CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: https://orca.cardiff.ac.uk/id/eprint/139790/

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Manzo, Marc A., Wang, Dian-Shi, Li, Winston W., Pinguelo, Arsène, Popa, Mariana O., Khodaei, Shahin, Atack, John R. ORCID: https://orcid.org/0000-0002-3410-791X, Ross, Ruth A. and Orser, Beverley A. 2021. 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. British Journal of Anaesthesia 126 (3) , pp. 674-683. 10.1016/j.bja.2020.11.032 file

Publishers page: http://dx.doi.org/10.1016/j.bja.2020.11.032 <http://dx.doi.org/10.1016/j.bja.2020.11.032>

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See
http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.

information services
gwasanaethau gwybodaeth

Inhibition of a tonic inhibitory conductance in mouse hippocampal neurons by negative allosteric modulators of α5GABA$_A$ receptors: implications for treating cognitive deficits

Running Title: Comparing the effect of α5GABA$_A$ receptor NAMs

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

[1] Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[2] Department of Anesthesiology and Pain Medicine, University of Toronto, Toronto, ON M5G 1E2, Canada
[3] Department of Anesthesia, Sunnybrook Health Sciences Centre, Toronto, ON M4N 3M5, Canada
[4] The Medicines Discovery Institute, Cardiff University, Cardiff, CF10 3AT, Wales
[5] Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON M5S 1A8, Canada

[†]M.A.M. and D.S.W. contributed equally to this work.

*Corresponding author:

Beverley A. Orser, MD, PhD, FRCPC, FCAHS, FRSC
Department of Anesthesiology and Pain Medicine
University of Toronto
Room 3318, Medical Sciences Building
1 King's College Circle
Toronto, Ontario M5S 1A8, Canada
Tel: 416-978-0574
Email: beverley.orser@utoronto.ca

Keywords: α5GABA$_A$ receptor; hippocampus; negative allosteric modulator; synaptic inhibition; tonic inhibition

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 ($\alpha5GABA_A$) receptors. Negative allosteric modulators that inhibit $\alpha5GABA_A$ receptors (α5-NAMs) are being developed as treatments for these disorders. The effects of α5-NAMs have been intensely 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 neurons. Thus, the goal of this study was to compare α5-NAM modulation of tonic inhibitory current in primary neurons.

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

Results: The α5-NAMs caused a concentration-dependent decrease in tonic current. The potencies varied as the IC_{50} of basmisanil (126.8 nM) was significantly higher than that of the other compounds (0.4 – 0.8 nM). In contrast, the maximal efficacies of the drugs were similar (35.5% to 51.3% inhibition). The α5-NAMs did not modify current evoked by saturating GABA, or mIPSCs.

Conclusions: Basmisanil was markedly less potent than the other α5-NAMs, an unexpected result based on studies of recombinant $\alpha5GABA_A$ receptors. Studying the effects of $\alpha5GABA_A$ receptor-

selective drugs on the tonic inhibitory current in neurons could inform the selection of compounds for future clinical trials.

Introduction

An 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 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 anesthesia and surgery, may result in part from an increase in the tonic inhibitory conductance.[5,6] Preclinical models have shown that commonly used general anesthetic drugs can trigger a sustained increase in the tonic inhibitory conductance in 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. The 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 extrasynaptic regions of neurons.[7,8] $GABA_A$ 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 specific location within neurons, the conditions of agonist-dependent activation, and receptor phosphorylation.[7-9]

Extrasynaptic $GABA_A$ 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 the glia.[7,8,10] These receptors are thought to mediate a paracrine or slow form of inhibition. There are two major classes of extrasynaptic receptors: those containing a δ subunit

(α4βδ, α6βδ, 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 subunits, and are transiently activated by high concentrations of GABA that are released from presynaptic terminals.[7,8]

α5 subunit-containing $GABA_A$ ($α5GABA_A$) receptors have gained particular attention due to their roles in learning and memory processes.[4] The most widely expressed $α5GABA_A$ receptors are α5β3γ2 complexes, although α5 subunits also associate with β1 or β2 subunits and γ1 or γ3 subunits.[13,14] Interestingly, $α5GABA_A$ receptors are also expressed, albeit at lower levels, in synapses where they primarily contribute to slowly decaying inhibitory postsynaptic currents (IPSCs).[15-19] $α5GABA_A$ 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 $α5GABA_A$ receptor activity typically impairs cognition, whereas reducing $α5GABA_A$ receptor function through either pharmacological or genetic approaches, enhances cognitive performance.[6,22-24]

Given the well-established role of $α5GABA_A$ receptors in cognition, drugs that act as negative allosteric modulators (NAMs) of these receptors, the 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 the 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 recombinant expression systems including HEK293 cells, Xenopus oocytes and mouse L(tk-) cells;[25] however, no study has directly compared the properties of α5-NAMs on the tonic inhibitory conductance in primary neurons.

α5-NAM modulation of the tonic current in neurons may better reflect the *in vivo* pharmacodynamic properties of the drugs for several reasons. First, $GABA_A$ receptor populations that generate current in neurons are heterogeneous. Although the tonic current in hippocampal neurons is predominantly generated by $α5GABA_A$ 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 increase the function of α3β3γ2, α4β3γ2 and α6β3γ2 $GABA_A$ receptors.[30] Overall, the net effect of α5-NAMs on the tonic current in neurons will be determined by both the inhibitory and potentiating drug actions on multiple subpopulations of $GABA_A$ receptors. Finally, intracellular signaling pathways such as kinases and phosphatases regulate $GABA_A$ receptor function and receptor pharmacology, and the activity of second messenger systems can differ between neurons 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 hippocampal neurons. These particular drugs were selected because they have been investigated in *in vitro* studies and several have been studied in clinical trials.[25,26] We also studied current evoked by a saturating concentration of GABA as well as miniature inhibitory postsynaptic currents (mIPSCs) as $α5GABA_A$ receptors can also contribute to synaptic currents. Overall, we expected to

demonstrate a utility of our experimental approach for screening and selection of α5-NAMs for future clinical trials.

Methods

Selection of five α5-NAMs

The five α5-NAMs investigated here (basmisanil; Ono-160; L-655,708; α5IA; and MRK-016; Supplementary Fig. 1), were selected because they have high binding affinities for α5GABA$_A$ receptors (Supplementary Table 1). In fact, three of the drugs had progressed to clinical trials namely 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 (www.clinicaltrials.gov; NCT02024789, NCT02928393, NCT02953639). Both α5IA and MRK-016 progressed to clinical trials, but these studies were halted in Phase 1 due to adverse side-effects.[33,34] Ono-160 was described in a most recent patent (WO 2015/115673 A1), and L-655,708 has been widely investigated in preclinical studies.[6,23,24,35]

Primary hippocampal neuronal culture

All experimental procedures were approved by the local Animal Care Committee at the University of Toronto. Timed-pregnant CD1 mice (Charles River, Montreal, Quebec, Canada) were used to prepare cultures of primary hippocampal neurons, as previously described.[36,37] Briefly, fetuses (embryonic days 16-18) were removed from a pregnant mouse that was euthanized by cervical dislocation. Hippocampi were dissected and cells were dissociated through mechanical titration. Cells were then plated at a density of approximately 1 x 10^6 cells per 35-mm culture dish coated with poly-D-lysine (Sigma-Aldrich, Oakville, Ontario, Canada). Cultures were maintained in neurobasal medium (Gibco, Burlington, Ontario, Canada) that was supplemented with B-27

(Gibco) and L-glutamate (Gibco) for 14 to 20 days before use. Cell cultures prepared under these conditions primarily contain neurons. At the time of the recordings, the neurons became polarized, had extensive axonal and dendritic arbors and formed numerous, functional synapses. Thus, the cultured cells resemble mature neurons *in vivo*.[38,39] Neurons that were adhered to the bottom of the culture dish with pyramid-shaped soma and clearly visible dendrites were selected for the recordings. Culture dishes were prepared from at least two different mice for each experiment, and a maximum of two cells was recorded from each dish.

Electrophysiology

Whole-cell voltage clamp recordings were performed in cultured hippocampal neurons using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, California, USA) that was controlled with the pClamp 8.0 software (Molecular Devices) via a Digidata 1322 interface (Molecular Devices). Neurons 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 to 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 to 330 mOsm). Drugs dissolved in extracellular solution were applied to the patched neuron by a computer-controlled, multibarrelled perfusion system (SF-77B, Warner Instruments, Hamden, Connecticut, 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 (2R)-amino-5-phosphonovaleric acid (APV, 20 μM) were added to the extracellular

solution. Tetrodotoxin (0.2 μM) was used to block voltage-dependent 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. mIPSCs 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}) of the drug. The concentrations for L-655,708, α5IA and MRK-016 were selected based on the results from studies of the binding affinity and efficacy of $\alpha5GABA_A$ 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, New Jersey, USA); and GABA from Sigma-Aldrich (Oakville, Ontario, Canada). Four of the five α5-NAMs: basmisanil, Ono-160, α5IA and MRK-016, were synthesized in-house at the Medicines Discovery Institute (Cardiff University, Cardiff, Wales). L-655,708 was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). 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 1% DMSO, stored at -20 ^{o}C. For the *in vitro* studies, the secondary stock was

further diluted in the extracellular solution to obtain the desired α5-NAM concentration in ≤ 0.1% DMSO.

Data analyses

Currents were analyzed 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 normalized to the amplitude of the bicuculline inhibitory response in that cell. The effects of each α5-NAM on the tonic current were reported as "percent inhibition," which was calculated as $(I_{\alpha5NAM}/I_{bicuculline}) \times 100\%$, where $I_{\alpha5NAM}$ is the current amplitude of the α5-NAM response and $I_{bicuculline}$ for that of bicuculline. The concentration-response curve of each α5-NAM was fitted with GraphPad Prism 6.01 (GraphPad, San Diego, CA) using nonlinear regression of log (agonist) *vs.* response (three parameters): Y = Bottom + (Emax-Bottom)/(1+10^((LogIC$_{50}$-X))), where Y is the "percent inhibition" and X is the concentration. The fit yielded values for the bottom (lowest percent inhibition), Emax (maximal percent inhibition) and IC$_{50}$; with SEM and 95% confidence intervals.

At least 30 seconds of mIPSC recordings under each experimental condition were analyzed with MiniAnalysis 6.0.3 (Synaptosoft Inc., Fort Lee, New Jersey, USA). Analyses were performed in recordings without bursting and compound events to accurately determine the parameters of mIPSCs. Each file was also 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 that were averaged to obtain the mean values presented in Table 3 ranged from 335 – 1170. All graphs in the report were created with GraphPad Prism 6.01 (Graphpad Software Inc., La Jolla, California, USA).

Statistical analyses

Data are presented as 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, the one-way ANOVA followed by the Tukey's multiple comparisons test was used. If normality was not met, the Kruskal-Wallis test followed by the Dunn's multiple comparisons test was used. A paired Student's *t* test was used to compare two groups, and when normality assumptions were not satisfied, the nonparametric Wilcoxon matched-pairs signed rank test was utilized. 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 the tonic current with similar efficacies but different potencies

We first investigated the effects of five α5-NAMs (basmisanil; Ono-160; L-655,708; α5IA and MRK-016) on the amplitude of the tonic current. The tonic current in neurons *in vivo* is primarily generated by extrasynaptic $GABA_A$ receptors that are activated by low, ambient concentrations of GABA.[7,8] To mimic such agonist conditions,[7,10] the neurons 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). Following the washout of bicuculline and the return of current to baseline, a α5-NAM was co-applied with GABA. The decrease in holding current caused by the α5-NAM was compared to the decrease caused by bicuculline. The results were described as "percent 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 nM to 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 following 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, the data were fitted with sigmoidal concentration-response curves (Fig. 2). The fittings generated the maximal inhibitory effect (E_{max}) and the half-maximal inhibitory concentration (IC_{50}). The E_{max} values did not differ between the

five α5-NAMs, as evidenced by the overlap in the 95% confidence intervals (Table 1). In contrast, the IC_{50} of basmisanil (126.8 nM, 95% confidence intervals of 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 consistently in the sub-nanomolar range (0.4 – 0.8 nM). The lower potency for basmisanil compared to the other four compounds was reflected by the rightward shift of the concentration-response curve, as shown in Fig. 2f.

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

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 next examined whether α5-NAMs modulated currents evoked by a saturating concentration of GABA, a condition that would activate both low-affinity and high-affinity $GABA_A$ receptors.

A saturating concentration of GABA (1 mM) was applied for 16 seconds to activate a peak current and a lower steady-state current (due to receptor desensitization, 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-desensitized 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 seconds 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, $n = 8$; also see Table 2). Similarly, the other four α5-NAMs had no effect

(Table 2). Together, the results show that 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 miniature inhibitory postsynaptic currents

mIPSCs are generated by postsynaptic $GABA_A$ receptors that are activated by GABA released from presynaptic terminals.[7] While α5$GABA_A$ receptors are predominantly located extrasynaptically, they are also expressed at lower levels in synaptic regions of neurons.[16,42] Thus, we investigated the effects of α5-NAMs on mIPSCs. Basmisanil had no effect on the amplitude or frequency of mIPSCs, as shown in 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 hippocampal neurons. The results showed that the maximal inhibitory effects of the five α5-NAMs were similar (Emax = 35.5% – 51.3%) whereas the potencies differed. Basmisanil (IC_{50} = 126.8 nM) was less potent than the other four compounds, all of which exhibited similar potencies (IC_{50} = 0.4 – 0.8 nM).

None of the α5-NAMs modified the amplitude of current evoked by a saturating concentration of GABA. While $\alpha5GABA_A$ 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 $\alpha5GABA_A$ receptors to the 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 $\alpha5GABA_A$ receptors do not contribute substantially to synaptic currents,[20,28] although they are expressed in synaptic regions on the dendrites of hippocampal neurons and can contribute to slowly decaying synaptic current.[7,16,17,19,43]

All five α5-NAMs inhibited the tonic current with similar efficacy. The Emax values (35.5% – 51.3%) are quite similar to those from recombinant α5β3γ2 receptors, but only for four of the five compounds (39% – 55%, Supplementary Table 1). The similarities between efficacies of basmisanil, Ono-160, α5IA and MRK-016 for inhibiting the tonic current in neurons and inhibiting recombinant $\alpha5GABA_A$ receptors are consistent with evidence that suggests the tonic current in neurons is primarily generated by $\alpha5GABA_A$ receptors.[4,6,20,37]

Interestingly, L-655,708 caused a greater inhibition of the tonic current in neurons (35.5%) than recombinant α5β3γ2 $GABA_A$ receptors expressed in mouse L (tk-) cells (17%, Supplementary

Table 1). Two factors, which are related to the experimental condition, could contribute to this discrepancy. GABA, applied at a low concentration as used in our study (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 due to different conformational changes that occur in the mono-liganded state.[25] Also, primary neurons and heterologous systems may differ in their intracellular environment and cell signaling 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 signaling 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 ranged from 0.4 to 0.8 nM, which are similar to those reported with recombinant α5β3γ2 $GABA_A$ receptors (0.66 – 1.4 nM, Supplementary Table 1). Surprisingly, basmisanil (IC_{50} = 126.8 nM) was clearly less potent than the other compounds. The potency of basmisnal also differed by more than an order of magnitude from the binding affinity for recombinant α5β3γ2 receptors (Ki = 4.7 nM, Supplementary Table 1). 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 neurons yet high binding affinity for α5β3γ2 receptors in recombinant systems.

The unexpected lower potency of basmisanil for inhibiting the tonic current is of considerable interests 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 (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.

Overall, we have identified two unexpected findings from the study: the greater efficacy of L-655,708 and the lower potency of basmisanil for inhibiting the tonic current in hippocampal neurons. These results suggest that α5-NAM development programs would benefit from *in vitro* studies of native $GABA_A$ receptors. Screening α5-NAM effect on tonic current in cultured hippocampal neurons, 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 labor-intensive clinical trials.

Despite the challenges faced in recent clinical trials, the development of α5-NAMs has considerable potential and should continue, given the extent to which hyperactivity of $\alpha 5GABA_A$ receptors is implicated in many devastating cognitive and psychiatric disorders.

Author 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

Declaration of interests

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

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

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. $\alpha5GABA_A$ 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. $\alpha5GABA_A$ 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 $GABA_ARs$ 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-1H-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**: 397-42

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

Table 1. Maximal percent inhibition (Emax) and IC_{50} values were obtained from the fitted curves, shown in Fig. 2. Data 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. The asterisks indicate a significant difference between the IC_{50} value for basmisanil and those for the other compounds.

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

Table 2. α5-NAMs do not modify the amplitude of the peak or steady-state current 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

	Peak (pA)	Steady-state (pA)
Control (n = 8)	5264 ± 950	644 ± 134
Basmisanil (1 μM)	5321 ± 943	693 ± 141
Control (n = 7)	4563 ± 1099	646 ± 226
Ono-160 (10 nM)	4252 ± 1091	640 ± 212
Control (n = 7)	4998 ± 1005	752 ± 186
L-655,708 (200 nM)	4945 ± 1025	746 ± 184
Control (n = 6)	4271 ± 764	567 ±140
α5IA (100 nM)	4286 ± 849	549 ± 136
Control (n = 5)	3514 ± 894	349 ± 98
MRK-016 (100 nM)	3390 ± 799	375 ± 84

Table 3. α5-NAMs do not modify miniature inhibitory postsynaptic currents. For all the 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 the Wilcoxon matched-pairs signed rank test was used. Data are presented as mean ± SEM

	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

Figure Legends

Fig 1. Tonic current is inhibited by α5-NAMs. (a) Representative traces (left) showing the effects of basmisanil on the tonic current (10 nM and 1 μM) in comparison to the effect of bicuculline (20 μM). A single α5-NAM concentration was tested on each cell. Summarized data for basmisanil (right) illustrate a concentration-dependent effect. n = 7, 7, 7, 7, 8, 5 (*left to right*). One-way ANOVA, $F_{(5,35)} = 11.1$, $P < 0.0001$. (b-e) Summarized data for the remaining four α5-NAMs, which also show concentration-dependent effects. One-way ANOVA for all except MRK-016, where the Kruskal-Wallis test was used. (b) Ono-160, n = 7, 7, 8, 8, 9 (*left to right*). $F_{(4, 34)} = 7.1$, $P = 0.0003$. (c) L-655,708, n = 6, 8, 8, 8, 8, 6 (*left to right*). $F_{(5, 38)} = 5.3$, $P = 0.0009$. (d) α5IA, n = 7, 7, 9, 9, 9, 7, $F_{(5, 42)} = 9.9$, $P < 0.001$. (e) MRK-016, n = 6, 8, 10, 8, 10, 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 the Dunn's multiple comparisons test was used.

Fig 2. The concentration-response curve of each α5-NAM is fitted from the same data 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.

Fig 3. Basmisanil does not modulate the peak and steady-state current evoked by a saturating concentration of GABA, nor affects mIPSCs. (a, left panel) Representative traces showing the effect of basmisanil on current evoked by GABA (1 mM). (a, right panel) Summarized data for the peak and steady-state current. n = 8; P = 0.8 and P = 0.1 for the peak and steady-state current,

respectively; Student's paired *t* test. Data are mean ± SEM. (b, top panel) Representative traces of mIPSCs, in the absence and presence of basmisanil. (b, 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.