± 4.9	93.6 ± 6.6
RMP, mV	-81.8 ± 1.2	-81.3 ± 1.2
I_{50} , pA	169.0	164.2
k	48.0	51.9
n	19	20

Values are mean ± SE. I_{50} , amplitude of current injection eliciting 50% of maximum response; k , slope factor; n , number of mice; RMP, resting membrane potential.