BJA

British Journal of Anaesthesia, 126 (3): 674–683 (2021)

doi: 10.1016/j.bja.2020.11.032
Advance Access Publication Date: 30 December 2020
Neuroscience and Neuroanaesthesia

Inhibition of a tonic inhibitory conductance in mouse hippocampal neurones by negative allosteric modulators of α5 subunit-containing γ-aminobutyric acid type A receptors: implications for treating cognitive deficits

Marc A. Manzo[1,†], Dian-Shi Wang[1,†], Winston W. Li[1], Arsène Pinguelo[1], Mariana O. Popa[2], Shahin Khodaei[1], John R. Atack[2], Ruth A. Ross[3] and Beverley A. Orser[1,4,5,*]

[1]Department of Physiology, University of Toronto, Toronto, ON, Canada, [2]Medicines Discovery Institute, Cardiff University, Cardiff, Wales, [3]Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada, [4]Department of Anesthesiology and Pain Medicine, University of Toronto, Toronto, ON, Canada and [5]Department of Anesthesia, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

*Corresponding author. E-mail: beverley.orser@utoronto.ca

[†]MAM and DSW contributed equally to this work.

Abstract

Background: Multiple cognitive and psychiatric disorders are associated with an increased tonic inhibitory conductance that is generated by α5 subunit-containing γ-aminobutyric acid type A (α5 $GABA_A$) receptors. Negative allosteric modulators that inhibit α5 $GABA_A$ receptors (α5-NAMs) are being developed as treatments for these disorders. The effects of α5-NAMs have been studied on recombinant $GABA_A$ receptors expressed in non-neuronal cells; however, no study has compared drug effects on the tonic conductance generated by native $GABA_A$ receptors in neurones, which was the goal of this study.

Methods: The effects of five α5-NAMs (basmisanil, Ono-160, L-655,708, α5IA, and MRK-016) on tonic current evoked by a low concentration of GABA were studied using whole-cell recordings in cultured mouse hippocampal neurones. Drug effects on current evoked by a saturating concentration of GABA and on miniature inhibitory postsynaptic currents (mIPSCs) were also examined.

Results: The α5-NAMs caused a concentration-dependent decrease in tonic current. The potencies varied as the inhibitory concentration for 50% inhibition (IC_{50}) of basmisanil (127 nM) was significantly higher than those of the other compounds (0.4–0.8 nM). In contrast, the maximal efficacies of the drugs were similar (35.5–51.3% inhibition). The α5-NAMs did not modify current evoked by a saturating GABA concentration or mIPSCs.

Conclusions: Basmisanil was markedly less potent than the other α5-NAMs, an unexpected result based on studies of recombinant α5 $GABA_A$ receptors. Studying the effects of α5 $GABA_A$ receptor-selective drugs on the tonic inhibitory current in neurones could inform the selection of compounds for future clinical trials.

Keywords: α5 $GABA_A$ receptor; hippocampus; negative allosteric modulator; neurocognitive disorder; synaptic inhibition; tonic inhibition

Received: 6 April 2020; **Accepted:** 16 November 2020

Editor's key points

- Increased tonic inhibitory conductance generated by α5 subunit-containing γ-aminobutyric acid type A (α5 $GABA_A$) receptors is associated with multiple cognitive and psychiatric disorders.
- The effects of negative allosteric modulators that inhibit α5 $GABA_A$ receptors (α5-NAMs), which are being developed as treatments for these disorders, were tested on isolated mouse hippocampal neurones.
- Basmisanil was less potent than the other α5-NAMs in intact neurones in contrast to studies of recombinant α5 $GABA_A$ receptors.
- The effects of α5 $GABA_A$ receptor-selective drugs on the tonic inhibitory current in neurones could inform selection of compounds for future clinical trials of neurocognitive disorders.

Increased tonic inhibitory conductance generated by γ-aminobutyric acid type A ($GABA_A$) receptors has been implicated in various cognitive and psychiatric disorders, including Alzheimer's disease, Down syndrome, and schizophrenia.[1–4] In addition, perioperative neurocognitive disorders, which are some of the most common adverse events that occur in older patients after anaesthesia and surgery, may result in part from an increase in this tonic inhibitory conductance.[5,6] Preclinical models have shown that commonly used general anaesthetic drugs can trigger a sustained increase in the tonic inhibitory conductance in neurones from brain regions that regulate cognition, including the hippocampus.[6] These cognitive disorders are associated with poor long-term outcomes and impose a tremendous burden on patients, their families, and the healthcare system; yet, no effective pharmacological treatments exist. Development of drugs that reduce the tonic inhibitory conductance is therefore of great interest.

The tonic inhibitory conductance is generated by $GABA_A$ receptors that are predominantly expressed in extra-synaptic regions of neurones.[7,8] γ-Aminobutyric acid type A receptors are heteropentameric ion channels that are formed from 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3). The combination of the various subunits confers unique physiological and pharmacological properties to the pentameric receptor complexes.[7,8] The properties of $GABA_A$ receptors are also influenced by their location within neurones, the conditions of agonist-dependent activation, and receptor phosphorylation.[7–9]

Extra-synaptic $GABA_A$ receptors have a relatively high affinity for GABA and are activated by persistent, low ambient levels of GABA, which either spills over from the synaptic cleft or is released from glia.[7,8,10] These receptors are thought to mediate a paracrine or slow form of inhibition. There are two major classes of extra-synaptic receptors: those containing a δ subunit (α4βδ, α6βδ, and α1βδ) and those containing α5 subunits (α5βγ).[8] Combinations of αβ, αβε, and α3βγ2 subunits also exist at lower levels.[11,12] In contrast, synaptic $GABA_A$ receptors have lower affinity for GABA, often contain α1βγ or α2βγ subunit, and are transiently activated by high concentrations of GABA that are released from presynaptic terminals.[7,8]

The α5 subunit-containing $GABA_A$ (α5 $GABA_A$) receptors have gained particular attention because of their roles in learning and memory processes.[4] The most widely expressed α5 $GABA_A$ receptors are α5β3γ2 complexes, although α5 subunits also associate with β1 or β2 subunit and γ1 or γ3 subunit.[13,14] Interestingly, α5 $GABA_A$ receptors are also expressed, albeit at lower levels, in synapses, where they primarily contribute to slowly decaying inhibitory postsynaptic currents.[15–19] The α5 $GABA_A$ receptors are highly expressed in the hippocampus and, to a lesser extent, in the neocortex, where they regulate cognition.[20,21] Animal models have shown that increasing α5 $GABA_A$ receptor activity typically impairs cognition, whereas reducing α5 $GABA_A$ receptor function through either pharmacological or genetic approaches enhances cognitive performance.[6,22–24]

Given the well-established role of α5 $GABA_A$ receptors in cognition, drugs that act as negative allosteric modulators (NAMs) of these receptors, so-called α5-NAMs, are being developed and tested in clinical trials as nootropic agents.[4,25,26] Similar to positive allosteric modulators (PAMs), such as midazolam and diazepam, α5-NAMs bind at a high-affinity benzodiazepine-binding site on $GABA_A$ receptors.[26] The binding site is located at the interface between α and γ subunits, when the α subunit is an α1, α2, α3, or α5 subunit.[27] In contrast to the actions of PAMs, α5-NAMs reduce the affinity of GABA, and thus reduce GABA-dependent channel opening. The resultant decrease in anion permeability of the cell membrane increases neuronal excitability and enhances synaptic plasticity.[28,29]

To date, more than a dozen α5-NAMs have been developed and several have progressed to clinical trials.[4,25,26] The pharmacological properties of α5-NAMs have been primarily studied using heterologous expression systems[25]; however, no study has directly compared the properties of α5-NAMs on the tonic inhibitory conductance in primary neurones.

The α5-NAM modulation of the tonic current in neurones may better reflect the *in vivo* pharmacodynamic properties of the drugs for several reasons. First, $GABA_A$ receptor populations that generate current in neurones are heterogeneous. Although the tonic current in hippocampal neurones is predominantly generated by α5 $GABA_A$ receptors, other $GABA_A$ receptor subtypes also contribute.[12,20] Second, α5-NAMs can both inhibit and enhance the function of different $GABA_A$ receptor subtypes. For example, L-655,708 inhibits α5β3γ2, α1β3γ2, and α2β3γ2 $GABA_A$ receptors, but increases the function of α3β3γ2, α4β3γ2, and α6β3γ2 $GABA_A$ receptors.[30] Overall, the net effect of α5-NAMs on the tonic current in neurones will be determined by both the inhibitory and potentiating drug actions on multiple subpopulations of $GABA_A$ receptors. Finally, intracellular signalling pathways, such as protein kinases and phosphatases, regulate $GABA_A$ receptor function and receptor pharmacology, and the activity of second messenger systems can differ between neurones and non-neuronal cells.[31,32]

The goal of this study was to directly compare the effect of five α5-NAMs (basmisanil, Ono-160, L-655,708, α5IA, and MRK-016) on the tonic inhibitory conductance in isolated mouse hippocampal neurones as an approach for screening and selection of α5-NAMs for future clinical trials. These drugs were selected because they have been investigated *in vitro* and several have been studied in clinical trials.[25,26] We also studied current evoked by a saturating concentration of GABA and miniature inhibitory postsynaptic currents (mIPSCs), as α5 $GABA_A$ receptors can also contribute to synaptic currents.

Methods

Selection of five α5-NAMs

The five α5-NAMs investigated (basmisanil, Ono-160, L-655,708, α5IA, and MRK-016; Supplementary Fig. 1) were selected because they have high binding affinities for α5 $GABA_A$ receptors (Supplementary Table 1), and three of the drugs have progressed to clinical trials (basmisanil, α5IA, and MRK-016). Basmisanil is the most widely studied α5-NAM in humans to date. At least three Phase 2 clinical trials have enrolled participants with either Down syndrome, stroke, or schizophrenia, with the common goal of improving cognitive function (https://www.clinicaltrials.gov/: NCT02024789, NCT02928393, and NCT02953639). Both α5IA and MRK-016 have progressed to clinical trials, but these studies were halted in Phase 1 because of adverse side-effects.[33,34] Ono-160 was described in a recent patent (WO 2015/115673 A1), and L-655,708 has been widely investigated in preclinical studies.[6,23,24,35]

Primary hippocampal neurone culture

All experimental procedures were approved by the local Animal Care Committee at the University of Toronto. Timed pregnant CD1 mice (Charles River, Montreal, QC, Canada) were used to prepare cultures of primary hippocampal neurones.[36,37] Briefly, fetuses (embryonic Days 16–18) were removed from a pregnant mouse that was euthanised by cervical dislocation. Hippocampi were dissected and cells were dissociated through mechanical trituration. Cells were then plated at a density of approximately 1×10^6 cells per 35 mm culture dish coated with poly-D-lysine (Sigma-Aldrich, Oakville, ON, Canada). Cultures were maintained in neurobasal medium (Gibco, Burlington, ON, Canada) that was supplemented with B-27 (Gibco) and L-glutamate (Gibco) for 14–20 days before use. Cell cultures prepared under these conditions primarily contain neurones. At the time of recording, the neurones were polarised, had extensive axonal and dendritic arbours, and formed numerous functional synapses resembling mature neurones *in vivo*.[38,39] Neurones that adhered to the bottom of the culture dish with pyramidal soma and clearly visible dendrites were selected for recordings. Culture dishes were prepared from at least two different mice for each experiment, and a maximum of two cells were recorded from each dish.

Electrophysiology

Whole-cell voltage clamp recordings were performed in cultured hippocampal neurones using an Axopatch™ 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) that was controlled with pCLAMP® 8.0 software (Molecular Devices) via a Digidata 1322 interface (Molecular Devices). Neurones were voltage-clamped at a holding potential of −60 mV. Patch pipettes were pulled from thin-walled borosilicate glass capillary tubes and had an open-tip resistance of 2–4 MΩ. The intracellular solution contained (in mM) 140 CsCl, 10 HEPES, 11 EGTA, 4 MgATP, 2 $MgCl_2$, 1 $CaCl_2$, and 2 TEA (pH 7.3 with CsOH; 285–295 mOsm). Extracellular recording solution contained (in mM) 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 5.4 KCl, 25 HEPES, and 28 glucose (pH 7.4; 320–330 mOsm). Drugs dissolved in extracellular solution were applied to the patched neurone by a computer-controlled, multibarrelled perfusion system (SF–77B; Warner Instruments, Hamden, CT, USA) that allowed fast solution exchange. All electrophysiological recordings were performed at room temperature (22–24°C).

Ionotropic glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and (2*R*)-amino-5-phosphonovaleric acid (APV; 20 μM) were added to the extracellular solution. Tetrodotoxin (0.2 μM) was used to block voltage-gated sodium channels. Tonic current was recorded by adding exogenous GABA (0.5 μM) to the extracellular solution. This concentration of GABA was selected because it is similar to the low extracellular concentration of GABA that occurs *in vivo*.[7,10] The competitive $GABA_A$ receptor antagonist bicuculline (20 μM) was applied to reveal the amplitude of the tonic current. Miniature IPSCs were recorded in the presence of CNQX (10 μM), APV (20 μM), and tetrodotoxin (0.2 μM) without exogenous GABA.

Each of the α5-NAMs (basmisanil, Ono-160, L-655,708, α5IA, and MRK-016) was studied at multiple concentrations to obtain the maximal efficacy and the half-maximal inhibitory concentration (IC_{50}). Concentrations for L-655,708, α5IA, and MRK-016 were selected based on the results from studies of the binding affinity and efficacy of α5 $GABA_A$ receptors expressed recombinantly.[23,33,34,40] For some drugs, the concentrations were selected based on the results reported in patents (US8835425B2 for basmisanil and WO 2015/115673 A1 for Ono-160).

Drugs and chemicals

Tetrodotoxin was purchased from Alomone Labs (Jerusalem, Israel). CNQX, APV, and bicuculline were obtained from Hello Bio Inc. (Princeton, NJ, USA), and GABA from Sigma-Aldrich. Four of the five α5-NAMs (basmisanil, Ono-160, α5IA, and MRK-016) were synthesised in-house at the Medicines Discovery Institute (Cardiff University, Cardiff, UK). L-655,708 was obtained from Sigma-Aldrich. All α5-NAMs were dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution of 10 mM. The stock solution was subsequently diluted in ultrapure water to create a secondary stock of 0.1 mM in DMSO 1%, stored at −2°C. For *in vitro* studies, the secondary stock was further diluted in the extracellular solution to obtain the desired α5-NAM concentration in DMSO ≤0.1%.

Data analyses

Currents were analysed with pClamp10 software (Molecular Devices). For the tonic current experiments, only a single concentration of the α5-NAM was applied to each cell, and the values were normalised to the amplitude of the bicuculline inhibitory response in that cell. The effects of each α5-NAM on the tonic current were reported as '% inhibition', which was calculated as $(I_{\alpha5NAM}/I_{bicuculline}) \times 100\%$, where $I_{\alpha5NAM}$ is the current amplitude of the α5-NAM response and $I_{bicuculline}$ that of bicuculline. The concentration–response curve of each α5-NAM was fitted with GraphPad Prism 6.01 (GraphPad, San Diego, CA, USA) using non-linear regression of log (agonist) *vs* response (three parameters): $Y=\text{bottom}+(E_{max}-\text{bottom})/(1+10^{\wedge}((\log IC_{50}-X)))$, where *Y* is the % inhibition and *X* is the concentration. The fit yielded values for the bottom (lowest % inhibition), E_{max} (maximal % inhibition), and IC_{50} values with SEM and 95% confidence intervals (CIs).

At least 30 s of mIPSC recordings under each experimental condition was analysed with Mini Analysis 6.0.3 (Synaptosoft, Inc., Fort Lee, NJ, USA). Analyses were performed in recordings without bursting and compound events to determine the

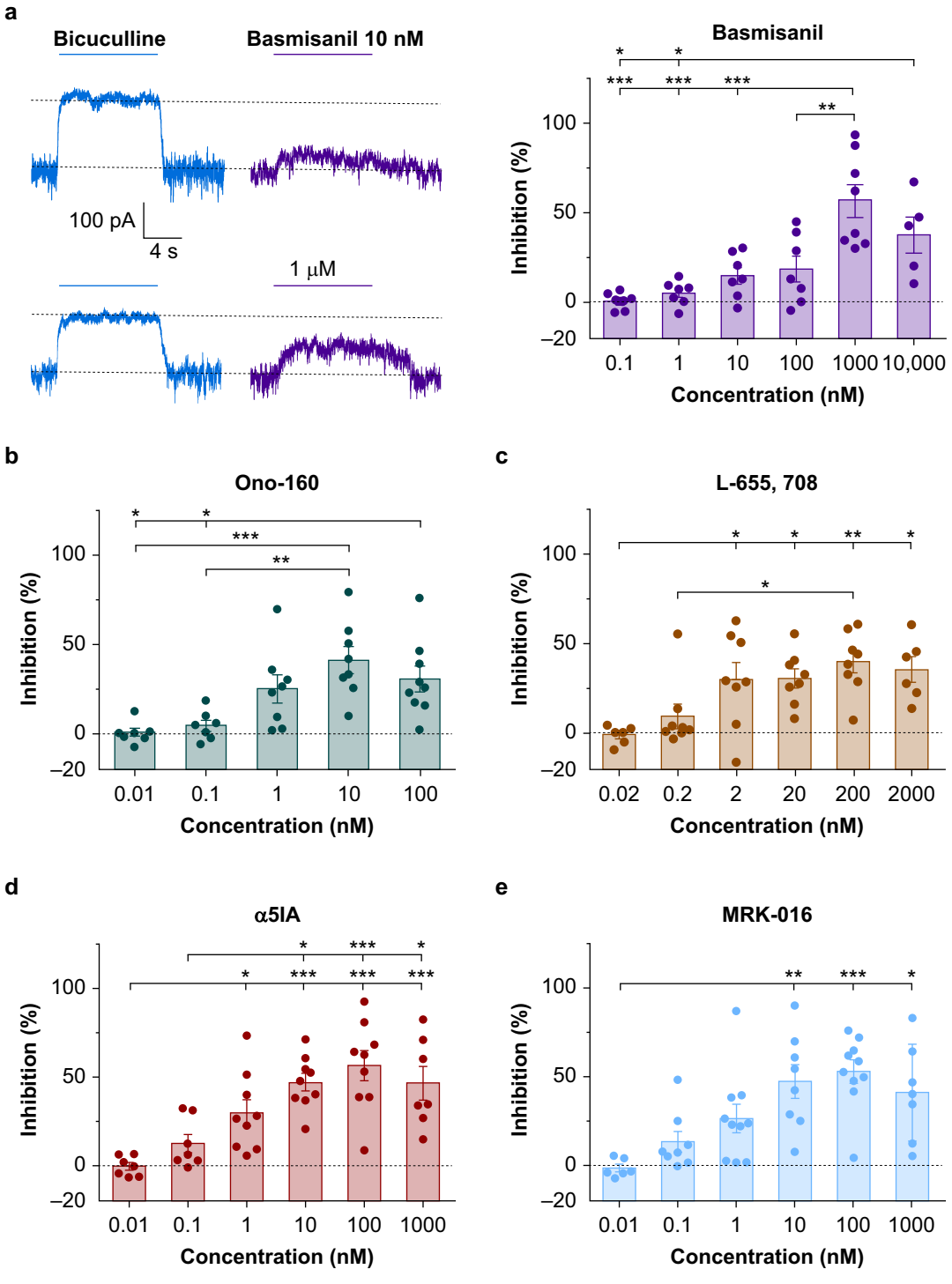

Fig 1. Tonic current inhibition by α5-NAMs. (a) Representative traces (left) showing the effects of basmisanil (10 nM and 1 μM) on the tonic current in comparison with the effect of bicuculline (20 μM). A single α5-NAM concentration was tested on each cell. Summarised data for basmisanil (right) illustrate a concentration-dependent effect. n=7, 7, 7, 7, 8, and 5 (left to right). One-way ANOVA; $F_{(5,35)}$=11.1; P<0.0001. (b–e) Summarised data for the remaining four α5-NAMs, which also show concentration-dependent effects. One-way ANOVA for all except MRK-016, where Kruskal–Wallis test was used. (b) Ono-160: n=7, 7, 8, 8, and 9 (left to right); $F_{(4,34)}$=7.1; P=0.0003. (c) L-655,708: n=6, 8, 8, 8, 8, and 6 (left to right); $F_{(5,38)}$=5.3; P=0.0009. (d) α5IA: n=7, 7, 9, 9, 9, and 7; $F_{(5,42)}$=9.9; P<0.001. (e) MRK-016: n=6, 8, 10, 8, 10, and 7 (left to right); Kruskal–Wallis statistic value $H_{(5)}$=24.3; P=0.0002. Data are mean [SEM]. *P<0.05; **P<0.01; ***P<0.001; Tukey's multiple comparisons test except for MRK-016, where Dunn's multiple comparisons test was used. ANOVA, analysis of variance; SEM, standard error of the mean; α5-NAM, negative allosteric modulators that inhibit α5 subunit-containing γ-aminobutyric acid type A receptor.

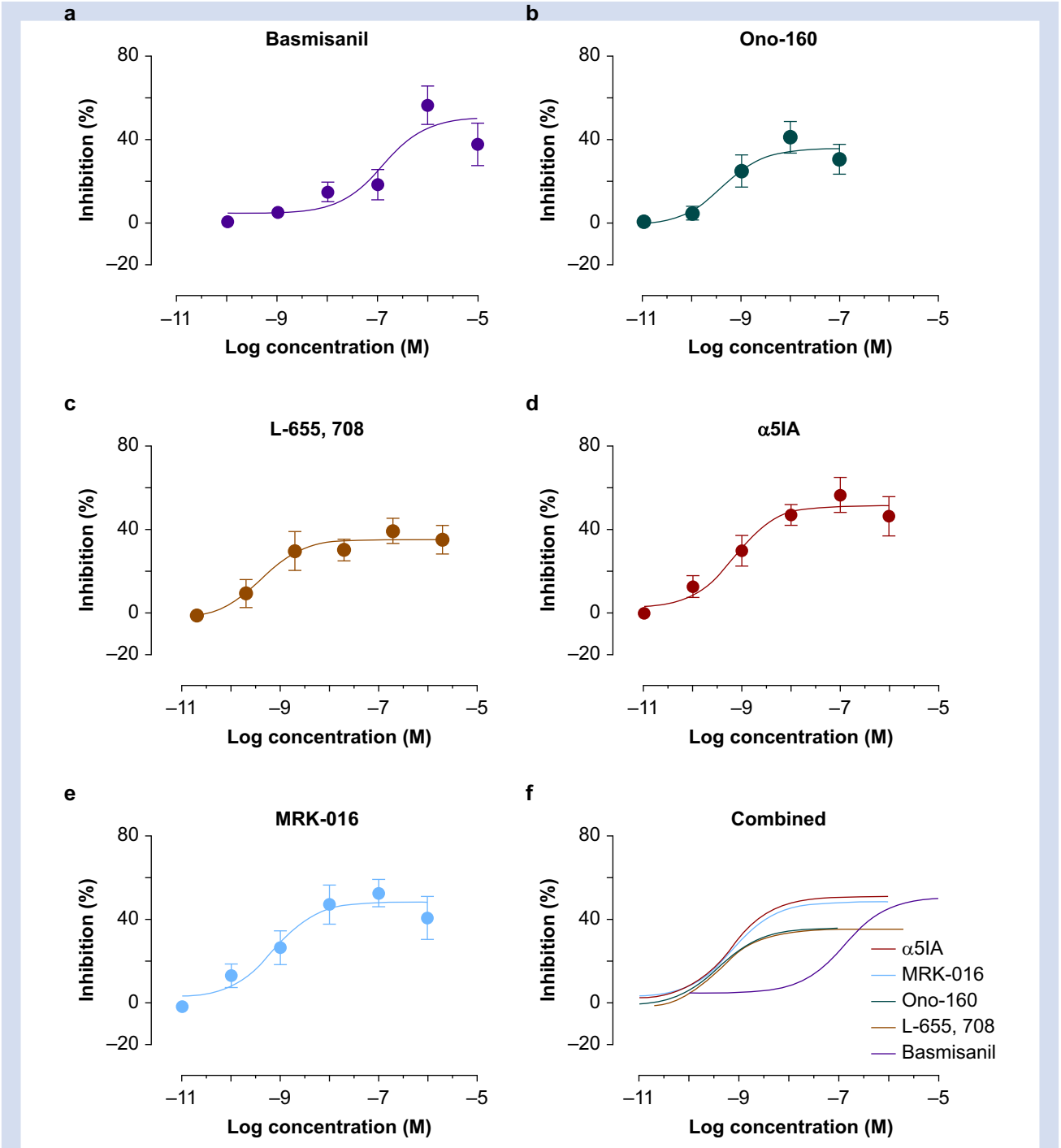

Fig 2. Concentration–effect curves for each α5-NAM fitted from the same data as in Fig 1. All α5-NAMs have similar efficacy, but basmisanil is the least potent, as the curve is shifted to the right. Data are mean [SEM]. Sample size is shown in Fig 1 SEM, standard error of the mean; α5-NAM, negative allosteric modulators that inhibit α5 subunit-containing γ-aminobutyric acid type A receptor.

parameters of mIPSCs. Each file was manually inspected to reject false events caused by noise and to include events that were not automatically detected. The total number of events under each experimental condition ranged from 335 to 1170. All graphs were created with GraphPad Prism 6.01 (GraphPad Software, Inc.).

Statistical analyses

Data are presented as mean (standard error of the mean [SEM]). The normality of data sets was tested with the D'Agostino–Pearson omnibus test ($n \geq 8$) or the Kolmogorov–Smirnov test ($n < 8$). For comparing three or more groups, one-way analysis of variance followed by Tukey's multiple

Table 1 Maximal inhibition (E_{max}) and IC_{50} values for α5-NAM effects. Data taken from the fitted curves shown in Fig 2 are presented as mean [SEM] (95% confidence intervals). The SEM values for IC_{50} are only available in log form and shown in the bottom row. Asterisks (*) indicate a significant difference between the IC_{50} value for basmisanil and those for the other compounds. $GABA_A$, γ-aminobutyric acid type A; IC_{50}, inhibitory concentration for 50% inhibition; SEM, standard error of the mean; α5-NAM, negative allosteric modulators that inhibit α5 $GABA_A$ receptor.

	Basmisanil	Ono-160	L-655,708	α5IA	MRK-016
E_{max}	50.6 [6.5]% (37.5–63.8)	36.1 [4.7]% (26.6–45.5)	35.5 [3.8]% (28.0–43.1)	51.3 [4.2]% (42.8–59.7)	48.5 [4.8]% (38.9–58.1)
IC_{50} (nM)	126.8* (27.5–583.4)	0.4 (0.06–2.9)	0.4 (0.06–2.9)	0.7 (0.2–2.7)	0.8 (0.2–3.7)
Log IC_{50} (M)	−6.9 [0.3]*	−9.4 [0.4]	−9.4 [0.4]	−9.2 [0.3]	−9.1 [0.3]

comparisons test was used. If normality was not met, Kruskal–Wallis test followed by Dunn's multiple comparisons test was used. Paired Student's *t*-test was used to compare two groups, and when normality assumptions were not satisfied, the non-parametric Wilcoxon matched-pairs signed rank test was utilised. Cumulative distributions of the amplitude and frequency of mIPSCs were compared using the Kolmogorov–Smirnov test. A two-tailed hypothesis test was used and statistical significance was set to $P<0.05$.

Results

α5-NAMs inhibited tonic current with similar efficacies but different potencies

The tonic current in neurones *in vivo* is primarily generated by extra-synaptic $GABA_A$ receptors that are activated by low, ambient concentrations of GABA.[7,8] To mimic such agonist conditions,[7,10] neurones were continuously perfused with a low concentration of GABA (0.5 μM). A competitive $GABA_A$ receptor antagonist, bicuculline (20 μM), was then co-applied with GABA. The amplitude of the tonic current was revealed, as indicated by a reduction in the holding current (Fig. 1a; $I_{bicuculline}$=122.7 [5.3] pA; n=167). After washout of bicuculline and return of current to baseline, α5-NAM was co-applied with GABA. The decrease in holding current caused by the α5-NAM was compared with the decrease caused by bicuculline. The results were described as % inhibition of the bicuculline response.

Basmisanil caused a concentration-dependent decrease in the amplitude of the tonic current, where 1 μM was significantly more effective than 0.1–100 nM (Fig. 1a). Increasing the concentration to 10 μM failed to further reduce the tonic current. The inhibitory effect of basmisanil was rapidly reversed after drug washout. Similarly, Ono-160, L-655,708, α5IA, and MRK-016 inhibited the tonic current, albeit at lower concentrations (Fig. 1b–e). The tonic current returned to baseline after washout of the α5-NAMs (Supplementary Fig. 2).

To compare the efficacy and potency of the α5-NAMs, data were fitted with sigmoidal concentration–effect curves (Fig. 2). Fittings generated the maximal inhibitory effect (E_{max}) and IC_{50}. The E_{max} values did not differ between the five α5-NAMs, as evidenced by the overlap in the 95% CIs (Table 1). In contrast, the IC_{50} value of basmisanil (126.8 nM; 95% CIs: 27.5–583.4 nM) was significantly greater than the other four compounds (Table 1). Notably, the IC_{50} values of Ono-160, L-655,708, α5IA, and MRK-016 were in the sub-nanomolar range (0.4–0.8 nM). The lower potency for basmisanil compared with the other four compounds was reflected by the rightward shift of the concentration–effect curve (Fig. 2f).

α5-NAMs did not inhibit currents evoked by a saturating GABA concentration

The α5-NAMs can have both positive and negative modulatory effects on other $GABA_A$ receptor subtypes.[25,30] Some of these receptor subtypes have a lower affinity for GABA than α5 $GABA_A$ receptors, yet higher single-channel conductance.[41] Therefore, we examined whether α5-NAMs modulated currents evoked by a saturating concentration of GABA, a condition that activates both low-affinity and high-affinity $GABA_A$ receptors.

A saturating concentration of GABA (1 mM) was applied for 16 s to activate peak current and a lower steady-state current (attributable to receptor desensitisation; Fig. 3a). The peak current was used as an indicator of maximal $GABA_A$ receptor activation, whereas the magnitude of the steady-state current revealed receptor subpopulations that resided in non-desensitised states. The effects of each α5-NAM were studied at a concentration that produced the maximal reduction in the amplitude of the tonic current, as shown in Fig. 1. The α5-NAM was pre-applied for 10 s before being co-applied with GABA.

Basmisanil did not alter the amplitude of the peak or steady-state current, as shown in Fig. 3a ($P>0.05$ for both; n=8; see also Table 2). Similarly, the other four α5-NAMs had no effects (Table 2). Thus, the α5-NAMs did not alter the function of $GABA_A$ receptors when activated by a saturating concentration of GABA.

α5-NAMs did not inhibit mIPSCs

MIPSCs are generated by postsynaptic $GABA_A$ receptors that are activated by GABA released from presynaptic terminals.[7] Although α5 $GABA_A$ receptors are predominantly located extra-synaptically, they are also expressed at lower levels in synaptic regions of neurones.[16,42] Basmisanil had no effect on the amplitude or frequency of mIPSCs (Fig. 3b). Similarly, the time course and charge transfer of mIPSCs were unaffected by basmisanil (Table 3). Likewise, Ono-160, L-655,708, α5IA, and MRK-016 did not modulate mIPSCs (Table 3).

Discussion

The primary goal of this study was to compare the effects of five α5-NAMs on the tonic inhibitory current in mouse hippocampal neurones. The maximal inhibitory effects of the five α5-NAMs were similar, whereas the potencies differed. Basmisanil was less potent than the other four compounds, all of which exhibited similar potencies.

None of the α5-NAMs modified the amplitude of current evoked by a saturating concentration of GABA. Although

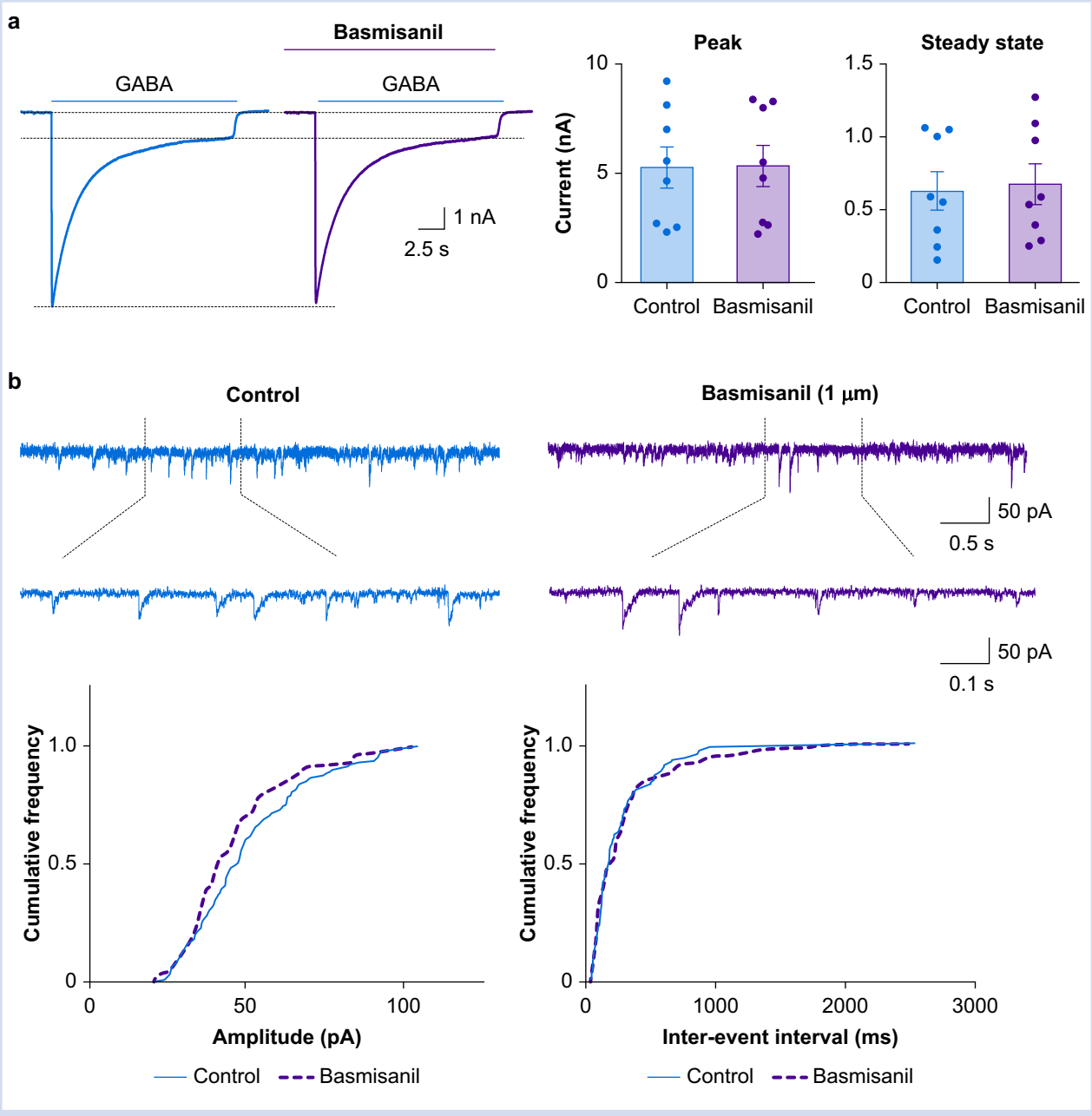

Fig 3. Basmisanil does not modulate the peak or steady-state current evoked by a saturating concentration of GABA, nor affect mIPSCs. (a) Left panel: representative traces showing the effect of basmisanil on current evoked by GABA (1 mM); right panel: summarised data for peak and steady-state currents. n=8; P=0.8 and P=0.1 for the peak and steady-state currents, respectively; Student's paired t-test. Data are mean [SEM]. (b) Top panel: representative traces of mIPSCs in the absence and presence of basmisanil; bottom panel: cumulative distributions of the amplitude (left) and frequency (right) of mIPSCs show that both were not altered by basmisanil. P=0.1 and P=0.7 for the amplitude and frequency, respectively; Kolmogorov–Smirnov test. GABA, γ-aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; SEM, standard error of the mean.

α5 $GABA_A$ receptors contribute to 25% of $GABA_A$ receptors in the hippocampus,[4] our results suggest that the current generated by other $GABA_A$ receptors masks the contribution of low-conductance α5 $GABA_A$ receptors to peak and steady-state responses. In addition, α5-NAMs did not change the amplitude, frequency, or time course of mIPSCs. This latter result is consistent with the notion that α5 $GABA_A$ receptors do not contribute substantially to synaptic currents,[20,28] although they are expressed in synaptic regions on the dendrites of hippocampal neurones and can contribute to slowly decaying synaptic currents.[7,16,17,19,43]

Table 2 Effects of α5-NAMs on the amplitude of peak or steady-state currents evoked by a saturating concentration of GABA (1 mM). $P>0.05$; Student's paired *t*-test. The steady-state current for Ono-160 was assessed using the Wilcoxon matched-pairs signed rank test. Data are presented as mean [SEM]. GABA, γ-aminobutyric acid; SEM, standard error of the mean; α5-NAM, negative allosteric modulators that inhibit α5 subunit-containing γ-aminobutyric acid type A receptor.

	Peak currents (pA)	Steady-state currents (pA)
Control (n=8)	5260 (950)	644 (134)
Basmisanil (1 μM)	5320 (943)	693 (141)
Control (n=7)	4560 (1100)	646 (226)
Ono-160 (10 nM)	4250 (1090)	640 (212)
Control (n=7)	5000 (1000)	752 (186)
L-655,708 (200 nM)	4940 (1020)	746 (184)
Control (n=6)	4271 (764)	567 (140)
α5IA (100 nM)	4290 (849)	549 (136)
Control (n=5)	3510 (894)	349 (98)
MRK-016 (100 nM)	3390 (799)	375 (84)

All five α5-NAMs inhibited the tonic current with similar efficacy. The E_{max} values were similar to those from recombinant α5β3γ2 receptors for four of the five compounds. The similarities between efficacies of basmisanil, Ono-160, α5IA, and MRK-016 for inhibiting the tonic current in neurones and inhibiting recombinant α5 $GABA_A$ receptors are consistent with evidence that suggests the tonic current is primarily generated by α5 $GABA_A$ receptors.[4,6,20,37]

Interestingly, L-655,708 caused a greater inhibition of the tonic current in neurones than recombinant α5β3γ2 $GABA_A$ receptors expressed in mouse L (tk-) cells. Two factors, which are related to the experimental condition, could contribute to this discrepancy. γ-Aminobutyric acid, applied at the low concentration we used (0.5 μM ≈ EC_3), could predominantly occupy only one of the two GABA binding sites on $GABA_A$ receptors, whereas GABA at higher concentrations, such as those used in studies of recombinant receptors (EC_{20}), might occupy both ligand binding sites.[25] L-655,708 may be more effective at a lower GABA concentration because of different conformational changes that occur in the mono-liganded state.[25] Also, primary neurones and heterologous systems may differ in their intracellular environment and cell signalling pathways that regulate $GABA_A$ receptor function and their responses to drugs. For example, phosphorylation modulates the effects of benzodiazepines through regulation of ligand binding at the benzodiazepine-binding site.[44,45] Such a difference in cell signalling pathways could alter the efficacy of L-655,708.

The potencies of the five α5-NAMs were similar with the exception of basmisanil. Their IC_{50} values were similar to those reported with recombinant α5β3γ2 $GABA_A$ receptors. Surprisingly, basmisanil was less potent than the other compounds. The potency of basmisanil also differed by more than an order of magnitude from its binding affinity for recombinant α5β3γ2 receptors. It will be of interest to determine the reasons why basmisanil has a low potency (or functional affinity) for inhibition of α5 $GABA_A$ receptors in neurones, yet high binding affinity for α5β3γ2 receptors in recombinant systems.

The unexpected lower potency of basmisanil for inhibiting the tonic current is of considerable interest, given the results from several clinical trials. Basmisanil is the most widely studied α5-NAM in humans to date. Three Phase 2 trials have enrolled participants with either Down syndrome, stroke, or schizophrenia, with the common goal of improving cognitive function. No improvement in cognition was observed in participants with Down syndrome, as measured by a battery of neuropsychological tests (https://www.clinicaltrials.gov/; NCT02024789). The study of stroke patients was terminated because of low recruitment of participants (NCT02928393). The clinical trial of patients with schizophrenia has been completed, but results have not yet been reported (NCT02953639). Our results, which show a low potency for basmisanil, raise the possibility that the drug might not reach sufficiently high concentrations in the human brain to inhibit the tonic current.

Table 3 Effects of α5-NAMs on miniature inhibitory postsynaptic currents. For all parameters, $P>0.05$; Student's paired *t*-test except for α5IA (frequency, rise, and area), basmisanil (frequency), L-655,708 (amplitude and area), and MRK-016 (frequency), where Wilcoxon matched-pairs signed rank test was used. Data are presented as mean [SEM]. SEM, standard error of the mean; α5-NAM, negative allosteric modulators that inhibit α5 subunit-containing γ-aminobutyric acid type A receptor.

	Amplitude (pA)	Frequency (Hz)	Rise time (ms)	Decay time (ms)	Area (pA.ms)
Control (n=7)	45.5 [4.7]	3.7 [0.2]	5.0 [0.3]	11.0 [1.0]	552.6 [88.7]
Basmisanil (1 μM)	41.7 [3.2]	4.6 [0.7]	5.3 [0.4]	9.9 [0.8]	468.1 [48.2]
Control (n=6)	39.0 [3.2]	4.0 [0.9]	5.8 [0.6]	9.0 [1.2]	399.0 [53.2]
Ono-160 (10 nM)	40.2 [1.8]	4.8 [0.8]	5.6 [0.4]	8.4 [1.1]	399.6 [49.3]
Control (n=7)	47.2 [6.5]	5.3 [0.8]	5.2 [0.3]	9.9 [0.8]	533.0 [109.0]
L-655,708 (200 nM)	42.8 [4.7]	4.8 [1.0]	5.3 [0.4]	9.2 [1.2]	446.7 [78.2]
Control (n=7)	43.8 [2.6]	1.6 [0.3]	5.4 [0.3]	10.3 [0.2]	514.3 [75.8]
α5IA (100 nM)	43.0 [2.3]	2.2 [0.4]	5.9 [0.2]	10.7 [1.3]	510.2 [72.6]
Control (n=8)	40.3 [4.0]	2.5 [0.5]	6.7 [0.4]	13.7 [1.5]	596.8 [69.6]
MRK-016 (100 nM)	40.0 [3.9]	2.8 [0.7]	6.4 [0.5]	13.8 [1.0]	591.9 [75.8]

Overall, we identified two unexpected findings: the greater efficacy of L-655,708 and the lower potency of basmisanil for inhibiting the tonic current in hippocampal neurones. These results suggest that α5-NAM development programmes would benefit from *in vitro* studies of native $GABA_A$ receptors. Screening α5-NAM effects on tonic current in cultured hippocampal neurones, where heterogeneous populations of $GABA_A$ receptors are expressed, may offer critical information that either supports or rejects a decision to embark on costly, time-consuming, and labour-intensive clinical trials. Despite the challenges faced in recent clinical trials, the development of α5-NAMs has considerable potential, given the extent to which hyperactivity of α5 $GABA_A$ receptors is implicated in many devastating cognitive and psychiatric disorders.

Authors' contributions

Study design: MAM, DSW, MOP, JRA, BAO
Data collection: MAM, DSW, WWL, AP
Data analysis: MAM, DSW, SK, RAR
Compound synthesis: MOP, JRA
Manuscript preparation: DSW, MAM, MOP, JRA, RAR, BAO

Declarations of interest

BAO is an inventor named on a Canadian patent (2,852,978), a US patent (9,517,265), and a pending US patent (62/268,137). JRA received personal fees from Ono Pharmaceutical Co. MAM, DSW, WWL, AP, MOP, SK, and RAR have no competing interests.

Funding

Canadian Institutes of Health Research (FDN-154312) to BAO; Ontario Graduate Scholarship to MAM, WWL, and SK; Frederick Banting and Charles Best Canada Graduate Scholarship—Master's Program to MAM and WWL; Dr Kirk Weber Research Award in Anaesthesia to AP; Canada Graduate Scholarship—Doctoral Program from the Canadian Institutes of Health Research to SK; Wellcome Trust to MOP and JRA; Welsh Government/European Regional Development Fund to JRA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2020.11.032.

References

1. Redrobe JP, Elster L, Frederiksen K, et al. Negative modulation of $GABA_A$ α5 receptors by RO4938581 attenuates discrete sub-chronic and early postnatal phencyclidine (PCP)-induced cognitive deficits in rats. *Psychopharmacology (Berl)* 2012; **221**: 451–68
2. Wu Z, Guo Z, Gearing M, Chen G. Tonic inhibition in dentate gyrus impairs long-term potentiation and memory in an Alzheimer's disease model. *Nat Commun* 2014; **5**: 4159
3. Martinez-Cue C, Martinez P, Rueda N, et al. Reducing $GABA_A$ α5 receptor-mediated inhibition rescues functional and neuromorphological deficits in a mouse model of Down syndrome. *J Neurosci* 2013; **33**: 3953–66
4. Jacob TC. Neurobiology and therapeutic potential of α5-GABA type A receptors. *Front Mol Neurosci* 2019; **12**: 179
5. Subramaniyan S, Terrando N. Neuroinflammation and perioperative neurocognitive disorders. *Anesth Analg* 2019; **128**: 781–8
6. Zurek AA, Yu J, Wang DS, et al. Sustained increase in α5$GABA_A$ receptor function impairs memory after anesthesia. *J Clin Invest* 2014; **124**: 5437–41
7. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci* 2005; **6**: 215–29
8. Brickley SG, Mody I. Extrasynaptic $GABA_A$ receptors: their function in the CNS and implications for disease. *Neuron* 2012; **73**: 23–34
9. Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, Orser BA. Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. *Mol Pharmacol* 2001; **59**: 814–24
10. Bright DP, Smart TG. Methods for recording and measuring tonic $GABA_A$ receptor-mediated inhibition. *Front Neural Circuits* 2013; **7**: 193
11. Mortensen M, Smart TG. Extrasynaptic αβ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons. *J Physiol* 2006; **577**: 841–56
12. Lee V, Maguire J. The impact of tonic $GABA_A$ receptor-mediated inhibition on neuronal excitability varies across brain region and cell type. *Front Neural Circuits* 2014; **8**: 3
13. Sur C, Quirk K, Dewar D, Atack J, McKernan R. Rat and human hippocampal α5 subunit-containing γ-aminobutyric $acid_A$ receptors have α5β3γ2 pharmacological characteristics. *Mol Pharmacol* 1998; **54**: 928–33
14. Ju YH, Guzzo A, Chiu MW, et al. Distinct properties of murine α5 γ-aminobutyric acid type A receptors revealed by biochemical fractionation and mass spectroscopy. *J Neurosci Res* 2009; **87**: 1737–47
15. Glykys J, Mann EO, Mody I. Which $GABA_A$ receptor subunits are necessary for tonic inhibition in the hippocampus? *J Neurosci* 2008; **28**: 1421–6
16. Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL. Synaptic and nonsynaptic localization of $GABA_A$ receptors containing the α5 subunit in the rat brain. *J Comp Neurol* 2006; **499**: 458–70
17. Hausrat TJ, Muhia M, Gerrow K, et al. Radixin regulates synaptic $GABA_A$ receptor density and is essential for reversal learning and short-term memory. *Nat Commun* 2015; **6**: 6872
18. Magnin E, Francavilla R, Amalyan S, et al. Input-specific synaptic location and function of the α5 $GABA_A$ receptor subunit in the mouse CA1 hippocampal neurons. *J Neurosci* 2019; **39**: 788–801
19. Vargas-Caballero M, Martin LJ, Salter MW, Orser BA, Paulsen O. α5 Subunit-containing $GABA_A$ receptors mediate a slowly decaying inhibitory synaptic current in CA1 pyramidal neurons following Schaffer collateral activation. *Neuropharmacology* 2010; **58**: 668–75
20. Caraiscos VB, Elliott EM, You-Ten KE, et al. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* 2004; **101**: 3662–7
21. Sur C, Fresu L, Howell O, McKernan RM, Atack JR. Autoradiographic localization of α5 subunit-containing $GABA_A$ receptors in rat brain. *Brain Res* 1999; **822**: 265–70

22. Collinson N, Kuenzi FM, Jarolimek W, et al. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. *J Neurosci* 2002; **22**: 5572–80
23. Atack JR, Bayley PJ, Seabrook GR, Wafford KA, McKernan RM, Dawson GR. L-655,708 enhances cognition in rats but is not proconvulsant at a dose selective for α5-containing $GABA_A$ receptors. *Neuropharmacology* 2006; **51**: 1023–9
24. Zurek AA, Bridgwater EM, Orser BA. Inhibition of α5 γ-aminobutyric acid type A receptors restores recognition memory after general anesthesia. *Anesth Analg* 2012; **114**: 845–55
25. Sieghart W, Savic MM. International Union of Basic and Clinical Pharmacology. CVI: $GABA_A$ receptor subtype- and function-selective ligands: key issues in translation to humans. *Pharmacol Rev* 2018; **70**: 836–78
26. Maramai S, Benchekroun M, Ward SE, Atack JR. Subtype selective γ-aminobutyric acid type A receptor ($GABA_AR$) modulators acting at the benzodiazepine binding site: an update. *J Med Chem* 2020; **63**: 3425–46
27. Sigel E, Ernst M. The benzodiazepine binding sites of $GABA_A$ receptors. *Trends Pharmacol Sci* 2018; **39**: 659–71
28. Bonin RP, Martin LJ, MacDonald JF, Orser BA. α5$GABA_A$ receptors regulate the intrinsic excitability of mouse hippocampal pyramidal neurons. *J Neurophysiol* 2007; **98**: 2244–54
29. Martin LJ, Zurek AA, MacDonald JF, Roder JC, Jackson MF, Orser BA. α5$GABA_A$ receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. *J Neurosci* 2010; **30**: 5269–82
30. Ramerstorfer J, Furtmuller R, Vogel E, Huck S, Sieghart W. The point mutation γ2F77I changes the potency and efficacy of benzodiazepine site ligands in different $GABA_A$ receptor subtypes. *Eur J Pharmacol* 2010; **636**: 18–27
31. Nakamura Y, Darnieder LM, Deeb TZ, Moss SJ. Regulation of $GABA_A$Rs by phosphorylation. *Adv Pharmacol* 2015; **72**: 97–146
32. Houston CM, Smart TG. CaMK-II modulation of $GABA_A$ receptors expressed in HEK293, NG108-15 and rat cerebellar granule neurons. *Eur J Neurosci* 2006; **24**: 2504–14
33. Atack JR. Preclinical and clinical pharmacology of the $GABA_A$ receptor α5 subtype-selective inverse agonist α5IA. *Pharmacol Ther* 2010; **125**: 11–26
34. Atack JR, Maubach KA, Wafford KA, et al. In vitro and in vivo properties of 3-*tert*-butyl-7-(5-methylisoxazol-3-yl)-2-(1-methyl-1*H*-1,2,4-triazol-5-ylmethoxy)-pyrazolo [1,5-*d*]-[1,2,4]triazine (MRK-016), a $GABA_A$ receptor α5 subtype-selective inverse agonist. *J Pharmacol Exp Ther* 2009; **331**: 470–84
35. Khodaei S, Avramescu S, Wang DS, et al. Inhibiting α5 subunit-containing γ-aminobutyric acid type A receptors attenuates cognitive deficits after traumatic brain injury. *Crit Care Med* 2020; **48**: 533–44
36. MacDonald JF, Mody I, Salter MW. Regulation of *N*-methyl-D-aspartate receptors revealed by intracellular dialysis of murine neurones in culture. *J Physiol* 1989; **414**: 17–34
37. Wang DS, Zurek AA, Lecker I, et al. Memory deficits induced by inflammation are regulated by α5-subunit-containing $GABA_A$ receptors. *Cell Rep* 2012; **2**: 488–96
38. Banker GA, Cowan WM. Rat hippocampal neurons in dispersed cell culture. *Brain Res* 1977; **126**: 397–442
39. Kaech S, Banker G. Culturing hippocampal neurons. *Nat Protoc* 2006; **1**: 2406–15
40. Dawson GR, Maubach KA, Collinson N, et al. An inverse agonist selective for α5 subunit-containing $GABA_A$ receptors enhances cognition. *J Pharmacol Exp Ther* 2006; **316**: 1335–45
41. Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF, Orser BA. Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol Pharmacol* 2003; **63**: 2–8
42. Brunig I, Scotti E, Sidler C, Fritschy JM. Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol* 2002; **443**: 43–55
43. Sun MY, Shu HJ, Benz A, et al. Chemogenetic isolation reveals synaptic contribution of δ $GABA_A$ receptors in mouse dentate granule neurons. *J Neurosci* 2018; **38**: 8128–45
44. Hodge CW, Mehmert KK, Kelley SP, et al. Supersensitivity to allosteric $GABA_A$ receptor modulators and alcohol in mice lacking PKCε. *Nat Neurosci* 1999; **2**: 997–1002
45. Churn SB, Rana A, Lee K, Parsons JT, De Blas A, Delorenzo RJ. Calcium/calmodulin-dependent kinase II phosphorylation of the $GABA_A$ receptor α1 subunit modulates benzodiazepine binding. *J Neurochem* 2002; **82**: 1065–76

Handling editor: Hugh C Hemmings Jr