A Substituted Anilino Enaminone Acts as a Novel Positive Allosteric Modulator of GABA<sub>A</sub> Receptors in the Mouse Brain

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Running head: Anilino enaminone activity through GABAR

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Discussion: 1460

Abbreviations: GABA, γ-Aminobutyric acid; scPTZ, subcutaneous pentylenetetrazol; MES, maximal electroshock seizure; HT, serotonin receptors; ACSF, artificial cerebrospinal fluid; mGluR1, metabotropic glutamate receptor 1; GluR, glutamate receptor; NMDA, N-methyl-D-aspartate; MOB, main olfactory bulb; MC, mitral cells; KRS-5Me-4OCF3, 5-Methyl-3-(4-trifluoromethoxy-phenylamino)-cyclohex-2-enone; KRS-5Me-4F, 3-(4-Fluoro-phenylamino)-5-methyl-cyclohex-2-enone; KRS-5Me-3Cl, 3-(3-Chloro-phenylamino)-5-methyl-cyclohex-2-enone; DMSO, dimethyl sulfoxide; D-AP5, L-2-amino-5-phosphonopentanoic acid (APV); CNQX, 6-cyano-7-nitroquinoxaline-2-3-dione; gabazine, 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (SR-95531); LY367385, (S)-(+)‐α-amino-4-carboxy-2-methylbenzeneacetic acid; CGP55845, (2S)-3'(((1S0-1-(3,4-
Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid; (R)-baclofen, (R)-4-amino-3-(4-chlorophenyl) butanoic acid; NNC 711, [1,2,5,6-Tetrahydro-1-[2-[[((diphenylmethylene)amino]oxy]ethyl]-3-pyridinecarboxylic acid hydrochloride; Vigabatrin, (±)-γ-Vinyl GABA; Flumazenil, 8-Fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid, ethyl ester; (S)-SNAP 5114, 1-[2-[(tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3piperidinecarboxylic acid.

Section: Neuropharmacology
Abstract

A small library of anilino enaminones was analyzed as potential anticonvulsant agents. We examined the effects of three anilino enaminones on neuronal activity of output neurons, mitral cells (MC), in an olfactory bulb brain slice preparation using whole-cell patch-clamp recording. These compounds are known to be effective in attenuating pentylenetetrazol (PTZ) induced convulsions. Among the three compounds tested, KRS-5Me-4-OCF₃ showed potent inhibition of MC activity with an EC₅₀ of 24.5 μM. It hyperpolarized the membrane potential of MCs accompanied by suppression of spontaneous firing. Neither ionotropic glutamate receptor blockers nor a GABA_B receptor blocker prevented the KRS-5Me-4-OCF₃-evoked inhibitory effects. In the presence of GABA_A receptor antagonists, KRS-5Me-4-OCF₃ completely failed to evoke inhibition of MC spiking activity, suggesting that KRS-5Me-4-OCF₃-induced inhibition may be mediated by direct action on GABA_A receptors or by indirect action through the elevation of tissue GABA levels. Neither vigabatrin (a selective GABA-T inhibitor) nor NNC-711 (a selective inhibitor of GABA uptake by GAT-1) eliminated the effect of KRS-5Me-4-OCF₃ on neuronal excitability, indicating that the inhibitory effect of the enaminone resulted from direct activation of GABA_A receptors. The concentration-response curves for GABA are left-shifted by KRS-5Me-4-OCF₃ demonstrating that KRS-5Me-4-OCF₃ enhanced GABA affinity and acted as a positive allosteric modulator of GABA_A receptors. The effect of KRS-5Me-4-OCF₃ was blocked by applying a benzodiazepine site antagonist suggesting that KRS-5Me-4-OCF₃ binds at the classical benzodiazepine site to exert its pharmacological action. The results suggest clinical use
of enaminones as anticonvulsants in seizures, and as a potential anxiolytic in mental disorders.
INTRODUCTION

A diverse series of anilino enaminones was recently synthesized and investigated as potential anticonvulsant compounds. Structurally, these compounds are uniquely different from currently available antiepileptic drugs (Edafiogho et al., 1992, 2007, 2009; Kombian et al., 2005; Abdel-Hamid et al., 2002; Foster et al., 1999). This class of enaminones has shown good to moderate protection in the traditional preclinical animal models, the subcutaneous pentylenetetrazol (scPTZ) test and the maximal electroshock seizure (MES) test. Their anticonvulsant activities are comparable to that of some clinically used agents in animal models of seizures with a minimal side effect profile as well as a wider margin of safety than conventional antiepileptic drugs such as carbamazepine, valproate and phenytoin (Eddington et al., 2000; Mulzac and Scott, 1993). The unique pharmacophoric structure of the anilino enaminones and the variety of bioactivities provide an excellent opportunity to develop new drugs.

We previously reported the anticonvulsant activity of anilino enaminones in vivo and the possible mechanisms of action of these compounds by which they elicit their response (Edafiogho et al., 1992; Mulzac and Scott, 1993; Ananthalakshmi et al., 2007). The anilino enaminone E139, inhibited EPSCs in the rat nucleus accumbens and hippocampus by enhancing extracellular GABA levels (Kombian et al., 2005; Ananthalakshmi et al., 2007), and by inhibiting tetrodotoxin-sensitive sodium currents to modulate excessive firing in individual neurons (Ananthalakshmi et al., 2006). A recent study aimed at elucidating the essential structural parameters necessary for anticonvulsant activity found that some benzylamino enaminones, which possess a similar chemical structure to anilino enaminones with benzyl-substitution at the NH-moiety, produced
anticonvulsant effects in rats and mice neurons by suppressing glutamate-mediated excitation and action potential firing (Edafiogho et al., 2006). The different substitutions at the NH-moiety change the target protein to which enaminones bind. These studies indicate that enaminones with similar chemical structure may possess different modes of action. Here, we hypothesize that the substituted site in enaminones may contribute to the mode of action of these compounds. To study the structure-activity relationships of enaminones, three enaminone compounds with non-ortho-substituted cyclohexenone were synthesized and used to determine the mechanism of their anticonvulsant action.

Epileptic seizures result from poorly controlled neuronal activity at a seizure focus and the subsequent spread of electrical excitation in brain circuits (Rall and Schleifer, 1990). It is not surprising that most effective anti-seizure medications have been demonstrated to inhibit neuronal excitability through modulating the function of several types of proteins such as sodium channels, NMDA receptors and GABA receptors (Rho and Sankar, 1999). The excitability of neurons in the brain is an integral of intrinsic membrane conductances and synaptic inputs. Both excitatory and inhibitory inputs regulate the resting excitability (Traynelis and Dingledine, 1988). Output neurons such as mitral cells (MCs) in the mouse main olfactory bulb (MOB) display their neuronal activity as spontaneous action potential firing, which can be modulated by intrinsic membrane receptors as well as synaptic inputs (Shepherd et al., 2004; Ennis et al., 2007). In the rodent MOB, MCs express high levels of different receptors such as GABA receptors (GABA_A, GABA_B), ionotropic and metabotropic glutamate receptors (NMDA, AMPA, mGluR1, kainate), and serotonin receptors (5-HT_{1A}, 5-HT_{2A/C}). Most of these receptor proteins are thought to be strongly epilepsy-related (Ennis et al., 2007;
McNamara JO, 1996; Snell et al., 2000; Wang et al., 2002). The functional modulation of these proteins may change synaptic input and neuronal excitability. Thus, in this study, we used acute slices of the mouse MOB and electrophysiological recordings from MCs to determine the effects of enaminones on the activity of MCs and the mechanisms underlying the inhibitory or excitatory actions of these compounds. Our results show that enaminone compounds with non-ortho-substituted cyclohexenone suppress neuronal excitability through activation of GABA\(_A\) receptors and display the characteristics of a positive allosteric modulator.
Methods

Synthesis of Anilino Enaminones. Anilino enaminones KRS-5Me-4-OCF₃, KRS-5Me-4-F and KRS-5Me-3-Cl were recently synthesized. Their chemical structures are shown in Fig. 2. The mono methyl anilino enaminones (3) were prepared from the 5-methylcyclohexane-1,3-dione (2) form by the decarboxylation of 4-carbo-tert-butoxy-5-methylcyclohexane-1,3-dione (1), and were refluxed with appropriate substituted aniline derivatives under standard conditions (Fig. 1) (Eddington et al., 2003). The β-hydroxy keto tert-butoxy ester was prepared as previously reported (Friary et al., 1973; Edafiogho et al., 1992; Scott et al., 1993). The enaminone structures were confirmed via NMR analyses at 400 MHz.

Slice Preparation. Wild type mice (C57BL/6J, Jackson Laboratory, Bar Harbor, ME) were used in agreement with Institutional Animal Care and Use Committee and NIH guidelines. Juvenile (16–25 day old) mice were decapitated, and the MOBs were dissected out and immersed in artificial cerebrospinal fluid (ACSF, see below) at 4°C, as previously described (Heinbockel et al. 2004). Horizontal slices (400 μm-thick) were cut parallel to the long axis using a vibratome (Vibratome Series 1000, Ted Pella Inc., Redding, CA). After 30 min at 30°C, slices were incubated in a holding bath at room temperature (22°C) until use. For recording, a brain slice was placed in a recording chamber mounted on a microscope stage and maintained at 30 ± 0.5°C by superfusion with oxygenated ACSF flowing at 2.5–3 ml/min.

Electrophysiological Recording and Data Acquisition. Visually-guided recordings were obtained from cells in the mitral cell layer with near-infrared differential interference contrast optics and a BX51WI microscope (Olympus Optical, Tokyo, Japan)
equipped with a camera (C2400-07, Hamamatsu Photonics, Japan). Images were
displayed on a Sony Trinitron Color Video monitor (PVM-1353MD, Sony Corp. Japan).
Recording pipettes (5-8 MΩ) were pulled on a Flaming-Brown P-97 puller (Sutter
Instrument Co., Novato, CA) from 1.5 mm O.D. borosilicate glass with filament. Seal
resistance was routinely >1GΩ and liquid junction potential was 9-10 mV; reported
measurements were not corrected for this potential. Data were obtained using a
Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Signals were low-pass
Bessel filtered at 2 kHz and digitized on computer disc (Clampex 10.1, Molecular
Devices). Data were also collected through a Digidata 1440A Interface (Molecular
Devices) and digitized at 10 kHz. Holding currents were generated under control of the
Multiclamp 700B Commander.

The ACSF consisted of (in mM): NaCl 124, KCl 3, CaCl2 2, MgSO4 1.3, glucose
10, sucrose NaHCO3 26, NaH2PO4 1.25 (pH 7.4, 300 mOsm), saturated with 95 O2/5%
CO2 (modified from Heyward et al. 2001). The standard pipette-filling solution consisted
of (mM) K gluconate 125, MgCl2 2, HEPES 10, Mg2ATP 2, Na3GTP 0.2, NaCl 1, EGTA
0.2.

**Chemicals and Solutions.** Drugs were bath perfused at the final concentration as
indicated by dissolving aliquots of stock in ACSF. The three enaminone compounds that
we tested were recently synthesized: (a) KRS-5Me-4-OCF3; (b) KRS-5Me-4F; (c) KRS-
5Me-3Cl. All enaminones were dissolved in DMSO to make 20 mM stock solution (final
concentration of DMSO in bath <0.1%). For all the experiments, the drugs were applied
by bath perfusion. Control recordings showed that 0.1% DMSO had no detectable effects
on the firing rate and membrane potential. The following drugs were also bath applied:
D-AP5, CNQX, gabazine, LY367385, CGP55845, (R)-baclofen, NNC 711, vigabatrin, flumazenil and (S)-SNAP 5114. Chemicals and drugs were supplied by Tocris (Ellisville, MO) and Sigma-Aldrich (St. Louis, MO).

Numerical data are expressed as the mean ± SEM. Tests for statistical significance were performed using paired Student's *t*-tests, and one-way ANOVA followed by the Bonferroni test for multiple comparisons.
RESULTS

Recordings were obtained from 166 MCs with whole-cell recordings in mouse MOB slices from 94 animals. All recorded cells showed measurable responses to enaminone KRS-5Me-4-OCF$_3$. MCs were identified visually by their soma location and relatively large soma size, and by their input resistance ($297 \pm 19.2$ M$\Omega$, $n = 46$). The membrane potential of MCs in this study was $-52.2 \pm 0.7$ mV ($n = 46$).

1. Enaminones KRS-5Me-3Cl and KRS-5Me-4F slightly depressed activity of mitral cells

MCs are principal neurons and play a crucial role in processing sensory information in MOB. They receive direct synaptic inputs from the axons of olfactory receptor neurons, send excitatory projections to olfactory cortical areas, and receive strong feedback inhibition primarily through reciprocal dendrodendritic synapses with local interneurons (Shepherd et al., 2004; Ennis et al., 2007). MCs generate spontaneous action potentials (1–6 Hz) in slices. Here, we made use of the intrinsic properties of MCs such as spontaneous firing, membrane potential and membrane conductance to test the effect of enaminones on MC activity and to determine the possible binding target of enaminones.

Bath application of either KRS-5Me-3Cl or KRS-5Me-4F modulated the spike rate of MCs (Fig. 3). Compared to control conditions, 20 $\mu$M KRS-5Me-3Cl slightly reduced the MC firing rate (in control: $3.1 \pm 0.5$ Hz; in drug: $2.6 \pm 0.5$ Hz; $n = 5$; $p < 0.05$, paired $t$ test). The other compound, KRS-5Me-4F (20 $\mu$M), decreased MC firing
from 4.8 ± 0.8 Hz to 4.0 ± 0.7 Hz (n = 5; p < 0.05, paired t test) and slightly
hyperpolarized the membrane potential by -0.4 ± 0.1 mV (n = 5; p < 0.05, paired t test).

2. KRS-5Me-4-OCF₃ inhibited spontaneous spiking of mitral cells and hyperpolarized the membrane potential

For the concentration tested, KRS-5Me-4-OCF₃ showed a difference in potency of inhibition of MC activity compared to the above two compounds (Fig. 3A). KRS-5Me-4-OCF₃ (20 μM) reversibly decreased MC firing from 4.4 ± 0.4 Hz to 2.9 ± 0.3 Hz (n = 50; p < 0.001, paired t test). The reduction of the firing rate was accompanied by hyperpolarization of the MC membrane potential by -0.9 ± 0.2 mV (n = 50; p < 0.001, paired t test). Fig. 3B illustrates the inhibitory effect in an original recording from a MC.

Even though, we did not perform a detailed comparison, the findings indicated that, at 20 μM, KRS-5Me-4-OCF₃ was the most potent compound compared to the other two enaminones, KRS-5Me-3Cl and KRS-5Me-4F, in terms of depressing spiking activity of MCs. Therefore, enaminone KRS-5Me-4-OCF₃ was selected for the remainder of the study to characterize the cellular actions of an enaminone and the mechanism underlying its inhibitory effect on neuronal activity.

3. Ionotropic glutamate receptors were not involved in the KRS-5Me-4-OCF₃-induced inhibition of neuronal activity

Ionotropic glutamate receptors play a critical role in the regulation of neuronal excitability in the MOB (Ennis et al., 2007). Blockade of ionotropic glutamate receptors may result in neuronal inhibition. To determine if the inhibitory effect of the
anticonvulsant agent KRS-5Me-4-OCF₃ was mediated through interaction with ionotropic glutamate receptors, we examined the effects of neuronal inhibition evoked by KRS-5Me-4-OCF₃ in the presence of AMPA/kainate and NMDA receptor inhibitors. In the presence of CNQX (10 µM), a potent AMPA/kainate receptor antagonist, the inhibitory effects of KRS-5Me-4-OCF₃ persisted as seen by a reduction of the firing rate (in CNQX: 3.2 ± 0.56 Hz; in CNQX plus KRS-5Me-4-OCF₃: 2.3 ± 0.50 Hz) (n = 4; p < 0.05, paired t test), and hyperpolarization of MCs by -0.8 ± 0.2 mV (n = 4; p < 0.05, paired t test) (Fig. 4). In comparison with the results shown in Fig. 3A, these values indicated that CNQX had no any additional effect on KRS-5Me-4-OCF₃-induced suppression of MC activity (p > 0.05; ANOVA and Bonferroni post-hoc analysis).

D-AP5 is a potent NMDA receptor antagonist. Blockade of both AMPA/kainate and NMDA receptors antagonizes the excitatory activities of ionotropic glutamate receptors. In the presence of both CNQX and D-AP5, KRS-5Me-4-OCF₃ reduced the firing rate (in CNQX+D-AP5: 3.4 ± 0.4 Hz; in CNQX+D-AP5 plus KRS-5Me-4-OCF₃: 2.2 ± 0.40 Hz) (n = 4; p < 0.05, paired t test) and hyperpolarized MCs by -0.9 ± 0.2 mV (n = 4; p < 0.05, paired t test) (Fig. 4). These values were not significantly different from the values of KRS-5Me-4-OCF₃-induced suppression recorded in ACSF control condition (see Fig. 3A) (p > 0.05 determined by ANOVA and Bonferroni post-hoc analysis). These results showed that ionotropic glutamate receptors were not involved in the KRS-5Me-4-OCF₃-induced MC inhibition.

4. The KRS-induced inhibition of MC excitability was not influenced by GABA_B receptors
GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs) are mostly restricted to the glomerular layer in the MOB (Bowery et al., 1987; Chu et al., 1990). GABA<sub>B</sub>Rs are metabotropic trans-membrane receptors for gamma-aminobutyric acid (GABA) and linked via G-proteins to potassium channels (Chen et al., 2005). Activation of GABA<sub>B</sub>Rs can stimulate the opening of K<sup+</sup> channels that will hyperpolarize the neuron, quiet down excitable cells, and hence stop neurotransmitter release. In the MOB, activation of GABA<sub>B</sub>Rs has been observed to reduce MC excitability (Palouzier-Paulignan et al., 2002; Isaacson and Vitten, 2003).

In the presence of GABA<sub>B</sub>R antagonist CGP55845 (10 μM), the KRS-5Me-4-OCF<sub>3</sub>-evoked modulation of MC firing persisted (in CGP55845: 3.7 ± 0.6 Hz; in CGP55845 plus KRS-5Me-4-OCF<sub>3</sub>: 2.8 ± 0.5 Hz) (n = 5; p < 0.05, paired t test), and MCs were hyperpolarized -1.0 ± 0.2 mV (n = 5; p < 0.05, paired t test). Fig. 5A shows a representative recording from one MC. No significant difference was observed for KRS-5Me-4-OCF<sub>3</sub>-evoked inhibition in control condition (see Fig. 3A) and in the presence of CGP55845 (p > 0.05 determined by ANOVA and Bonferroni post-hoc analysis).

Compared to control conditions, the GABA<sub>B</sub>R agonist (R)-baclofen (50 μM) evoked a strong decrease in firing rate of MCs (in control: 2.6 ± 0.4 Hz; in baclofen: 1.3 ± 0.3 Hz) (n = 4; p < 0.01, paired t test) and hyperpolarization of MCs by -1.6 ± 0.7 mV (n = 4; p < 0.05, paired t test). In the presence of GABA<sub>B</sub>R agonist baclofen, KRS-5Me-4-OCF<sub>3</sub> further reduced the MC firing rate to 0.7 ± 0.1 Hz (n = 4; p < 0.05, paired t test), and hyperpolarized MCs by -0.9 ± 0.3 mV (n = 4; p < 0.05, paired t test). In comparison with the inhibitory effects of KRS-5Me-4-OCF<sub>3</sub> in control conditions (% of control firing, see Fig. 3A), the effects of KRS-5Me-4-OCF<sub>3</sub> recorded in the presence of (R)-
baclofen (% of values recorded in (R)-baclofen) did not significantly change ($p > 0.05$ determined by ANOVA and Bonferroni post-hoc analysis). These results indicated that the inhibitory effect of KRS-5Me-4-OCF$_3$ persisted irrespective of GABA$_B$R activation or blockade, suggesting that neither a GABA$_B$R antagonist nor an agonist influenced the enaminone-induced MC inhibition.

5. Blockade of GABA$_A$ receptor reversed KRS-induced inhibition of MC excitability

GABA$_A$Rs play an important role in regulating MC excitability (Panzanelli et al., 2005; Laurie et al., 1992) by suppressing neuronal activity. Bath application of GABA (50 µM) dramatically decreased the firing rate of MCs (in control: 5.0 ± 0.8 Hz; in GABA: 1.7 ± 0.3 Hz) ($n = 6$; $p < 0.001$, paired $t$ test) and hyperpolarized MCs by -1.1 ± 0.3 mV ($n = 6$; $p < 0.05$, paired $t$ test), indicating that GABA induced a large direct inhibition of MC activity via GABARs (Fig. 6B). The potent inhibition by GABA is consistent with previous reports showing that GABA receptors are abundant in MCs (Persohn et al., 1992; Laurie et al., 1992). In the presence of GABA, KRS-5Me-4-OCF$_3$ reduced the MC firing rate to 0.7 ± 0.2 Hz ($n = 4$; $p < 0.01$, paired $t$ test). The enhancement of the KRS-5Me-4-OCF$_3$-evoked inhibition in the presence of increased extracellular GABA levels (50 µM) suggested that KRS-5Me-4-OCF$_3$-evoked inhibition may be mediated by direct activation of GABA receptors rather than by increased GABA levels. Potentially, increased endogenous GABA levels could result from increasing GABA release or from reducing GABA re-absorption and GABA degradation in the slice.
Blockade of GABA\textsubscript{A} receptors can result in over-excitation of neurons. In the presence of the GABA\textsubscript{A} receptor antagonist picrotoxin, the inhibition of MC activity by KRS-5Me-4-OCF\textsubscript{3} was completely blocked (in picrotoxin: 6.1 ± 1.0 Hz; in picrotoxin plus KRS-5Me-4-OCF\textsubscript{3}: 6.2 ± 1.0 Hz) \((n = 9, p > 0.05, \text{paired } t \text{ test})\) (Fig. 6A, B). Gabazine is another selective GABA\textsubscript{A} receptor antagonist. Similarly, in the presence of gabazine, the KRS-5Me-4-OCF\textsubscript{3}-induced inhibition of MC activity was completely abolished (in gabazine: 5.4 ± 0.7 Hz; in gabazine plus KRS-5Me-4-OCF\textsubscript{3}: 5.7 ± 0.7 Hz) \((n = 6, p > 0.05, \text{paired } t \text{ test})\) (Fig. 6B). These results indicated that blockade of GABA\textsubscript{A} receptors prevented KRS-5Me-4-OCF\textsubscript{3}-evoked inhibition of MC activity and suggested that KRS-5Me-4-OCF\textsubscript{3} acted through enhanced GABA levels or direct action on GABA\textsubscript{A} receptors.

Further evidence for the involvement of GABA\textsubscript{A} receptors came from measurements of MC ionic currents induced by KRS-5Me-4-OCF\textsubscript{3} with or without blockade of GABA\textsubscript{A} receptors using the antagonist gabazine. In voltage-clamp mode at a holding potential of -60 mV, KRS-5Me-4-OCF\textsubscript{3} produced an outward current in MCs of 14.7 ± 3.5 pA \((n = 5; \text{the steady state current at -60 mV in KRS-5Me-4-OCF}3 \text{ was measured and subtracted from that in ACSF})\). In the presence of gabazine, KRS-5Me-4-OCF\textsubscript{3}-induced outward currents were blocked (-0.2 ± 1.6 pA, \(n = 4; \text{the current at -60 mV in KRS-5Me-4-OCF}3 \text{ plus gabazine was subtracted from that in gabazine})\).

6. Enhancement of extracellular GABA levels did not block the KRS-5ME-4-OCF\textsubscript{3}-induced inhibition of neuronal excitability
The above results (Fig. 6) showed that inhibition of MC firing evoked by KRS-5Me-4-OCF₃ could be blocked by GABAₐ receptor antagonists. This result suggested that the enaminone either binds to GABAₐ receptors to produce the inhibitory effects or acts to enhance extracellular GABA levels. Extracellular GABA levels are in part controlled by GABA re-uptake and degradation (Errante et al., 2002). GABA released from synaptic terminals may be removed from the extracellular space by GABA uptake back into synaptic terminals and/or into glial cells by plasma membrane transporters (GATs). Subsequently, the captured GABA is degraded by the enzyme GABA transaminase (GABA-T). To test the possible involvement of endogenous GABA in the KRS-5Me-4-OCF₃-evoked neuronal inhibition, we examined the role of the enzyme GABA-T and membrane transporters, GATs, in enaminone-mediated inhibition of MC activity.

Bath application of vigabatrin (200 µM), an irreversible and selective GABA-T inhibitor that results in extracellular accumulation of GABA in the synaptic cleft, induced a reduction in MC firing rate (in control: 4.3 ± 0.5 Hz; in vigabatrin: 3.4 ± 0.4 Hz) \( n = 4, p < 0.05 \), paired \( t \) test. In the presence of vigabatrin, bath application of KRS-5Me-4-OCF₃ (20 µM) resulted in further reduction of the firing rate (in vigabatrin: 3.5 ± 0.5 Hz; in vigabatrin plus KRS: 2.0 ± 0.2 Hz) \( [n = 7, p < 0.001, \text{paired } t \text{ test}] \) accompanied by hyperpolarization of the membrane potential \( (\Delta V_m = -0.8 ± 0.3 \text{ mV}, n = 7, p < 0.05, \text{paired } t \text{ test}) \) (Fig. 7A, C). The persistence of the KRS-5Me-4-OCF₃ effect in the presence of vigabatrin indicated that enaminone-evoked neuronal inhibition was not mediated by GABA-T.
To determine if enaminones interacted with GATs to regulate GABA re-uptake and if GATs modulate KRS-5Me-4-OCF₃-evoked neuronal inhibition, we applied NNC-711, a potent and selective inhibitor of GABA uptake by GAT-1, and (S)-SNAP 5114, a selective inhibitor by GAT-3 and GAT-2. Bath application of NNC-711 (10 µM) reduced MC spiking (in control: 4.4 ± 0.5 Hz; in NNC-711: 3.8 ± 0.3 Hz) \( (n = 5, p < 0.05, \text{paired } t \text{ test}) \) but did not significantly modulate the membrane potential (\( \Delta V_m = -0.38 \pm 0.09 \text{ mV}, n = 5; p > 0.05 \)). In the presence of NNC-711, KRS-5Me-4-OCF₃ further reduced the firing rate (in NNC-711: 3.9 ± 0.3 Hz; in NNC-711 plus KRS: 2.3 ± 0.2 Hz) \( (n = 22, p < 0.001, \text{paired } t \text{ test}) \) and hyperpolarized MCs by -0.8 ± 0.1 mV \( (n = 22, p < 0.01) \). Similarly, bath application of (S)-SNAP 5114 (20 µM) reduced MC spiking (in control: 3.5 ± 0.6 Hz; in SNAP: 2.7 ± 0.4 Hz) \( (n = 4, p < 0.05, \text{paired } t \text{ test}) \), hyperpolarized membrane potential (\( \Delta V_m = -0.6 \pm 0.1 \text{ mV}, n = 4; p < 0.05 \)). In the presence of (S)-SNAP 5114, KRS-5Me-4-OCF₃ further reduced the firing rate (in SNAP: 3.0 ± 0.5 Hz; in SNAP 5114 plus KRS: 1.8 ± 0.3 Hz) \( (n = 7, p < 0.001, \text{paired } t \text{ test}) \) and hyperpolarized MCs by -0.9 ± 0.1 mV \( (n = 7, p < 0.05) \). The results indicate that the inhibitory effects of KRS-5Me-4-OCF₃ persisted in the presence of GABA reuptake inhibitors (Fig. 7B, C).

The effects of KRS-5Me-4-OCF₃ on MC activity were also tested in the presence of both GABA reuptake inhibitor NNC-711 (10 µM) and GABA-T inhibitor vigabatrin (100 µM). Under this condition, application of 20 µM KRS-5Me-4-OCF₃ produced firing inhibition (in NNC711+vigabatrin: 3.8 ± 0.5 Hz; in NNC-711+vigabatrin plus KRS: 2.1 ± 0.4 Hz) \( (n = 9, p < 0.001, \text{paired } t \text{ test}) \) and hyperpolarization by -0.7 ± 0.2 mV \( (n = 9, p < 0.05, \text{paired } t \text{ test}) \) (Fig. 7C). These results indicated that neither GABA
reuptake transporters, GATs, nor GABA transaminase (GABA-T) blocked KRS-5Me-4-OCF₃-evoked neuronal inhibition, suggesting that extracellular enhancement of GABA levels did not contribute to the pharmacological effects of KRS-5Me-4-OCF₃.

7. **Substituted anilino enaminone exhibited characteristics of a positive allosteric modulator of GABA<sub>A</sub> receptor**

We showed that KRS-5Me-4-OCF₃ significantly enhanced the inhibitory effect of GABA on MC activity (Fig. 6B), i.e., the enaminone inhibited the firing rate to 2.9 ± 0.3 Hz in ACSF (Fig. 3) and it suppressed the firing rate to 0.7 ± 0.2 Hz in the presence of 50 µM GABA (Fig. 6B) (p < 0.05 determined by ANOVA and Bonferroni post-hoc analysis). The enhancement of the inhibitory effect of bath-applied GABA suggested that KRS-5Me-4-OCF₃ acted as a positive allosteric modulator of GABA<sub>A</sub> receptors.

It has been established that the GABA<sub>A</sub> receptor is the main target for positive allosteric modulators such as benzodiazepines (Munro et al., 2008; Fisher, 2009; Möhler et al., 2002). By binding at a site distinct from the GABA binding site and by increasing GABA affinity for the GABA<sub>A</sub> receptor, positive allosteric modulators facilitate an augmentation of GABA<sub>A</sub> receptor function. To examine the concentration-dependence of the inhibitory effect of KRS-5Me-4-OCF₃ on neuronal activity and to test if KRS-5Me-4-OCF₃ behaved like a positive allosteric modulator, the concentration-response relationships of KRS-5Me-4-OCF₃ and of GABA in the absence and presence of KRS-5Me-4-OCF₃ (0 µM, 5 µM, 20 µM) were measured (Fig. 8). The averaged inhibitory effects evoked by varying concentrations of the enaminone (Fig. 8A) and of GABA (Fig. 8B) were well fit by the Hill equation and allowed to estimate an EC<sub>50</sub>. Based on a fitted
Hill coefficient \((n)\) value of 1.11 in Fig. 8A, it appeared that the stoichiometry of drug
and receptor interaction was 1:1. The inhibitory effect of KRS-5Me-4-OCF₃ on neuronal
activity was concentration-dependent with the estimated value of 24.5 \(\mu\)M of EC₅₀. Fig.
8B showed that GABA evoked concentration-dependent inhibition, and the
concentration-response curves were left shifted by KRS-5Me-4-OCF₃. The shift of the
concentration-response relationships suggested that KRS-5Me-4-OCF₃ mostly likely
bound at non-GABA binding sites on the GABA receptor. The EC₅₀ of GABA fitted by
the Hill equation was 28.8 \(\mu\)M for GABA only, 19.9 \(\mu\)M for GABA plus 5 \(\mu\)M KRS-
5Me-4-OCF₃, and 10.5 \(\mu\)M for GABA plus 20 \(\mu\)M KRS-5Me-4-OCF₃. The affinity of
GABA for GABA binding sites was enhanced by KRS-5Me-4-OCF₃ which suggested an
action of KRS-5Me-4-OCF₃ as a positive allosteric modulator of the GABAₐ receptor.
The property provides a cellular mechanism that accounts for the anticonvulsant effects
of KRS-5Me-4-OCF₃ in vivo.

8. The enhancement of GABA by KRS-5Me-4-OCF₃ is through binding at the classical
benzodiazepine site

Previous in vivo studies reported that enaminones show potent anti-convulsion
effects in chemical-induced epilepsy animal models but fewer side-effects such as
sedation, drowsiness and dizziness (Mulzac and Scott, 1993; Eddington et al., 2003).
Based on these in vivo results and our in vitro results, we hypothesized that KRS-5Me-4-
OCF₃ might bind to benzodiazepine sites to exert its pharmaceutical actions. Therefore,
an antagonist of the benzodiazepine site was used to ascertain whether the enhancement of GABA by KRS-5Me-4-OCF₃ was mediated at the classical benzodiazepine site.

Bath application of flumazenil (10 µM), a benzodiazepine site antagonist, slightly increased MC spiking from $3.3 \pm 0.6$ Hz (in ACSF) to $3.8 \pm 0.8$ Hz ($n = 6$, $p < 0.05$, paired t test), but did not significantly modulate the membrane potential ($\Delta V_m = -0.02 \pm 0.08$ mV, $n = 6$; $p > 0.05$). In the presence of flumazenil, KRS-5Me-4-OCF₃ failed to evoke the inhibitory effect on the firing rate (in flumazenil: $3.7 \pm 0.7$ Hz; in flumazenil plus KRS: $3.7 \pm 0.8$ Hz ($n = 19$, $p > 0.05$, paired t test) and on the membrane potential ($V_m = 0.01 \pm 0.1$ mV, $n = 19$; $p > 0.05$). The blocked inhibitory effects of KRS-5Me-4-OCF₃ by flumazenil suggested that KRS-5Me-4-OCF₃ binds at the benzodiazepine site to exert its pharmacological actions.
Discussion

Here, we provide the first report that the enaminone KRS-5Me-4-OCF₃ acted as a novel positive allosteric modulator to decrease neuronal activity via direct regulation of GABA<sub>A</sub> receptors. Our study showed that anilino enaminone KRS-5Me-4-OCF₃ and its analogues displayed inhibitory effects on neuronal activity with different potencies. The inhibitory potency was dependent on the chemical structure and concentration of the enaminone. Among the three compounds, at the concentration tested, KRS-5Me-4-OCF₃ showed the most potent inhibition of spiking of MCs and evoked hyperpolarization of the membrane potential. These results are consistent with previous in vivo results that KRS-5Me-4-OCF₃ is the most potent anticonvulsant agent (Eddington et al., 2003). Neither excitatory ionotrophic glutamate receptors (NMDA and non-NMDA receptors) nor inhibitory GABA<sub>B</sub> receptors were involved in KRS-5Me-4-OCF₃-evoked inhibition of neuronal activity. The KRS-5Me-4-OCF₃-induced inhibition of activity was abolished by GABA<sub>A</sub> receptor antagonists, suggesting that the inhibition may act directly through activation of GABA<sub>A</sub> receptors or indirectly through an increase of extracellular GABA levels. Our results showed that neither blockade of GABA re-uptake nor blockade of GABA-T influenced KRS-5Me-4-OCF₃-evoked neuronal inhibition, indicating that the inhibition by KRS-5Me-4-OCF₃ was mediated through direct activation of GABA<sub>A</sub> receptors. The left-shift of the concentration-response relationship of enaminone KRS-5Me-4-OCF₃ in the presence of GABA implies that KRS-5Me-4-OCF₃ binds to a site distinct from the GABA binding site to enhance GABA activity. This property indicates that KRS-5Me-4-OCF₃ acted as a positive allosteric modulator of the GABA<sub>A</sub> receptor.
Thus, our results suggest that KRS-5Me-4-OCF₃ could be a potential medication as anxiolytic, anticonvulsant, anesthetic, and sedative-hypnotic.

Previously, nucleus accumbens (NAc) and coronal hippocampal slices in the rat brain have been used to study anticonvulsant enaminone suppression of excitatory synaptic transmission and epileptiform activity (Kombian et al., 2005; Ananthalakshmi et al., 2007). The MOB is anatomically different from NAc and hippocampus and provides three advantages to test cellular mechanisms of anilino enaminone action. Firstly, epilepsy-related proteins such as GABA<sub>A</sub> receptors, sodium channels, ionotropic glutamate receptors and metabotropic glutamate are expressed in MCs. Secondly, MCs show the property of spontaneous spiking, and epilepsy-related proteins participate in the regulation of neuronal spiking. Thirdly, the excitability of MCs can be regulated by synaptic input. Thus, MCs in MOB slices serve as a good model for testing the bioactivity of enaminone compounds and to explore the mechanisms underlying their activity. The strategy we used was to (1) test the effects of synthesized enaminones on neuronal activity and (2) determine the cellular basis of their pharmacological actions.

**Mechanism underlying the suppression of neuronal activity.** The subcutaneous pentylenetetrazol (scPTZ) seizure model identifies compounds that inhibit the GABA antagonistic effects of pentylenetetrazol or raise the seizure threshold (Stables and Kupferberg, 1997). Recent studies have shown that a number of enaminone compounds display inhibition against glutamate-mediated excitatory synaptic transmission by modulation of GABAergic transmission (Kombian et al., 2005; Ananthalakshmi et al., 2006). Based on the above findings and the results we present, we hypothesize the existence of an essential pharmacophore within the enaminone structure that possibly...
interacts with the GABA receptor, which is significant for achieving anticonvulsant activity. Even though the exact site and structural requirements for optimal binding are unknown, we believe, because of the molecular similarities between the enaminone analogues, the compounds we tested share a common binding pocket on the GABA receptor which explains the probability of eliciting similar biological responses.

The three compounds that we tested in the present study have been previously reported for their different potency of anti-convulsion in vivo (Eddington et al., 2003). The compound KRS-5Me-4-OCF₃, which is the most potent anticonvulsant in vivo (Eddington et al., 2003), possessed the most potent inhibitory effect on neuronal activity in vitro. The consistence of the neuronal inhibition in vitro and the anticonvulsant activity in vivo suggests that the anticonvulsant activity of KRS-5Me-4-OCF₃ results from preventing over excitability in epilepsy. The consistent in vitro and in vivo results also suggest that recording in MCs is an appropriate means to elucidate the bioactivity of enaminones.

Both GABAₐ and GABAₐ receptors are present in the MOB. They participate in the regulation of MC excitability in distinct ways. GABAₐ receptors directly regulate the excitability of MCs while GABAₐ receptors mediate the regulation of MC excitability via presynaptic inhibition in the MOB (Shepherd et al., 2004; Ennis et al., 2007). Anti-epileptic drugs like benzodiazepines are known to interact with GABAₐ receptors (Rall and Schleifer, 1990). This supports our results that the enaminone KRS-5Me-4-OCF₃ does not interact with GABAₐ receptors. Rather it interacts directly with GABAₐ receptors to decrease neuronal activity of MCs.
Enaminones with different chemical structure have been reported to display distinct mechanisms underlying their neuronal inhibition and anticonvulsant effects. Another anticonvulsant enaminone, E139 (Fig. 2), was reported to suppress excitatory synaptic transmission by (a) enhancing extracellular GABA levels (Kombian et al., 2005) and (b) blocking TTX-sensitive sodium channels and, thereby, directly inhibiting postsynaptic neuronal excitability (Ananthalakshmi et al., 2006). Meanwhile, several enaminones with chemical moieties different from KRS-5Me-4-OCF₃ were described to interact with GABA_A receptors (Hogenkamp et al., 2007; Yohannes et al, 2003, US patent: 6,653,471; Reitz et al., 1999, US patent: 5,922,731). Other enaminone derivatives that probably target GABA receptors were provided by using the comparative molecular field analysis (CoMFA) and comparative molecular similarity (CoMSIA) techniques, which can generate models to define the specific structural and electrostatic features essential for enhanced binding of enaminones to the putative GABA receptor (Jackson et al., 2009).

The GABA_A receptor is a ligand-gated ion channel responsible for mediating the effects of GABA, the major inhibitory neurotransmitter in the brain. The GABA_A receptor complex has been reported to have distinct binding sites for GABA, benzodiazepines, barbiturates, ethanol (Santhakumar et al., 2007), inhaled anaesthetics, and neuroactive steroids. Positive allosteric modulators enhance the affinity of GABA for the binding site. Allosteric modulators of GABA_A receptors such as benzodiazepines, neuroactive steroids, and barbiturates have been identified that are useful as anxiolytics, anticonvulsants, anesthetics, and sedative-hypnotics. Previous in vivo studies reported that enaminones show potent anti-convulsion effects in chemical-induced epilepsy animal
models but fewer side-effects such as sedation, drowsiness and dizziness (Mulzac and Scott, 1993; Eddington et al., 2003). Based on these in vivo results and our results, we hypothesized and confirmed that KRS-5Me-4-OCF₃ binds to benzodiazepine sites to exert its pharmaceutical actions. Therefore, in addition to the reported pharmacological action of KRS-5Me-4-OCF₃ as anticonvulsant, it is reasonable to speculate that KRS-5Me-4-OCF₃ might display an anxiolytic effect in a clinical setting.

A specific substituted site in the chemical structure of enaminones may be required for targeting GABA_A receptors and for conferring anticonvulsant activity. The three enaminones studied in the present paper, KRS-5Me-4-OCF₃, KRS-5Me-4F, and KRS-5Me-3Cl (Fig. 2), differed in their ability to suppress neuronal activity at the concentration tested. Our results indicated that a para-substitution of the phenyl group with –OCF₃ evoked the most potent suppression of neuronal excitability, while a para-, or meta- substitution of the phenyl group with fluoro, chloro decreased the potency of the inhibitory activity. This result suggests that a substitution in the phenyl group most strongly influences the potency of the inhibitory action of enaminones. The importance of the substitution group was also demonstrated in benzylamino enaminones in which unsubstituted benzylamine analog compound 30 (Fig. 2) showed the most potent activities in anti-convulsion and excitatory synaptic depression (Edafiogho et al., 2006).

The mechanism underlying the neuronal inhibition by the anilino enaminone KRS-5Me-4-OCF₃ is different from recent evidence obtained for another anilino enaminone, E139 (Fig. 2). The suppression of excitatory synaptic transmission evoked by E139 may be indirectly mediated through enhancement of GABA levels (Kombian et al., 2005). The most striking difference in the chemical structure of E139 and KRS-5Me-
4-OCF3 is in the ortho-substitution of the cyclohexenone moiety. E139 has an ortho-substitution of the cyclohexenone moiety, whereas the three compounds we tested in this study were not ortho-substituted in cyclohexenone. Our results indicate that non-substituted enaminones in ortho-cyclohexenone like KRS-5Me-4-OCF3 act as a positive allosteric modulator of GABA\textsubscript{A} receptors. Based on the chemical structure and our bioactivity analysis, we postulate that the ortho-site of cyclohexenone plays an important role in determining the interaction with a target protein. A recent study on benzylamino enaminones, which possess a similar structure to anilino enaminones, demonstrates a completely different cellular mechanism of action on excitatory synaptic depression and anti-convulsion (Edafiogho et al., 2006). Benzylamino enaminone compound 30 was found to depress glutamate-mediated excitatory synaptic transmission. In addition, enaminones without ortho-substitution of cyclohexenone that target GABA\textsubscript{A} receptors were described in several patented enaminones (Yohannes et al, 2003, US patent: 6,653,471; Reitz et al., 1999, US patent: 5,922,731). Therefore, we presume that substituted enaminones with different site substitutions may form different pharmacophores targeting specialized proteins. The chemical and pharmacological analysis of the structure-response relationship may provide a new means to guide rational drug design for potential anticonvulsant and anxiolytic compounds.

**Authorship Contributions**

Participated in research design: Heinbockel, Wang.

Conducted experiments: Wang, Sun.

Contributed new reagents: Jackson, Scott.
Performed data analysis: Wang, Heinbockel.

Wrote or contributed to the writing of the manuscript: Heinbockel, Wang, Jackson, Scott.

Other: Heinbockel acquired funding for the research.
References


Footnotes

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Figure Legends

**Figure 1:** Synthesis of aniline enaminones. Conditions: (a) H$_2$SO$_4$, Δ; (b) Δ, substituted amine.

**Figure 2.** Chemical structure of aniline enaminone analogues. The difference among the three mono methyl compounds is the anilino substitution represented by *meta*-Chloro; *para*-Fluoro, and *para*-trifluoro. E139 is a *para*-bromo anilino enaminone derivative with *ortho*-methyl ester substituted moiety at cyclohexenone and Compound 30 is the benzylamino analogue.

**Figure 3.** Anilino enaminones depressed the spiking activity of MCs. **A.** Normalized bar graph shows inhibition of spiking of MCs in response to bath application of KRS-5Me-4-OCF$_3$, KRS-5Me-3Cl, and KRS-5Me-4F. Responses to enaminones were normalized with respect to control conditions (* $p < 0.05$, **$*$ $p < 0.001$). **B.** Original recording from a representative MC illustrated the inhibition in firing rate and hyperpolarization following application of KRS-5Me-4-OCF$_3$.

**Figure 4.** Neither AMPA/kainate nor NMDA receptor antagonists blocked the KRS-5Me-4-OCF$_3$-induced inhibition of MCs. **A.** KRS-5Me-4-OCF$_3$-induced suppression of neuronal firing recorded in a representative MC in the presence of CNQX. **B.** The normalized and averaged results showed the persistence of KRS-5Me-4-OCF$_3$-induced
inhibitory effects on spontaneous spiking of MCs in the presence of either CNQX or CNQX plus D-AP5 (*p < 0.05).

Figure 5. GABA_B receptors were not involved in KRS-5Me-4-OCF_3-evoked inhibition. 

A. Original recording from an MC during KRS-5Me-4-OCF_3 application in ACSF condition and in the presence of GABA_BR antagonist CGP55845. The upper trace (i) illustrates the effect of KRS-5Me-4-OCF_3 on spiking of an MC and the effect of adding CGP55845. The upper trace is shown at an extended time scale in the middle (ii) and lower trace (iii). B. The normalized results showed the persistence of KRS-5Me-4-OCF_3-evoked inhibitory effects on spiking of MCs in the presence of the GABA_BR antagonist CGP55845 (10 µM) and the GABA_BR agonist (R)-baclofen (50 µM) (*p < 0.05, ***p < 0.001). The data for the effect of KRS-5Me-4-OCF_3 on spiking of MCs were normalized with respect to ACSF, or CGP88545 or baclofen alone.

Figure 6. KRS-5Me-4-OCF_3-evoked inhibition of MC spiking activity was blocked by GABA_A receptor antagonists. A. Original recording obtained from a MC showed that picrotoxin (20 µM) blocked 20 µM KRS-5Me-4-OCF_3-evoked suppression of neuronal firing. B. The normalized and averaged results showed KRS-5Me-4-OCF_3-evoked inhibitory effects on spiking of MCs in the presence of GABA_A receptor agonist GABA (50 µM), and antagonist picrotoxin (20 µM) and gabazine (5 µM) (**p < 0.01, *** p < 0.001). The data for the effect of GABA or KRS-5Me-4-OCF_3 on spiking of MCs were normalized with respect to control condition.
**Figure 7.** Neither an inhibitor of GABA reuptake nor an inhibitor of GABA degradation enzyme GABA-T blocked the KRS-5Me-4-OCF₃-evoked MC inhibition.  

A. Original recording from a MC showing that spiking inhibition evoked by KRS-5Me-4-OCF₃ persisted in the presence of vigabatrin (200 µM).  

B. Original recording from a MC showing that spiking inhibition evoked by KRS-5Me-4-OCF₃ persisted in the presence of NNC-711 (10 µM).  

C. Summary of results from normalized and averaged data (*p < 0.05, **p < 0.001).

**Figure 8.** The concentration–response curves of KRS-5Me-4-OCF₃–evoked inhibition and of GABA in the presence of KRS-5Me-4-OCF₃.  

A. The KRS-5Me-4-OCF₃–evoked change in spiking rate was normalized to the control condition, and then averaged. Each point was the mean value ± SEM of 4 to 7 cells. The line is fit for the data to the Hill equation: \( y = Y_0 + Ax^n/(K_d^n + x^n) \), where \( y \) is the inhibition of spiking rate, \( Y_0 \) is minimal inhibition, \( A \) is maximal inhibition, \( K_d \) is the apparent dissociation constant for agents, and \( n \) is the Hill coefficient. \( K_d \) and \( n \) were estimated using a Marquadt nonlinear least-squares routine.  

B. Shift of the concentration–response curves of GABA in the presence of KRS-5Me-4-OCF₃ at different concentrations (0, 5, 20 µM). The lines are fits for the data to the above Hill equation.
Fig. 1

Chemical structures representing the transformation sequence:

1. \( \text{R}^1 \text{H} \)
2. \( \text{R}^1 \text{H} \)
3. \( \text{R}^1 \text{H} \)
Fig. 2

Chemical structures of compounds:

- **KRS-5Me-4-OCF$_3$**
- **KRS-5Me-4-F**
- **KRS-5Me-3-Cl**
- **E139**
- **Benzyllamino Enaminone (Compound 30)**
Fig. 3

A  

Spike rate (% of control)

<table>
<thead>
<tr>
<th>KRS-5Me-3CI</th>
<th>KRS-5Me-4F</th>
<th>KRS-5-Me-4OCF3</th>
</tr>
</thead>
</table>

B  

KRS-5Me-4-OCF3

60 mV
2 min
Figure 4

A) KRS

B) Spike rate (% of control)

- CNQX
- CNQX & D-AP5

KRS

* Significant difference
Fig. 5

A

(i) KRS  CGP  KRS+CGP  CGP

(ii) KRS

(iii) CGP  KRS+CGP  CGP

60 mV  4 min  2 min

B

<table>
<thead>
<tr>
<th></th>
<th>Spike rate (%)</th>
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<tbody>
<tr>
<td>ACSF</td>
<td>** ***</td>
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<tr>
<td>baclofen</td>
<td>*</td>
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<tr>
<td>CGP55845</td>
<td>*</td>
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Fig. 5
Fig. 6

Panel A: A graph showing the effect of picrotoxin on neural activity, marked with KRS.

Panel B: A bar graph comparing spike rates (% of control) under different conditions: ACSF, GABA, Picrotoxin, and Gabazine. The graph shows significant differences marked with ** and ***.
Inhibition of spike rate (%) vs. Concentration of KRS (µM)

Inhibition of spike rate (%) vs. Concentration of GABA (µM)

- GABA
- GABA + 5 µM KRS
- GABA + 20 µM KRS

Fig. 8