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Rescue of Deficient Amygdala Tonic γ-Aminobutyric Acidergic Currents in the *Fmr*$^{-/y}$ Mouse Model of Fragile X Syndrome by a Novel γ-Aminobutyric Acid Type A Receptor-Positive Allosteric Modulator

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Alterations in the ratio of excitatory to inhibitory transmission are emerging as a common component of many nervous system disorders, including autism spectrum disorders (ASDs). Tonic γ-aminobutyric acidergic (GABAergic) transmission provided by peri- and extrasynaptic GABA type A ($GABA_A$) receptors powerfully controls neuronal excitability and plasticity and, therefore, provides a rational therapeutic target for normalizing hyperexcitable networks across a variety of disorders, including ASDs. Our previous studies revealed tonic GABAergic deficits in principal excitatory neurons in the basolateral amygdala (BLA) in the *Fmr1*$^{-/y}$ knockout (KO) mouse model fragile X syndrome. To correct amygdala deficits in tonic GABAergic neurotransmission in *Fmr1*$^{-/y}$ KO mice, we developed a novel positive allosteric modulator of $GABA_A$ receptors, SGE-872, based on endogenously active neurosteroids. This study shows that SGE-872 is nearly as potent and twice as efficacious for positively modulating $GABA_A$ receptors as its parent molecule, allopregnanolone. Furthermore, at submicromolar concentrations ($\leq$1 μM), SGE-872 is selective for tonic, extrasynaptic α4β3δ-containing $GABA_A$ receptors over typical synaptic α1β2γ2 receptors. We further find that SGE-872 strikingly rescues the tonic GABAergic transmission deficit in principal excitatory neurons in the *Fmr1*$^{-/y}$ KO BLA, a structure heavily implicated in the neuropathology of ASDs. Therefore, the potent and selective action of SGE-872 on tonic $GABA_A$ receptors containing α4 subunits may represent a novel and highly useful therapeutic avenue for ASDs and related disorders involving hyperexcitability of neuronal networks.

Key words: fragile X syndrome; amygdala; $GABA_A$ receptor; GABA; positive allosteric modulator

Increasing evidence indicates that dysfunction in γ-aminobutyric acidergic (GABAergic) neurotransmission is a primary driver of neuronal network dysfunction in a variety of nervous system disorders. Pathological changes in GABAergic neurotransmission can alter excitatory/inhibitory (E/I) balance in local circuits and are associated with symptoms of a range of neurological and neurodevelopmental disorders, such as schizophrenia, epilepsy, and autism spectrum disorders (ASDs), including fragile X syndrome (FXS), Rett syndrome, tuberosclerosis, and idiopathic autism (Zoghbi, 2003; Maguire et al., 2005; Geschwind and Levitt, 2007; Wafford and Ebert, 2008; Maldonado-Avilés et al., 2009; Macdonald et al., 2010; Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011; Han et al., 2012, 2014; Fatemi et al., 2014). Furthermore,

SIGNIFICANCE:

This study shows the potential of targeting a particular type of neurotransmission, tonic γ-aminobutyric acidergic transmission, with a novel, selective compound, SGE-872, to treat neurophysiological symptoms such as social anxiety in individuals with fragile X syndrome. SGE-872 and related molecules may especially improve those symptoms related to the amygdala, a primary brain region responsible for the regulation of anxiety.

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extrasynaptic, tonically active GABA type A ($GABA_A$) receptors that contain the δ subunit in conjunction with α4 or α6 subunits are relatively ubiquitously expressed in key brain regions involved in the neuropathology of these disorders. These areas include the hippocampus (Stell et al., 2003), neocortex (Drasbek, 2005), cerebellum (Brickley et al., 2001), thalamus (Jia, 2005), and amygdala (Olmos-Serrano et al., 2010). Therefore, these receptors constitute a prime target for drug development to augment GABAergic tone, restore E/I balance, and improve cognitive and behavioral symptoms. In support of this concept, recent studies have revealed that pharmacological or genetic rescue of GABAergic neurotransmission can restore molecular and/or behavioral deficits in a variety of animal models of neurodevelopmental disorders, including ASDs (Olmos-Serrano et al., 2010; Han et al., 2012, 2014).

GABA is the main inhibitory neurotransmitter in the adult central nervous system (CNS) acting mainly through chloride-permeable, pentameric $GABA_A$ receptors composed of a variety of subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3). These receptors typically take the form of two α subunits and two β subunits, combined with either a γ or a δ subunit (Farrant and Nusser, 2005). The combination of $GABA_A$ receptor subunits that forms a functional receptor determines the regional and developmental expression, physiological and pharmacological properties, and the subcellular location (e.g., synaptic or extrasynaptic) of the receptor (Hevers and Lüddens, 1998; Pirker et al., 2000; Mody and Pearce, 2004; Brickley and Mody, 2012). Synaptically located receptors provide fast, phasic conductance by responding to large concentrations of GABA in the synapse (millimolar) and have high desensitization rates. GABA also displays relatively low affinity and high efficacy at these receptors. In contrast, peri- and extrasynaptic receptors, which most often contain δ, α4, α5, or α6 subunits, provide slow, tonic conductance by responding to relatively low concentrations of GABA (in the nanomolar to micromolar range) at lower desensitization rates, with GABA displaying very high affinity and lower efficacy than at their synaptic counterparts (Semyanov et al., 2004; Farrant and Nusser, 2005). These properties allow these $GABA_A$ receptors to modulate a potent, dynamic tonic conductance many times larger than that of the collective fast, phasic inhibition (Brickley et al., 1996; Rossi et al., 2003). Thus, tonic conductance crucially affects key cellular and neuronal network functions, such as intrinsic neuronal excitability (Bonin et al., 2007), integration of synaptic inputs (Mitchell and Silver, 2003; Semyanov et al., 2004), and synaptic plasticity (Martin et al., 2010).

Endogenous neurosteroid metabolites of progesterone, such as allopregnanolone, are potent positive allosteric modulators of $GABA_A$ receptors (Majewska, 1992) that can have therapeutic efficacy in disorders associated with E/I imbalances, such as epilepsy (Reddy and Rogawski, 2009), and in the BTBR animal model of autism (Han et al., 2014). Although neurosteroids can show particular efficacy at δ subunit-containing $GABA_A$ receptors (Wohlfarth et al., 2002), allopregnanolone and its 3β-methylated synthetic analog ganaxolone have potent positive modulation on a wide range of $GABA_A$ receptor subtypes, including those that express α1, α2, α3, and γ2L subunits (Carter et al., 1997). This "off-target" efficacy could contribute to unnecessary side effects associated with treatment.

To reduce this possibility and to target neurosteroid modulation specifically to tonic GABAergic transmission, we used allopregnanolone as a molecular platform to develop a novel neurosteroid compound, SGE-872. We show that SGE-872 has exceptional efficacy and selectivity for α4δ subunit-containing $GABA_A$ receptors. Furthermore, we tested the ability of this compound to rescue deficient tonic GABAergic currents in the basolateral amygdala (BLA) of the *Fmr1*$^{-/y}$ knockout (KO) mouse model of FXS, an ASD characterized by social withdrawal and emotional deficits associated with dysfunction of the amygdala (Markram and Markram, 2010). Principal excitatory neurons (PNs) of the BLA that crucially regulate sensory integration and fear modulation (Ehrlich et al., 2009) have deficient tonic GABAergic tone associated with neuronal hyperexcitability and E/I imbalance in *Fmr1*$^{-/y}$ KO mice (Olmos-Serrano et al., 2010; Martin et al., 2014). This study shows that application of SGE-872 to *Fmr1*$^{-/y}$ PNs strikingly corrects the tonic GABAergic deficit. Furthermore, at the concentration sufficient to rescue this defect, SGE-872 preferentially affects tonic $GABA_A$ receptors and not phasic receptors. Therefore, our data reveal that selective targeting of α4 subunits via a novel positive allosteric modulator can correct amygdala-based GABAergic deficits in an animal model of FXS and may serve to improve E/I balance more broadly across other critical brain regions involved in related syndromic and nonsyndromic etiologies of autism.

MATERIALS AND METHODS

Cell Cultures

The functional potency and efficacy of allopregnanolone and SGE-872 were assessed by whole-cell patch clamp of heterologously expressed $GABA_A$ receptors composed of either the α1β2γ2 or the α4β3δ subunits. LTK cells were stably transfected with the human α1β2γ2 subunits of the GABA receptor, and CHO cells were transiently transfected with the human α4β3δ subunits via the lipofectamine method (Dalby et al., 2004). 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. Although the two receptor subtypes were expressed in two different host cell systems, allopregnanolone showed similar pharmacology at α1β2γ2 and a4β3δ receptors, consistent with the literature on a variety of cell types (Belelli et al., 2002; Belelli and Lambert, 2005), suggesting that we did not see comparatively different pharmacology in the two populations resulting from differences in the host cell type.

Whole-Cell Patch Clamp of Transfected Cells

Whole-cell currents were measured with HEKA EPC-10 amplifiers in PatchMaster software or by using the high-throughput QPatch platform (Sophion, Stockholm, Sweden). Bath solution for all experiments contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, D-glucose 10, pH (NaOH) 7.4. Intracellular (pipette) solution contained (in mM) KCl 130, $MgCl_2$ 1, Mg-ATP 5, HEPES 10, EGTA 5, pH 7.2. During experiments, cells and solutions were maintained at room temperature (19–30°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, and whole-cell recordings were obtained by manual patch clamp. For experiments with the QPatch system, cells were transferred as a suspension to the QPatch system in the bath solution, and automated whole-cell recordings were performed.

Cells were voltage clamped at a holding potential of –80 mV. For the analysis of test articles, two successive applications of 2 μM GABA were first applied, and then, after wash out, GABA receptors were stimulated again by 2 μM GABA after sequential preincubation of increasing concentrations of the test article. Preincubation duration was 30 sec, and the duration of the GABA stimulus was 4 sec. Test compounds were dissolved in dimethylsulfoxide to form stock solutions (10 mM) and were diluted to 0.01, 0.1, 1.0, and 10 μM in bath solution. All concentrations of test compounds were tested on each cell. The relative percentage of potentiation was defined as the peak amplitude in response to GABA EC20 in the presence of the test article divided by the peak amplitude in response to GABA EC20 alone, multiplied by 100. The ability of SGE-872 to activate $GABA_A$ receptors directly was assessed in cells expressing the $\alpha4\beta3\delta$ subunits. Two successive applications of 2 μM GABA were first applied, and then, after wash out, 10 μM SGE-872 was applied. Data are expressed as the peak response to 2 μM GABA alone divided by the peak response to 10 μM SGE-872 alone.

Animal Use

Wild-type (control; strain name FVB.129P2; stock No. 4828) and *Fmr1* KO mice (strain name FVB.129P2-$Fmr1^{tm1Cgr}$/J; stock No. 4624) on the congenic FVB strain background were obtained from The Jackson Laboratory (Genetics Research, Bar Harbor, ME) and maintained as separate congenic stocks at Children's National Medical Center. Animals were housed and utilized in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Acute Brain Slice Preparation

Acute slices were prepared from postnatal day (P) 21–30 male control and $Fmr1^{-/y}$ mice. Animals were briefly anesthetized with CO_2 and decapitated. Brains were removed quickly and placed in cold (4°C) sucrose-based oxygenated (95% O_2/5% CO_2) cutting solution composed of (in mM) sucrose 234, glucose 11, $NaHCO_3$ 26, KCl 2.5, $NaH_2PO_4 \cdot H_2O$ 1.25, $MgSO_4 \cdot 7H_2O$ 10, and $CaCl_2 \cdot H_2O$ 0.5. Coronal slices containing the BLA were obtained with a slicing vibratome (VT1200s; Leica, Wetzlar, Germany) by removing the cerebellum with a perpendicular cut to the rostral–caudal plane and gluing the caudal side down on the vibratome stage submerged in cold cutting solution. Slice thickness was 300 μM for all experiments. The slices were immersed in oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (ACSF) at 34°C for 30–45 min. ACSF was composed of (in mM) NaCl 126, $NaHCO_3$ 26, glucose 10, KCl 2.5, $NaH_2PO_4 \cdot H_2O$ 1.25, $MgCl_2 \cdot 7H_2O$ 2, and $CaCl_2 \cdot 2H_2O$ 2, pH 7.4, with osmolarity maintained at 290–300 mOsm.

Slice Electrophysiology

For all experiments, slices were placed in a submerged slice chamber and continuously perfused with ACSF at 2–4 ml/min maintained at 26–28°C with an inline heater system (Warner Instruments, Hamden, CT). Slices were visualized on a fixed-stage upright microscope (Nikon, Tokyo, Japan) equipped with ×10 and ×60 objectives with differential interference contrast optics, infrared illumination, and an infrared-sensitive camera (Cohu, Poway, CA). Whole-cell patch clamp recordings were performed with glass pipettes with resistance of 2.0–4.0 MΩ when filled with intracellular solution. Access resistance of recordings was <25 MΩ and was monitored throughout the experiment with brief 5-mV steps every 20 sec. Data were discarded if the access resistance changed by >25%. Membrane potentials were adjusted for junction potential (12 mV). Data were acquired with a Multiclamp 700A amplifier and digitized with a Digidata 1322A in pClamp 9.2 (Molecular Devices, Sunnyvale, CA). All recordings were made from PNs initially identified visually as having a large, pyramid-like soma with two to seven primary dendrites and then identified physiologically as having long afterhyperpolarizing potentials (AHPs; Sah et al., 2003) induced by prolonged hyperpolarizing current injections (600 msec). Cesium chloride-based intracellular solution contained (in mM) CsCl 135, HEPES 10, EGTA 10, QX-314 5, $MgCl_2$ 2, Mg-ATP 4, and Na-GTP 0.3, $E_{Cl-} = 0$ mV. Because cesium interferes with potassium currents involved in AHPs, care was taken to assess the AHP within 4–5 sec of cell membrane rupture before cesium could sufficiently diffuse into the cell. GABAergic tonic currents and phasic spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in voltage clamp and isolated by blocking ionotropic glutamatergic transmission with 6,7-dinitroquinoxaline-2,3-dione (20 μM final concentration; AMPA/kainate antagonist; Tocris Bioscience, Ellisville, MO) and DL-2-amino-5-phosphonopentanoic acid (50 μM final concentration, NMDA receptor antagonist; Tocris Bioscience) in the ACSF. All experimental compounds were applied locally with a gravity-fed Y-tube following baseline vehicle application. For tonic current recordings, 2 μM exogenous GABA was bath and locally applied during baseline and experimental compound application to stimulate extrasynaptic $GABA_A$ receptors. Before application of any experimental compounds or gabazine to measure tonic currents in these experiments, a whole-cell configuration was achieved, followed by exogenous GABA application for at least 10 min to allow the cell to equilibrate and achieve a steady-state tonic current. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the absence of exogenous GABA.

Statistical Analysis

Tonic currents were acquired and analyzed as reported previously (Krook-Magnuson et al., 2008). Briefly, 10-sec

samples were taken from voltage clamp recordings (V_h = –70 mV) under each experimental condition (baseline [I_{BSLN}], compound [SGE-872 or allopregnanolone (I_{cmpd})] and gabazine [I_{GBZ}, $GABA_A$ receptor antagonist SR-95531, 50 μM]). To minimize bias from phasic events, a Gaussian distribution was fit to the right side of an all-points histogram from each sample from a point 1–3 pA left of the peak (Glykys and Mody, 2007). The Gaussian peak determined the mean current for the sample. Total tonic current was calculated from the difference in mean baseline and gabazine currents ($I_{GBZ} - I_{BSLN}$), and compound-induced current was calculated from the difference in mean baseline and compound currents ($I_{cmpd} - I_{BSLN}$). To control for differences in cell size/capacitance, calculated currents were expressed as tonic current densities for each cell based on cell capacitance (current density = current [pA]/capacitance [pF]). Capacitance was determined in voltage clamp with brief 10-mV biphasic voltage steps delivered immediately after establishing the whole-cell configuration.

Action-potential-dependent sIPSCs were analyzed for frequency, amplitude, and decay kinetics before and after the application of test compounds in the absence of exogenous GABA. Averaged sIPSCs were fit with the double exponential function $f(x) = A_{fast}e^{-t/\tau(fast)} + A_{slow}e^{-t/\tau(slow)}$. Fitted sIPSCs were then used to determine the weighted time constant $\tau_{d,w} = [(A_{fast}\ \tau_{fast}) + (A_{slow}\ \tau_{slow})]/(A_{fast} + A_{slow})$. Phasic synaptic efficacy was estimated by integrating total IPSC charge per 1-sec interval (Huntsman et al., 1999).

All recordings were analyzed off-line (pClamp, RRID:rid_000085; Mini Analysis Program, RRID:SciRes_000143; Patchmaster, RRID:SciRes_000168). Values are mean ± SE. Two-tailed Student's *t*-test was used for measures of evoked and tonic currents, sIPSC amplitude, frequency, weighted decay, and synaptic efficacy (Microcal Origin, RRID:rid_000069; Graphpad Prism, RRID:rid_000081). Paired Student's *t*-test was used to determine significance for within-group comparisons between conditions (i.e., baseline and drug), and ANOVA with Bonferroni correction for multiple comparisons was used to test differences in baseline and drug conditions across genotypes (control and $Fmr1^{-/y}$) or between compounds (allopregnanolone and SGE-872).

RESULTS

SGE-872 Is a Potent and Selective Modulator of $GABA_A$ Receptors

We first wanted to assess the pharmacology of SGE-872 compared with allopregnanolone. To accomplish this we used patch-clamp electrophysiology in mammalian cells expressing recombinant human $GABA_A$ receptors. $GABA_A$ receptors composed of the α1β2γ2 subunits were used as surrogates for synaptic receptors, and $GABA_A$ receptors composed of the α4β3δ subunits were used as surrogates for extrasynaptic receptors (Belelli and Lambert, 2005).

Allopregnanolone potentiated GABA-evoked currents in a concentration-dependent manner with an EC50 of 185 nM and Emax of 476% in cells expressing α1β2γ2 and an EC50 of 80 nM and Emax of 418% in cells expressing α4β3δ (Fig. 1A, top, Fig. 1B, left). In contrast, SGE-872 displayed greater selectivity for $GABA_A$ receptors composed of α4β3δ subunits compared with receptors composed of α1β2γ2 subunits. In cells expressing the α4β3δ subunits, SGE-872 potentiated GABA-evoked currents with an EC50 of 160 nM and Emax of 838% (Fig. 1A, bottom, Fig. 1B, right). However, in cells expressing α1β2γ2 subunits, SGE-872 was substantially less potent, and the concentration–response curve did not reach an asymptote by the highest concentration tested, so potency and efficacy values could not be estimated. At 10 μM, SGE-872 potentiated GABA-evoked currents by 744% ± 261% (mean ± SD, n = 3).

The ability of SGE-872 to activate $GABA_A$ receptors directly was also investigated in cells that expressed α4β3δ subunits. 10 μM SGE-872 had minimal direct activation, evoking a current that was 22.9% ± 3.5% of the current evoked by an EC20 concentration of GABA (2 μM, n = 3; Fig. 1C).

SGE-872 Enhances Tonic $GABA_A$ Receptor-Mediated Currents in BLA PNs

GABAergic tone crucially regulates network function in the BLA (Ehrlich et al., 2009), and dysfunction in the amygdala has been associated with symptoms of ASDs (Baron-Cohen et al., 2000; Amaral et al., 2003; Markram and Markram, 2010). In animal models of ASDs, GABAergic alterations exist in the BLA in particular (Olmos-Serrano et al., 2010; Paluszkiewicz et al., 2011; Vislay et al., 2013) Furthermore, in the $Fmr1^{-/y}$ KO mouse model of FXS, PNs of the BLA display deficits in tonic GABAergic transmission in association with increases in E/I balance that can be rescued by tonic GABAergic augmentation (Olmos-Serrano et al., 2010). Specifically, $Fmr1^{-/y}$ PNs show a decreased physiological response to the $GABA_A$ receptor superagonist 4,5,6,7-tetrahydroisoxa-zolo[5,4-c]pyridin-3-ol (THIP), also known as *gaboxadol*, at a concentration shown to affect α4δ-containing receptors preferentially (Brown et al., 2002; Stórustovu and Ebert, 2006; Martin et al., 2014), consistent with decreased function of these receptors in these cells. With these findings, we next tested the ability of allopregnanolone and SGE-872 to enhance tonic $GABA_A$ receptor-mediated currents in BLA principal neurons in acute brain slices from both control and $Fmr1^{-/y}$ mice (Fig. 2).

First, we observed that baseline tonic $GABA_A$ receptor-mediated current densities across all experimental groups in the presence of exogenous GABA (2 μM) were significantly decreased in $Fmr1^{-/y}$ cells compared with control neurons (total control baseline, 0.21 ± 0.02 pA/pF, n = 22; $Fmr1^{-/y}$ baseline, 0.12 ± 0.02, n = 23; ANOVA with Bonferroni correction, $F[1,43] = 0.290$, n = 45, $P = 0.003$; Fig. 2C). Current densities were determined by the change in holding current following the application of the $GABA_A$ receptor antagonist gabazine (50 μM). This result is consistent with reduced tonic GABAergic tone in the $Fmr1^{-/y}$ BLA, as we have previously characterized (Olmos-Serrano et al., 2010). Application of SGE-872 at a low concentration (100 nM)

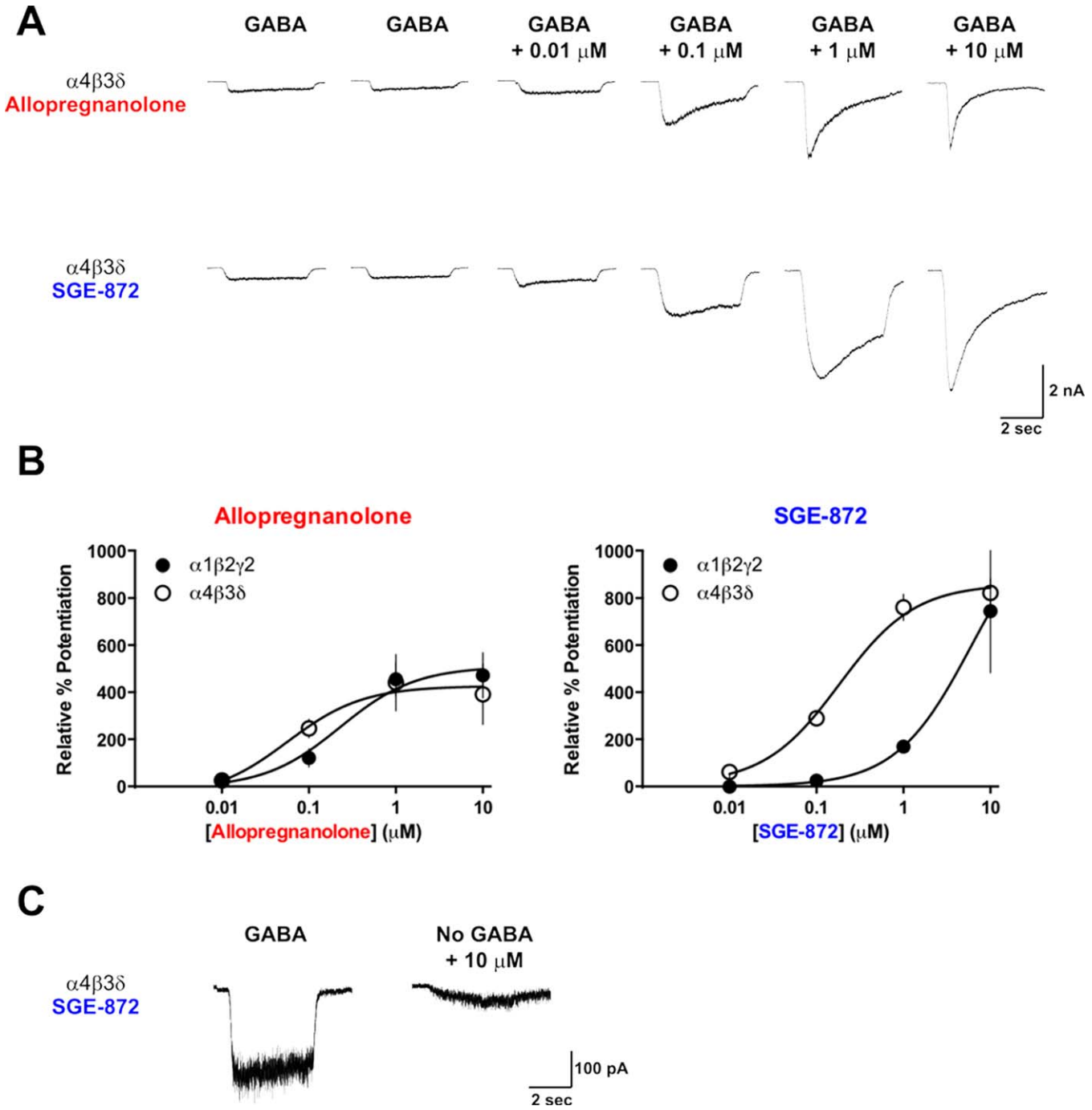

Fig. 1. SGE-872 is a more potent and selective positive allosteric modulator than allopregnanolone. **A:** Representative current responses from LTK cells transfected with α4β3δ $GABA_A$ receptors in response to 2 sec of stimulus with either GABA (2 μM) or GABA plus each modulator at increasing concentrations. **B:** Dose–response curves for allopregnanolone (at left) and SGE-872 (right) indicate that SGE-872 potentiates α4β3δ-mediated GABAergic currents more than allopregnanolone at concentrations greater than 0.1 μM. In addition, SGE-872 is more selective for α4β3δ-containing receptors vs. α1β2γ2 receptors compared with allopregnanolone. **C:** The highest concentration tested (10 μM) minimally directly activates α4β3δ $GABA_A$ receptors in the absence of GABA.

significantly enhanced tonic current density in control cells (control baseline, 0.17 ± 0.04 pA/pF; control SGE-872 100 nM, 0.24 ± 0.04 pA/pF; n = 8, paired *t*-test, $t[7] = -5.18$, $P = 0.001$) but not in $Fmr1^{-/y}$ cells ($Fmr1^{-/y}$ baseline, 0.12 ± 0.03 pA/pF; $Fmr1^{-/y}$ SGE-872 100 nM, 0.16 ± 0.04 pA/pF; n = 7, paired *t*-test, $t[6] = -1.75$,

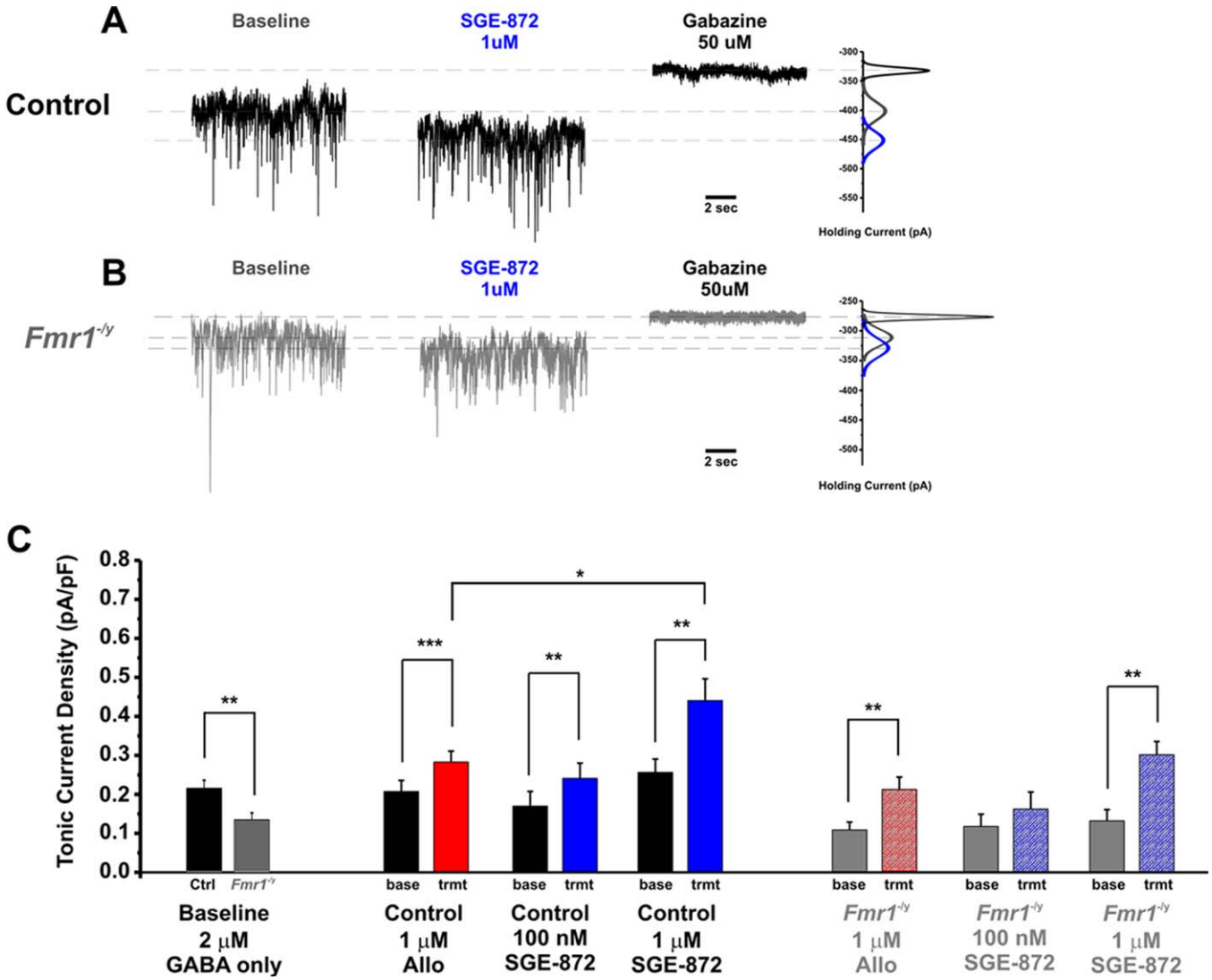

Fig. 2. Allopregnanolone and SGE-872 significantly potentiate tonic currents in principal neurons of the BLA. **A,B:** Representative whole-cell voltage clamp traces recorded from control (A) and *Fmr1*$^{-/y}$ (B) principal neurons show 10-sec samples before (baseline) and after (SGE-872) direct application of SGE-872 1 μM in the presence of 2 μM exogenous GABA, followed by application of gabazine 50 μM (V_{hold} = −70 mV). Gaussian distributions (right) for each sample indicate the differences in mean holding current at each condition. **C:** Baseline tonic density is decreased in *Fmr1*$^{-/y}$ cells in the presence of 2 μM GABA (left). Pairwise comparisons show that allopregnanolone and SGE-872 significantly potentiate baseline tonic current density in control and *Fmr1*$^{-/y}$ cells. However, *Fmr1*$^{-/y}$ cells require a higher concentration of SGE-872 (1 μM vs. 100 nM) to have a significant effect. Allopregnanolone also significantly potentiates tonic current density in both genotypes. However, in wild-type cells, SGE-872 (1 μM) has a stronger potentiation than allopregnanolone (ANOVA). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$P = 0.13$). In contrast, application of a higher concentration (1 μM) of SGE-872 significantly enhanced tonic current density in both control and *Fmr1*$^{-/y}$ BLA PNs (Fig. 2A,B; control baseline, 0.26 ± 0.03 pA/pF; control 1 μM SGE-872, 0.44 ± 0.06 pA/pF; n = 8, paired *t*-test, $t[7] = -4.62$, $P = 0.002$; *Fmr1*$^{-/y}$ baseline, 0.13 ± 0.03 pA/pF; *Fmr1*$^{-/y}$ 1 μM SGE-872, 0.30 ± 0.03 pA/pF; n = 8, paired *t*-test, $t[7] = -4.55$, $P = 0.003$; Fig. 2C). Furthermore, treatment with SGE-872 at 1 μM restored tonic current density in *Fmr1*$^{-/y}$ PNs to control baseline levels (*Fmr1*$^{-/y}$ 0.30 pA/pF vs. control 0.26 pA/pF, ANOVA with Bonferroni correction, $F[1,14] = 0.899$, n = 15, $P = 0.360$).

SGE-872 Is More Effective Than Allopregnanolone for Augmenting Tonic Current in BLA PNs

Allopregnanolone (1 μM) also significantly enhanced tonic $GABA_A$ receptor-mediated current density in both

control and *Fmr1*$^{-/y}$ PNs (control baseline, 0.20 ± 0.03 pA/pF; control allopregnanolone 1 μM, 0.28 ± 0.03; n = 9, paired *t*-test, *t*[8] = –5.30, *P* = 0.0007; *Fmr1*$^{-/y}$ baseline, 0.10 ± 0.02 pA/pF; *Fmr1*$^{-/y}$ allopregnanolone 1 μM, 0.21 ± 0.03 pA/pF; n = 7, paired *t*-test, *t*[6] = –4.87, *P* = 0.003). However, in control cells but not in *Fmr1*$^{-/y}$ cells, SGE-872 demonstrated a stronger potentiation of these current densities than allopregnanolone, consistent with higher efficacy of SGE-872 on recombinant $GABA_A$ receptors shown in Figure 1 (control allopregnanolone 1 μM, 0.28 ± 0.03 pA/pF; control SGE-872 1 μM, 0.44 ± 0.06 pA/pF; ANOVA with Bonferroni correction, F[1,15] = 7.02, *P* = 0.018; *Fmr1*$^{-/y}$ allopregnanolone 1 μM, 0.21 ± 0.03 pA/pF; *Fmr1*$^{-/y}$ SGE-872 1 μM, 0.30 ± 0.03 pA/pF; ANOVA with Bonferroni correction, F[1,13] = 4.02, *P* = 0.07; Fig. 2C).

SGE-872 Demonstrates Specificity for Tonic Over Phasic $GABA_A$ Receptors in BLA PNs

Characterization of GABA currents in a recombinant system demonstrated that SGE-872 is more active at putative extrasynaptic receptors (α4β3δ) than at putative synaptic receptors (α1β2γ2) at low concentrations (≤1 μM; Fig. 1B). Therefore, we wanted to test whether this preference also existed in BLA PNs. To accomplish this, we recorded sIPSCs in the absence of exogenous GABA and compared baseline sIPSC properties with those at low and high concentrations of SGE-872 (Fig. 3). Upon application of 1 μM SGE-872 to control BLA PNs, a concentration that significantly augmented tonic current density (Fig. 2A,C), we observed no significant change in sIPSC frequency, amplitude, weighted decay constant, or estimated phasic synaptic efficacy (frequency baseline, 18.3 ± 1.36 Hz; frequency SGE-872 1 μM, 16.4 ±1.45 Hz; *t*[8] = 2.11, *P* = 0.070; amplitude baseline, 43.8 ± 2.56 pA; amplitude SGE-872 1 μM, 40.6 ± 2.66 pA; *t*[8] = 2.10, *P* = 0.072; tau_ω baseline, 17.4 ± 1.53; tau_ω SGE-872 1 μM, 19.6 ± 2.05; *t*[8] = –1.27, *P* = 0.241; efficacy baseline, 13,571.3 ± 1,608.1 pC/sec; efficacy SGE-872, 1 μM 12,966.4 ± 1,820.3 pC/sec, *t*[8] = 0.292, *P* = 0.778; n = 9, paired *t*-test; Fig. 3B). However application of 10 μM SGE-872 caused significant changes in sIPSC kinetics and an associated increase in phasic GABAergic synaptic efficacy, indicating significant enhancement of phasic synaptic receptors in addition to tonically active receptors (frequency baseline, 16.1 ± 1.07 Hz; frequency SGE-872 1 μM, 15.7 ± 1.01 Hz; *t*[6] = 0.556, *P* = 0.598; amplitude baseline, 53.0 ± 4.56 pA; amplitude SGE872 1 μM, 50.4 ± 4.28 pA; *t*[6] = 1.47, *P* = 0.192; tau_ω baseline, 18.0 ± 1.68; tau_ω SGE-872 1 μM, 28.1 ± 3.63; *t*[6] = –2.91, *P* = 0.027; efficacy baseline, 15,514.0 ± 2,004.3 pC/sec; efficacy SGE-872 1 μM, 22,057.3 ± 3,352.8 pC/sec; *t*[6] = –2.38, *P* = 0.027; n = 7, paired *t*-test; Fig. 3A,C). Therefore, SGE-872 preferentially modulates tonically active $GABA_A$ receptors at low concentrations (≤1 μM) compared with high concentrations (10 μM) in mouse BLA PNs.

DISCUSSION

Tonic GABAergic Transmission in Neurodevelopmental Disorders

Alterations in GABAergic transmission are pervasive in ASDs and other related neurodevelopmental disorders such as schizophrenia and tuberosclerosis (Chao et al., 2010; Markram and Markram, 2010; Pizzarelli and Cherubini, 2011; Rudolph and Mohler, 2014). These disruptions affect local and regional network synchronization, synaptic plasticity, and neuronal excitability, all of which rely heavily on tonic GABAergic conductance (Pizzarelli and Cherubini, 2011; Brickley and Mody, 2012). The power of dysfunctional GABAergic neurotransmission has recently been exemplified by findings showing that rescue of GABAergic signaling alone can ameliorate dysfunction in animal models of autism (Han et al., 2012, 2014).

In FXS specifically, multiple studies have revealed hyperexcitability and/or hypersynchronization of neuronal networks in key brain regions such as sensory cortex (Gibson et al., 2008; Gonçalves et al., 2013), prefrontal cortex (Testa-Silva et al., 2012), and amygdala (Olmos-Serrano et al., 2010; Martin et al., 2014), brain regions that contribute to core symptoms of ASDs such as hypersensitivity, anxiety, social withdrawal, and cognitive impairment (Markram and Markram, 2010). Tonic GABAergic conductance mediated by δ subunit-containing $GABA_A$ receptors can modulate excitability (Semyanov et al., 2004) and regulate γ-frequency band oscillations (Mann and Mody, 2010) that are crucial for regional synchronization and cognitive processing (Fries, 2009). Therefore, given the widespread disruption in GABAergic tone in the FXS brain, potent and specific modulation of tonic inhibitory tone by SGE-872 has the potential to normalize both local and regional neuronal communication and improve overall brain function. Additional testing in behavioral paradigms is required to determine whether the enhancement of tonic GABAergic tone by SGE-872 can also improve behavioral symptoms in FXS and other animal models of ASDs.

Expression of the δ subunit (mRNA and protein) is broadly decreased in the FXS brain in association with the functional loss of fragile X mental retardation protein (D'Hulst et al., 2006; Gantois et al., 2006), the transcriptional regulator that underlies dysfunction in the syndrome (Bear et al., 2004). Furthermore, tonic GABAergic transmission is compromised in key brain regions, such as the subiculum (Curia et al., 2009) and the BLA (Olmos-Serrano et al., 2010; Martin et al., 2014). Here we confirm previous data revealing significantly lower tonic GABAergic currents in BLA PNs in *Fmr1*$^{-/y}$ mice compared with controls and extend this work to reveal that this deficit is rescued by application of SGE-872. Previous molecular and electrophysiological analyses have indicated that BLA PNs likely express at least three distinct tonically active, extrasynaptic $GABA_A$ receptor subtypes, the δ, α5, and α3 subunit-containing receptors (Olmos-Serrano et al., 2010; Marowsky et al., 2012; Martin et al., 2014). From the determined specificity of SGE-872, δ subunit-containing

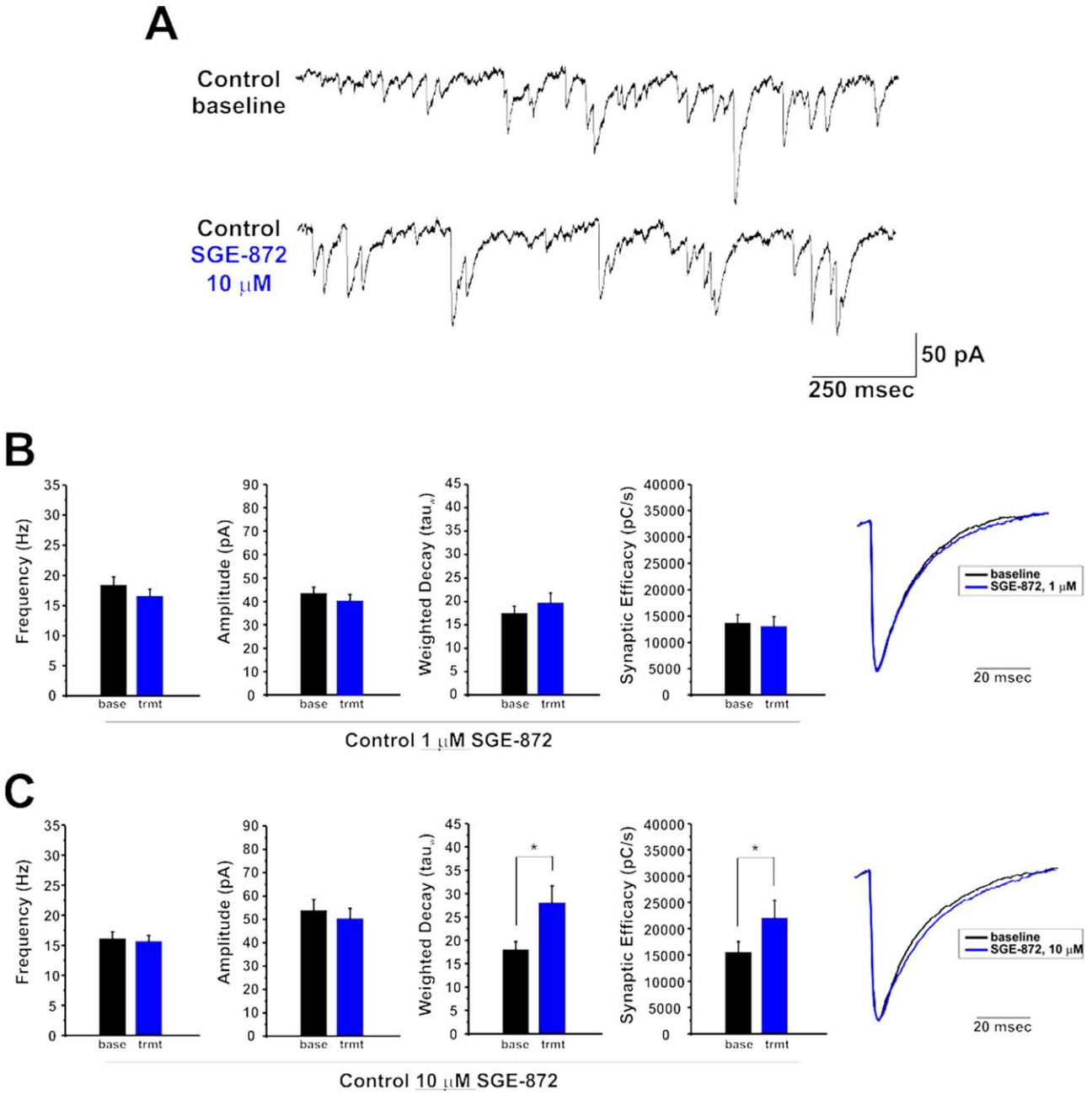

Fig. 3. SGE-872 is selective for tonic vs. phasic $GABA_A$ receptors at low concentrations. **A:** Representative voltage clamp traces of sIPSCs recorded in a principal neuron in the BLA (all measurements taken from control brain slices) before (top) and after (bottom) application of SGE-872 (10 μM) in the absence of exogenous GABA (V_{hold} = −70 mV). **B,C:** Quantification of changes in sIPSC properties of cells exposed to SGE-872 at 1 μM (B) and 10 μM (C) indicates average sIPSC frequency, amplitude, weighted decay, and overall GABAergic synaptic efficacy before and after SGE-872 treatment. Representative average sIPSC waveforms (far right, scaled for amplitude) demonstrate changes in weighted decay constant (tau_ω) after SGE-872 application. At 1 μM, SGE-872 had no significant effect on any sIPSC properties (B). In contrast, at 10 μM, SGE-872 significantly increased both the weighted decay constant (tau_ω) of sIPSCs and the total GABAergic synaptic efficacy (C). $\star P < 0.05$, paired *t*-test.

receptors expressed with α4 likely mediate the increased tonic currents that we observe. However, because δ subunit expression is probably reduced in the *Fmr1*$^{-/y}$ BLA (Gantois et al., 2006; Martin et al., 2014), we cannot discount the possible participation of uncharacteristically expressed $GABA_A$ receptors that contain α4 in the absence of δ in the augmented currents. Regardless of the mechanism, our results reveal that selective α4 targeting is highly efficacious

for correcting underlying GABAergic alterations in the *Fmr1*$^{-/y}$ KO BLA.

SGE-872 Potency and Specificity for Extrasynaptic $GABA_A$ Receptors

Our characterization of SGE-872 introduces a novel neurosteroid-based compound that acts as a potent, selective positive allosteric modulator at tonically active $GABA_A$ receptors. In transfected cells, SGE-872 clearly showed a preference for putative extrasynaptic ($\alpha4\beta3\delta$) $GABA_A$ receptors over putative synaptic receptors ($\alpha1\beta2\gamma2$) with an ability to gate $\alpha4\beta3\delta$ receptors directly at the highest concentration tested (10 μM). The compound also exhibited greater efficacy at lower concentrations than the endogenous neurosteroid allopregnanolone, at least at $\alpha4\beta3\delta$-containing receptors. In addition, we observed some desensitization of the GABA response in the presence of SGE-872 in these transfected cells, especially at higher concentrations (1 and 10 μM; Fig. 1). To assess these effects, we attempted an additional experiment to assess steady-state GABA current in $\alpha4\beta3\delta$-containing receptors in the presence of SGE-872 (data not shown) in which we first applied GABA for 90 sec to allow it to reach steady state, followed by application of SGE-872 for 90 sec to observe a second, higher potentiated steady state. Unfortunately, we did not obtain a sufficient number of stable recordings in this experiment to attain an accurate measurement of steady-state potentiation in $\alpha4\beta3\delta$-containing receptors.

We did, however, observe sustained augmentation of the tonic GABAergic current by SGE-872 in PNs in the mouse BLA in both controls and the *Fmr1*$^{-/y}$ KO mouse model of FXS. In the *Fmr1*$^{-/y}$ slices, SGE-872 enhanced tonic GABAergic currents to control levels at a concentration that preferentially modulated extrasynaptic rather than synaptic $GABA_A$ receptors.

When tested in the *Fmr1*$^{-/y}$ KO model of FXS, SGE-872 was less effective at lower concentrations (100 nM) in *Fmr1*$^{-/y}$ KO neurons than in control PNs. This result was expected given the reduced expression of $GABA_A$ receptor subunits, including the δ and the α4 subunits in the FXS brain (D'Hulst et al., 2006). Despite the decreased effectiveness of the compound to enhance tonic GABAergic current in *Fmr1*$^{-/y}$ PNs, application at 1 μM showed significant efficacy, indicating that SGE-872 can effectively rescue the tonic current deficit in the *Fmr1*$^{-/y}$ BLA. In addition, further evaluation showed that, despite potentiation of $\alpha1\beta2\gamma2$ receptors by ~200% in transfected cells, response of phasic events (sIPSCs) to 1 μM application in the acute control brain slice was not significantly affected by SGE-872 in control PNs. Several factors could account for the lack of enhancement of sIPSCs at this concentration (1 μM), including a differential $GABA_A$ receptor pool in the BLA PNs (i.e., more $\alpha2/\alpha3$ subunit-containing receptors than α1; Vislay et al., 2013), lack of penetration of the compound into the slice, strong activity of GABA transporters in the synaptic cleft resulting in reduced effect, and/or desensitization of the receptor to the presence of the modulator. However, at 10 μM, application of SGE-872, phasic receptors showed increased efficacy, accounted for by increased weighted decay of synaptic events but no significant increase in sIPSC amplitude, perhaps because neurosteroids likely primarily potentiate $GABA_A$ receptors by increasing receptor open time (Barker et al., 1987).

Potential Clinical Implications of Findings

Given the crucial role of tonic GABAergic transmission in regulating neuronal excitability and synaptic plasticity (Brickley and Mody, 2012), extrasynaptic receptors have become targets of drug development for a wide range of CNS disorders, such as insomnia, anxiety, depression, schizophrenia, and autism (Wafford and Ebert, 2006; Rudolph and Mohler, 2014). Accordingly, attempts to synthesize α4δ-selective molecules have produced δ subunit-selective agonists such as THIP and gaboxadol (Wafford and Ebert, 2008) and positive allosteric modulators with specificity for α4δ-$GABA_A$ receptors such as DS2 (Wafford et al., 2009) and AA29504 (Hoestgaard-Jensen et al., 2010). Although deficits in tonic GABAergic conductance represent only one of the many defects in GABAergic signaling in *Fmr1*$^{-/y}$ mice, including GABA production, inhibitory synapse number, receptor expression, and GABA production and release (Olmos-Serrano et al., 2010), augmentation of the tonic current acts as a powerful regulator of increased cellular excitability and a potential therapeutic tool. For instance, gaboxadol alone can physiologically rescue the hyperexcitable phenotype of *Fmr1*$^{-/y}$ PNs in the acute amygdala brain slice (Olmos-Serrano et al., 2010) and rescue at least one behavioral phenotype in the *Fmr1*$^{-/y}$ mouse, hyperactivity (Olmos-Serrano et al., 2011). However, determination of the full potential therapeutic value of tonic GABAergic modulators for FXS requires additional behavioral studies with SGE-872 and related compounds.

Here we show that SGE-872 consistently displays stronger positive allosteric modulation of tonic $GABA_A$ receptors than the principle endogenous neurosteroid allopregnanolone in both human transfected cells and in BLA PNs in acute mouse brain slices. In fact, SGE-872 displays efficacy at $\alpha4\beta3\delta$ $GABA_A$ receptors equal to or greater than that of DS2, AA29504, allopregnanolone, or its 3β-methylated homologue ganaxolone (Carter et al., 1997) at concentrations selective for α4δ subunit-containing receptors. This superior efficacy and selectivity signify a possible therapeutic advantage of SGE-872 for treatment of disorders that have significantly reduced expression of the α4δ $GABA_A$ receptors, such as schizophrenia (Maldonado-Avilés et al., 2009), FXS (D'Hulst et al., 2006; Gantois et al., 2006), idiopathic ASDs (Fatemi et al., 2010, 2014), and those disorders in which allopregnanolone has shown possible efficacy for improving symptoms, including generalized anxiety (Schüle et al., 2014), depression (Bäckström et al., 2014), posttraumatic stress disorder (Pinna, 2014), and epilepsy (Carver and Reddy, 2013).

In summary, this study shows that SGE-872 is a potent and selective positive allosteric modulator of α4 subunit-containing $GABA_A$ receptors that augments deficient tonic GABAergic tone in the BLA of the *Fmr1*$^{-/y}$ KO mouse model of FXS. Therefore, augmentation of tonic GABAergic neurotransmission may prove effective for improving amygdala-based symptoms in FXS and related ASDs.

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CONFLICT OF INTEREST STATEMENT

GM-B, CML, FGS, AJR, MAA, and JJD are currently or were formerly employed by Sage Therapeutics, Inc. Joshua G Corbin and Molly M Huntsman served as paid consultants on the Sage Therapeutics Scientific Advisory Board.

ROLE OF AUTHORS

BSM, MAA, and JJD performed the experiments and analyzed the data. The work was overseen and directed by JGC, MMH and JJD with intellectual contribution/data analysis input from GM-B, CML, FGS, AJR, MAA. All authors collaborated on editing and writing the article.

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