

Anticonvulsant enaminones depress excitatory synaptic transmission in the rat brain by enhancing extracellular GABA levels

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1 Enaminones are a novel group of compounds that have been shown to possess anticonvulsant activity in *in vivo* animal models of seizures. The cellular mechanism by which these compounds produce their anticonvulsant effects is not yet known. This study examined the effects of enaminones on excitatory synaptic transmission.

2 We studied the effects of 3-(4'-chlorophenyl)aminocyclohex-2-enone (E118), methyl 4-(4'-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate (E139) and ethyl 4-(4'-hydroxyphenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate (E169) on isolated evoked, glutamate-mediated excitatory synaptic responses by recording whole-cell currents and potentials in cells of the nucleus accumbens (NAc) contained in forebrain slices.

3 The anticonvulsant enaminones (E118 and E139), but not E169, depressed NMDA and non-NMDA receptor-mediated synaptic responses. The inhibition of the non-NMDA response was concentration-dependent (1.0–100 μ M) with a maximal depression of \sim –30%. E118 and E139 had similar potencies (EC_{50} = 3.0 and 3.5 μ M, respectively) in depressing this response but E139 was more efficacious (E_{max} = $-31.3 \pm 3.8\%$) than E118 (E_{max} = $-22.6 \pm 1.6\%$).

4 The excitatory postsynaptic current (EPSC) depression caused by 10 μ M E139 ($-27.7 \pm 3.8\%$) was blocked by 1 μ M CGP55845 ($6.3 \pm 8.1\%$), a potent GABA_B receptor antagonist.

5 Pretreatment of slices with γ -vinylGABA and 1-(2-(((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid (NO-711), an irreversible GABA transaminase (GABA-T) inhibitor and a GABA reuptake blocker, respectively, like the anticonvulsant enaminones, also caused a depression of the evoked EPSC (-38.1 ± 14.1 and $-24.1 \pm 8.9\%$, respectively). In the presence of these compounds, E139 did not cause a further depression of the EPSC. Our data suggest that anticonvulsant enaminones cause EPSC depression by enhancing extracellular GABA levels possibly through the inhibition of either GABA reuptake or GABA-T enzyme, or both.

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Abbreviations: AMPA, (s)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propanoate; DL-APV, DL-2-amino-5-phosphonovaleate; DMSO, dimethyl sulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; E118, 3-(4'-chlorophenyl)aminocyclohex-2-enone; E139, methyl 4-(4'-bromophenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate; E169, ethyl 4-(4'-hydroxyphenyl)aminocyclohex-3-en-6-methyl-2-oxo-1-oate; E_{max} , maximum effect; γ vG, gamma vinyl GABA; Nac, nucleus accumbens; NMDA, *N*-methyl-D-aspartate; NO-711, 1-(2-(((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid

Introduction

Epilepsy is a common neurological disorder characterized by recurrent seizures. This condition has been described since ancient times yet it still continues to afflict a substantial number of people (1–2%) in the world today (McNamara, 1999; Browne & Holmes, 2001) with no cure in sight. This may be due in part to a complex pathophysiology and/or etiology of this disorder (see reviews by McNamara, 1999; Löscher, 2002). Epilepsy is managed medically by either pharmacotherapy or surgery where this is possible. Currently available drugs have serious shortcomings as they only provide symptomatic

relief and their use is often accompanied by significant adverse or side effects. Furthermore, there is a significant proportion of patients (up to 40%) who do not respond to these agents (Regesta & Tanganelli, 1999; Kwan & Brodie, 2000; Löscher, 2002). There is therefore a need for continued research into this disorder and the development of newer agents for its management (McNamara, 1999; Brodie, 2001; Löscher, 2002).

A new class of compounds, called enaminones, with a unique structure different from currently available antiepileptic drugs has recently been synthesized (Edafiohgo *et al.*, 1992). Based on their anticonvulsant activity, they have been classified into three classes (1, 2 and 3) with class 1 being the

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most potent in these tests (Edafiogho *et al.*, 1994). E139, E121 and E118, all class I anticonvulsants, have shown promise in preventing seizures in mouse and rat models of epilepsy while others (e.g. E169) are inactive as anticonvulsants. Unlike conventional antiepileptic drugs, the anticonvulsant enaminones displayed no neurotoxicity such as ataxia, and had wider safety margins as determined by their higher protective indices when compared to conventional antiepileptic drugs such as valproate, carbamazepine and phenytoin (Mulzac & Scott, 1993). They may therefore present a unique opportunity to develop anticonvulsant drugs that have a superior side effect profile as well as a wider margin of safety than currently available antiepileptic drugs.

In order to understand the mechanism(s) underlying the actions of these compounds to produce anticonvulsant activity, their effects on neuronal physiology need to be clarified. Hyperexcitability of neurons at the epileptogenic focus underlies the spontaneous synchronous discharge of neurons that precipitates an epileptic seizure (McNamara, 1999). The excitability of neurons is an integral of intrinsic membrane conductances and synaptic inputs. Both excitatory and inhibitory inputs contribute to the resting excitability (Traynelis & Dingledine, 1988; McNamara, 1994). Since the role of glutamate in the pathogenesis of seizure disorders is quite well known (McNamara *et al.*, 1988; McBain *et al.*, 1989; Dingledine *et al.*, 1990; Kraus *et al.*, 1994; McNamara, 1994), we were interested to know if enaminones affected this transmitter system to produce their anticonvulsant effects.

We tested the hypothesis that enaminones may interact with glutamate and/or its receptors to produce the reported anticonvulsant effects in rats and mice (Edafiogho *et al.*, 1992; Abou-Zeid & Edafiogho, 2002). The rationale for this was based on the possible role of glutamate in the genesis of seizures (Dingledine *et al.*, 1990) as well as computer modeling of these compounds and crystallographic studies, which suggest that they would fit a putative glutamate receptor site (Abou-Zeid & Edafiogho, 2002; Edafiogho *et al.*, 2003). In this study, we hereby report our findings on the effects of three enaminones on pure NMDA and non-NMDA receptor-mediated responses recorded in neurons of the nucleus accumbens (NAc).

Methods

Synthesis of enaminones

The enaminones E118, E139 and E169 were synthesized for this study according to methods reported previously (Edafiogho *et al.*, 1992; 1994; 2003). Briefly, the condensation reaction between β -diketo intermediates and appropriate amino compounds, under carefully controlled reaction conditions, yielded the enaminones E118, E139 and E169 (Figure 1).

Animal experiments

All the rats used in this study were obtained from the Kuwait University Animal Resource Centre. International guidelines on humane handling of animals were followed throughout this study and the minimum number of animals necessary to produce the required results was used.

Slice preparation

Parasagittal forebrain slices containing the NAc and the cortex were generated using previously published techniques (Kombian *et al.*, 2003). Briefly, male Sprague–Dawley rats (75–150 g) were anesthetized with halothane before decapitation. The brain was quickly removed from the rat and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) that was bubbled with 95% O₂ and 5% CO₂. The composition of the ACSF was (in mM): 126 NaCl; 2.5 KCl; 1.2 NaH₂PO₄; 1.2 MgCl₂; 2.4 CaCl₂; 18 NaHCO₃; 11 glucose, producing a solution with osmolality of between 310 and 320 mosm. Thin slices (350- μ m thick) were cut in the ice-cold ACSF using a Leica (VT 1000S) tissue slicer. Slices were incubated in ACSF (bubbled with 95% O₂ and 5% CO₂) at room temperature and allowed to recover for at least 1 h before use.

Electrophysiological recording and data acquisition

One slice was trimmed and transferred into a 500 μ l capacity recording chamber and perfused submerged at a flow rate of 2–3 ml min⁻¹ (29–31°C) with ACSF that was bubbled with 95% O₂ and 5% CO₂. 'Blind patch' recordings were performed

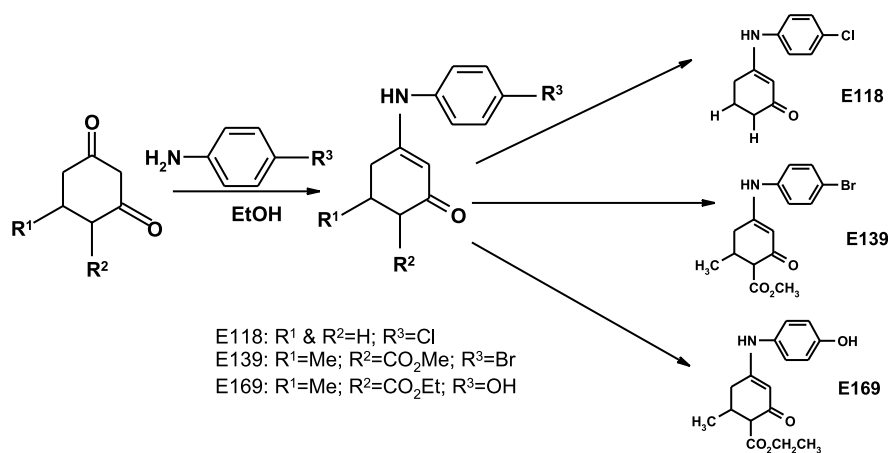


Figure 1 The synthetic pathway of obtaining the enaminones used in this study. The differences in the three compounds studied are in the substituents represented by R¹ to R³ on the basic enaminone structure.

in the conventional whole-cell mode using glass electrodes with tip resistance of 4.0–8.0 M Ω . The internal recording solution had the following composition (in mM): K-gluconate (135), NaCl (8), EGTA (0.2), HEPES (10), Mg-ATP (2) and GTP (0.2). pH and osmolarity were adjusted to 7.3 (with KOH) and 270–280 mosm, respectively. Bipolar tungsten stimulating electrodes were positioned at the prefrontal cortex–accumbens border to evoke synaptic responses. Recordings were made using the Axopatch 1D amplifiers in either voltage or current clamp modes. Cells were voltage clamped at -80 mV (holding potential, V_h), and input (R_{input}) and access (R_a) resistances of all cells were determined and monitored regularly throughout each experiment by applying a 75 ms, 20 mV hyperpolarizing pulse or 100 pA negative current in current clamp. All cells reported in this study had R_a of 10–30 M Ω . Data from cells that showed $>15\%$ changes in R_a during the experiment were excluded from further analysis.

All synaptic responses were recorded either as inward currents at V_h of -80 mV or depolarizing potentials at resting potential unless otherwise stated. All cells had a graded evoked response (EPSC and EPSP) to increasing stimulation intensity (ranging from 0.25 to 4.0 mA) and an intensity giving 50–60% of the maximum evoked synaptic response was used to evoke test responses. All data were acquired using pClamp Software (Clampex 7 or 8; Axon Instruments) at a sampling rate of 6.7 kHz, filtered at 1 kHz, digitized and stored for off-line analysis. Each stored trace was an average of two successive synaptic responses elicited at 10 s intervals.

Analysis and statistics

EPSC and EPSP amplitudes were measured from baseline to peak and taken as the synaptic strength at the chosen stimulus intensity. Responses were normalized by taking the mean of the last 3–4 responses prior to drug application and dividing the rest of the responses by this mean. These normalized values were then used for average plots. For these plots, all cells receiving the same treatment were aligned at the first minute of first drug application and averaged over the entire period. All values are stated as mean \pm standard error. One-way ANOVA and *post hoc* tests, as indicated in the Results section, were used to compare different values or treatments using the SigmaStat[®] software. Differences between groups were taken as significant at a probability level of $P \leq 0.05$. Graphing was performed using the SigmaPlot[®], GraphPad Prism[®] and CorelDraw[®] softwares.

Chemicals and solutions

All drugs were bath perfused at final concentrations indicated by dissolving aliquots of stock in the ACSF. All enaminones were synthesized in-house (see Figure 1) and were dissolved in dimethyl sulfoxide (DMSO; final concentration in bath $<0.1\%$), aliquoted and frozen at -20°C and used within 3 weeks. Most routine laboratory chemicals as well as, DL-2-amino-5-phosphonovaleric acid (DL-APV), γ -vinyl GABA (γ VG) and picrotoxin were purchased from Sigma Company (Germany). 6,7-Dinitroquinoxaline-2,3-dione (DNQX), NO-711 were obtained from RBI (U.S.A.), CGP55845 was from Tocris (U.K.).

Results

The results reported here were obtained from whole-cell recordings in 92 neurons of the rostral NAc. Out of this number, 83 neurons ($\sim 90\%$) responded to enaminones with a measurable effect on synaptic transmission. The majority of cells in this region ($\sim 95\%$) are medium spiny GABAergic neurons (Pennartz *et al.*, 1994) with relatively very negative resting potentials (-72 to -90 mV). The other passive and active membrane properties of these cells were similar to those previously reported (Kombian *et al.*, 2003).

Effects of anticonvulsant enaminones on synaptic transmission in the NAc

Under control conditions, stimulation of the cortico-accumbens afferents results in a mixed response mediated by glutamate and GABA (Pennartz & Kitai, 1991). The effects of enaminones on mixed synaptic responses were initially tested with E139. Figure 2 shows that bath applied E139 ($10 \mu\text{M}$) reversibly depresses this mixed response referred to as a depolarizing postsynaptic current (DPSC) by $-25 \pm 5.7\%$ ($n=4$). This effect was without any significant change in the holding current (-13.3 ± 12.0 pA, $P > 0.05$, $n=4$), although at higher concentrations ($\geq 100 \mu\text{M}$) E139 induced an inward current.

Isolation of synaptic responses

Glutamate-induced, pure EPSCs were isolated by applying $50 \mu\text{M}$ of picrotoxin, a GABA_A receptor-chloride channel

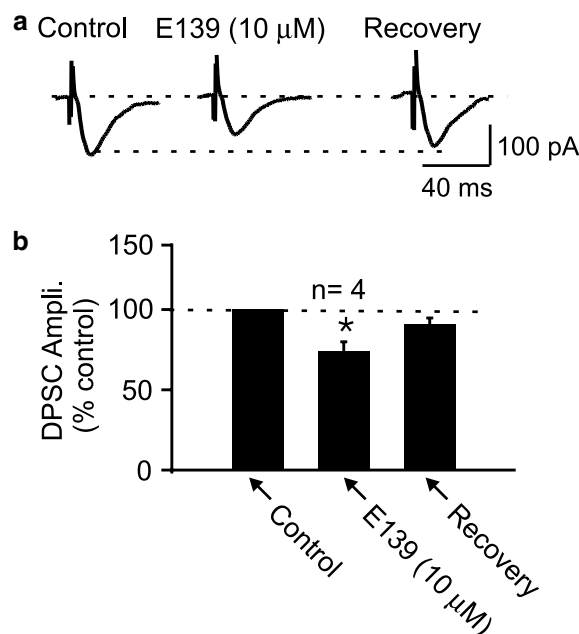


Figure 2 Enaminone, E139 depresses evoked depolarizing postsynaptic currents. (a) Sample depolarizing postsynaptic currents recorded in a NAc cell at a holding potential of -80 mV in control, after 5 min bath application of $10 \mu\text{M}$ E139 and following 10 min washout of E139. (b) Bar graph summarizing the above effect of $10 \mu\text{M}$ E139 ($n=4$). In this figure and in all other figures, * indicates statistical significance at $P \leq 0.05$ compared to control.

blocker. At a holding potential of -80 mV, the response was entirely non-NMDA receptor-mediated as it could be completely blocked by DNQX ($10 \mu\text{M}$, $n=8$; see Figure 3a). NMDA receptor-mediated pure EPSPs were isolated by combining pharmacological and biophysical approaches. In the presence of picrotoxin ($50 \mu\text{M}$), addition of $10 \mu\text{M}$ DNQX abolished the non-NMDA and GABA receptor-mediated responses recorded at rest. Cells were then depolarized to between -45 and -55 mV. Stimulation at slightly higher intensity at these potentials produced a slow rising and decaying response characteristic of the NMDA receptor kinetics ($n=8$). This response in the presence of picrotoxin and DNQX and at depolarized potentials was purely NMDA receptor-mediated as it was blocked by the selective NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid as previously reported ($n=3$; Kombian *et al.*, 2003).

Anticonvulsant enaminones inhibit glutamate-mediated synaptic responses

To characterize the effects of anticonvulsant enaminones on excitatory synaptic transmission, we pharmacologically and/or biophysically isolated and studied glutamate-mediated fast synaptic transmission. In this region, both NMDA and non-NMDA receptor-mediated responses can be recorded (Pen-

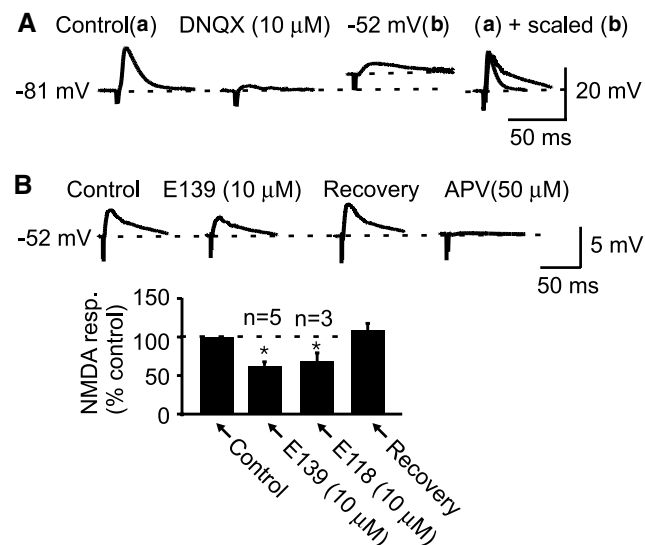


Figure 3 E139 depresses isolated, pure NMDA receptor-mediated excitatory postsynaptic potentials (EPSPs). (A) Sample control EPSP (a) recorded in a cell at its resting potential in the presence of only picrotoxin ($50 \mu\text{M}$). Application of DNQX ($10 \mu\text{M}$) completely abolishes this response (middle trace). The cell was then depolarized to -52 mV (by positive current injection) and stimulated at a slightly higher stimulus intensity to generate an NMDA receptor-mediated EPSP with the characteristic slow rise and decay rates (b). Far right panel: superimposed control EPSP (a) and the NMDA receptor-mediated response (b) scaled to the amplitude of (a) and baseline adjusted to show the differences in the kinetics of these two responses. (B) A summary bar graph showing that E139 and E118 reversibly depress the pure NMDA receptor-mediated EPSP ($n=5$ and 3, respectively). Above this are sample traces taken from the cell in (A) illustrating this effect (note the differences in scale here). Far right trace shows that application of DL-APV ($50 \mu\text{M}$) blocks this synaptic response. In these experiments and the rest of the study, picrotoxin ($50 \mu\text{M}$) was present throughout each experiment.

nartz *et al.*, 1990; Kombian & Malenka, 1994). Figure 3a shows the protocol used to isolate the NMDA receptor-mediated EPSP for the purpose of this study. We chose to record the NMDA response in current clamp mode because it was much easier to observe the characteristic slow rise and decay kinetics of the potential. Figure 3b reveals that $10 \mu\text{M}$ E139 and E118 both reversibly inhibit this pure, NMDA receptor-mediated EPSP by $-37.5 \pm 5.3\%$ ($n=5$) and $-28.2 \pm 8.1\%$ ($n=3$) respectively.

The other glutamate-mediated response is through the activation of AMPA/kainate or non-NMDA receptors, which are responsible for basal, fast excitatory synaptic transmission because unlike the NMDA receptors, they do not display voltage-dependent activation (Mayer *et al.*, 1984). These pure non-NMDA receptor-mediated currents were used to investigate the effects of three enaminones on fast excitatory synaptic transmission. Bath application of E118 or E139 caused a reversible depression of the evoked EPSC. The onset of action was about 2 min with a maximum effect observed at 5 min (Figure 4a and b). Recovery from the EPSC depression was complete and relatively quick, with responses returning to baseline levels in 5–10 min after the commencement of washout (Figure 4a and b). The synaptic depressant effects of both of these enaminones were concentration-dependent. The estimated EC_{50} of E139 was $3.5 \mu\text{M}$ with a maximum depression of $-31.3 \pm 3.8\%$ (Figure 4c; $n=4$) obtained at $100 \mu\text{M}$. For E118, the estimated EC_{50} was $3.0 \mu\text{M}$ but with a maximal response of $-22.6 \pm 1.6\%$ (Figure 4c; $n=5$) at $100 \mu\text{M}$. The E118- and E139-induced EPSC depression at concentrations $\leq 10 \mu\text{M}$ was without significant changes in holding current. E169, on the other hand, was inactive as it neither depressed the evoked EPSCs nor induced any currents at all concentrations tested. These findings indicate that while E118 and E139 are equipotent, E139 is more efficacious than E118 in depressing evoked EPSC, with E169 having no efficacy. E139 was used for the rest of the study because it was more efficacious and produced the most consistent responses. A concentration of $10 \mu\text{M}$ was selected as this concentration produced a robust effect on the evoked EPSC that could be subjected to pharmacological characterization. This concentration of E139 was shown to cause a consistent EPSC depression following repeated applications. In six cells, a second application of E139 ($10 \mu\text{M}$) shortly after recovery from the first application produced an almost identical level of EPSC depression ($-23.7 \pm 1.4\%$ in the first compared to $-23.3 \pm 2.5\%$ in the second; $P>0.05$, $n=6$; paired *t*-test).

To determine if the anticonvulsant enaminones produced the synaptic depression by interacting with the postsynaptic non-NMDA receptor, we analyzed the activation and inactivation kinetics of the non-NMDA receptor-mediated EPSC in the absence and presence of E139 ($10 \mu\text{M}$). The rise time constant τ_1 and the decay time constant τ_2 were fitted to a single exponential. E139 ($10 \mu\text{M}$) did not significantly alter τ_1 (3.9 ± 0.5 ms in control and 3.7 ± 0.6 ms in the presence of E139; $P>0.05$, paired *t*-test, $n=8$) or τ_2 (23.3 ± 5.3 ms in control compared to 23.0 ± 8.1 ms in the presence of E139, $P>0.05$, paired *t*-test, $n=8$). These values indicate that E139 does not affect the rise time or the decay rate of the non-NMDA receptor-mediated EPSC and hence may not cause the decrease in EPSC amplitude by altering the kinetics of this receptor channel.

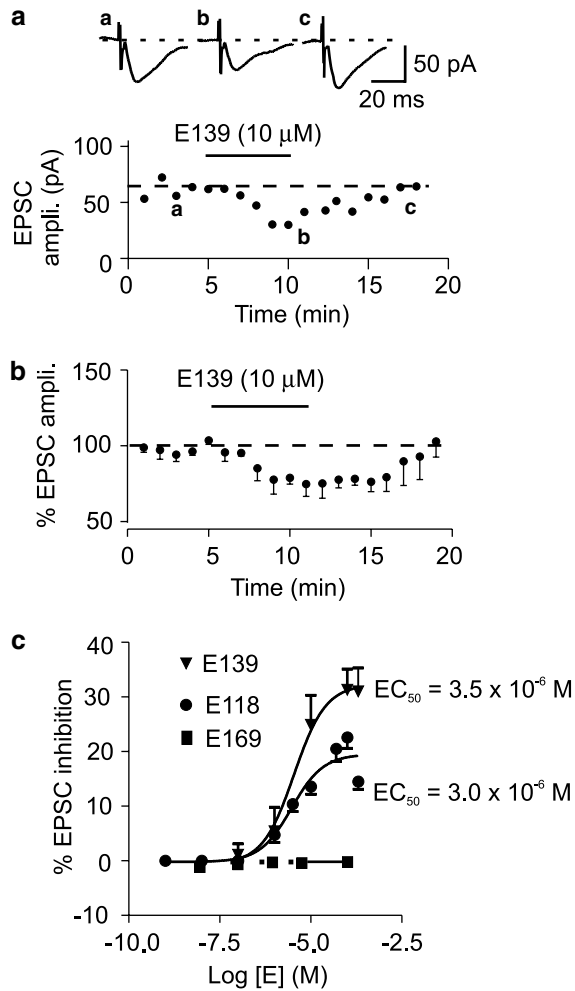


Figure 4 Anticonvulsant enaminones depress fast, non-NMDA receptor-mediated synaptic responses in a concentration-dependent manner. (a) A time-effect graph of EPSCs recorded in a representative cell that was treated with E139 (10 μM). In this graph and in all other graphs, above each are sample traces taken at the times indicated by letters. (b) A normalized and averaged time-effect plot of the effect of E139 on evoked EPSCs obtained from five cells that received the same concentration of E139 (10 μM). (c) Concentration-response curves generated by applying different concentrations of 3 different enaminones (E118, E139 and E169). Each point on the graph has an *n* value of 3–5 cells.

Enaminone-induced depression of evoked EPSC is blocked by a GABA_B receptor antagonist

To further characterize the mechanism of action of anti-convulsant enaminones in depressing EPSC amplitude, we hypothesized that it may act indirectly through an intermediate neuromodulator. Since several currently available anticonvulsants work by enhancing GABA mechanisms, we tested if GABA was a possible intermediate that these compounds employed to depress the evoked EPSC amplitude. We have previously reported that, GABA which is abundant in this nucleus depresses EPSCs through GABA_B receptors (Kombian *et al.*, 2004). Bath application of 1 μM CGP55845, a potent GABA_B receptor antagonist caused an increase in evoked EPSC amplitude (27.1 ± 8.2%; *n* = 5; *P* < 0.05; Figure 5) confirming the presence of a tonic GABA effect on excitation

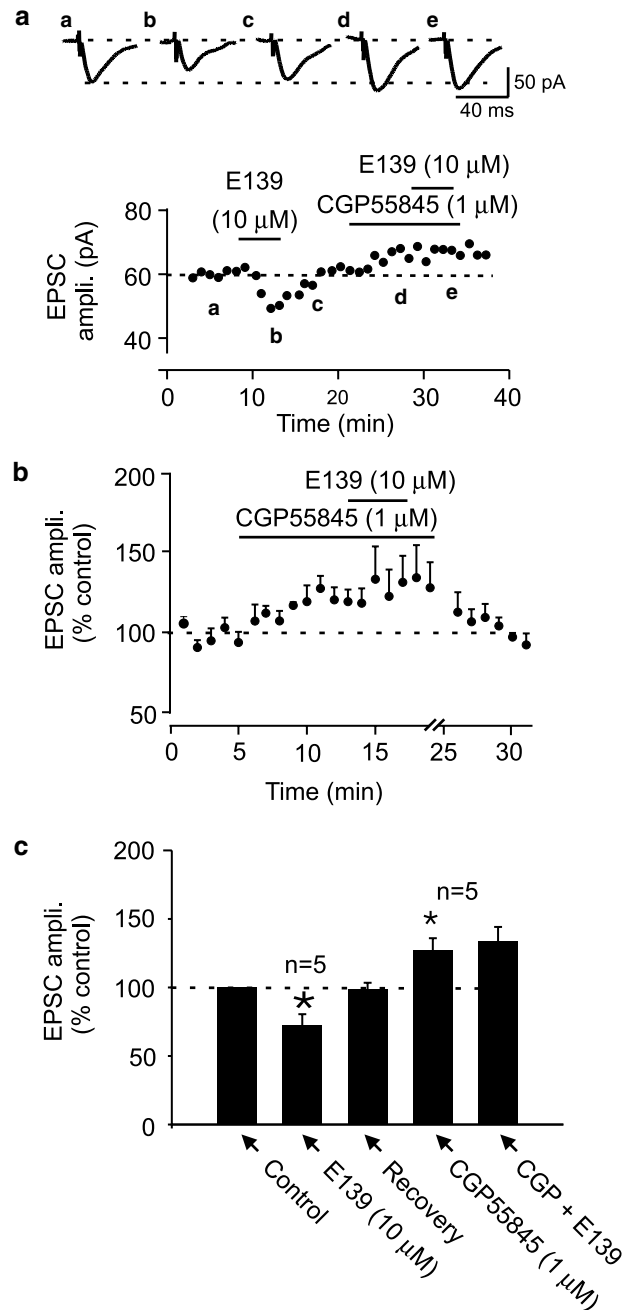


Figure 5 GABA_B receptor antagonist blocks E139-induced EPSC depression. (a) A time-effect plot of evoked EPSCs recorded in a representative cell showing the effect of E139 (10 μM) before and after pretreatment with 1 μM CGP55845, a GABA_B receptor antagonist. Note the unmasking by CGP55845 of an endogenous GABA effect on the EPSC. Subsequent application of E139 in the presence of CGP55845 produced no EPSC inhibition. (b) A normalized and averaged time-effect plot in five cells that were pretreated with CGP55845 prior to E139 (10 μM). (c) A bar graph summarizing the effect of E139 in control and in the presence of CGP55845 (*n* = 5).

in this nucleus (Kombian *et al.*, 2004). When E139 (10 μM) was applied at the peak of the CGP55845 effect, the change in EPSC amplitude was 6.3 ± 8.1% compared to a depression of -27.7 ± 3.8% observed in these same cells prior to CGP55845 pretreatment (*P* < 0.05; *n* = 5; paired *t*-test; Figure 5). The lack

of effect of E139 in the presence of CGP55845 was not due to desensitization of the E139 effect from the previous exposure as we had earlier shown that the effect of E139 on evoked EPSC in this region does not desensitize. Therefore, the complete block of the E139-induced synaptic depression by CGP55845 suggests that enaminones cause EPSC depression indirectly through GABA and GABA_B receptors.

Enhancement of extracellular GABA levels occludes enaminone effects on EPSCs

The fact that the EPSC depression produced by E139 was blocked by a GABA_B receptor antagonist suggests that anticonvulsant enaminones either bind to the GABA binding site on GABA_B receptors to produce the observed effect or act to increase extracellular GABA levels, which then mediates the synaptic depression. The former possibility is unlikely to be the case as an anticonvulsant enaminone, ADD 196022, with close structural similarity with E139, is reported not to affect [³H]-GABA binding to neuronal membranes (Mulzac & Scott, 1993). The other alternative, which is to alter extracellular GABA levels, was therefore examined in this study.

There is a tonic presence of GABA in the NAc (Pennartz *et al.*, 1994; Kombian *et al.*, 2004) and this appears to be controlled mainly by plasma membrane transporters called GATs that remove extracellular GABA back into terminals and glial cells, which may then subsequently be degraded by the enzyme GABA transaminase (GABA-T) (Borden *et al.*, 1992; Isaacson *et al.*, 1993; Minelli *et al.*, 1995; Brickley *et al.*, 1996; Rossi & Hamann, 1998). Blockade or inhibition of GATs and GABA-T usually results in the extracellular accumulation of GABA that may lead to prolonged GABA effects (Otis *et al.*, 1991; Isaacson *et al.*, 1993). In the CNS, change in extracellular GABA levels can be monitored by measuring its effect on other responses such as GABA_A receptor-mediated currents or GABA_B receptor-mediated synaptic depression (see Overstreet & Westbrook, 2001). To verify these facts electrophysiologically, we manipulated the extracellular levels of GABA with known pharmacological agents and asked if the effects of E139 remained unaltered under these conditions.

By monitoring EPSC amplitude, we first tested if γ vG, an irreversible GABA-T inhibitor (Löscher & Horstermann, 1994; Qume *et al.*, 1995; Qume & Fowler, 1997) and an activator of reverse GABA transport (Wu *et al.*, 2001), depressed evoked EPSC. When 500 μ M γ vG was bath applied to these cells, there was depression of the evoked EPSC amplitude ($-38.1 \pm 14.1\%$, $n = 4$; Figure 6). This effect of γ vG did not recover following 15–20 min washout consistent with the fact that it is an irreversible inhibitor of GABA-T. At the peak of the γ vG-induced EPSC depression, application of E139 (10 μ M) did not cause a further decrease in EPSC amplitude ($-39.1 \pm 9.4\%$; $P > 0.05$ compared to depression with γ vG alone, $n = 4$, paired *t*-test; Figure 6). The effect of γ vG was confirmed to be through GABA, acting on GABA_B receptors, as it was blocked when slices were pretreated with CGP55845 (1 μ M; $-6.4 \pm 5.7\%$; $P < 0.05$ compared to γ vG effect in control; $n = 4$; unpaired *t*-test; Figure 6c). The occlusion of the EPSC depressant effect of E139 by γ vG suggests that E139 may work to enhance extracellular GABA levels by either inhibiting GABA-T and/or inducing non-

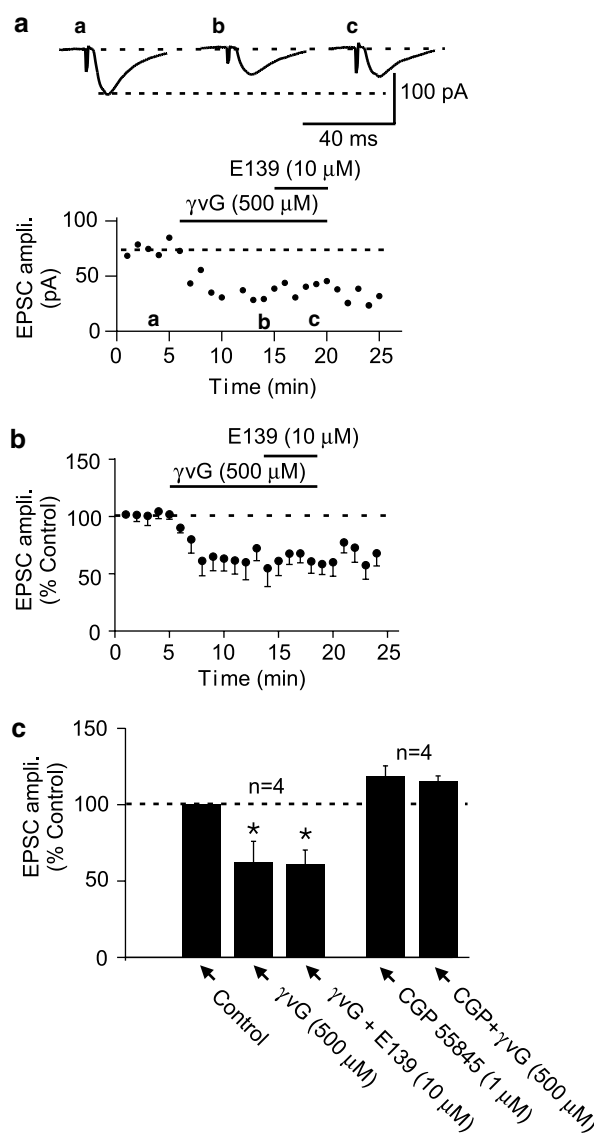


Figure 6 The EPSC depressing effect of E139 is occluded by pretreatment with γ -vinyl GABA (γ vG), an irreversible GABA transaminase inhibitor. (a) A representative time-effect plot of the effect of 500 μ M γ vG on evoked EPSCs and the subsequent application of E139 showing no additional synaptic depression. (b) A normalized averaged time-effect plot obtained from four cells that received the above treatment. (c) A summary bar graph of the effect of 500 μ M γ vG and the lack of effect of E139 in the presence of γ vG. This graph also summarizes the blockade of γ vG effect in four additional cells that were pretreated with 1 μ M CGP55845 prior to γ vG.

vesicular GABA release through reversal of GABA transporters (Levi & Raiteri, 1993; Wu *et al.*, 2001; 2003).

To determine if anticonvulsant enaminones also interacted with GABA reuptake to enhance extracellular GABA levels, we used a selective GABA reuptake inhibitor, NO-711 to test if we could also occlude the E139 effect. When NO-711 (50 μ M) was applied, it depressed the evoked EPSC amplitude ($-24.1 \pm 8.9\%$, $n = 5$; Figure 7). In contrast to γ vG however, this effect was reversible upon washout and was also shown to be *via* GABA as it was blocked by pretreatment with 1 μ M CGP55845 ($-1.9 \pm 2.2\%$; $P < 0.05$ compared to the effect of

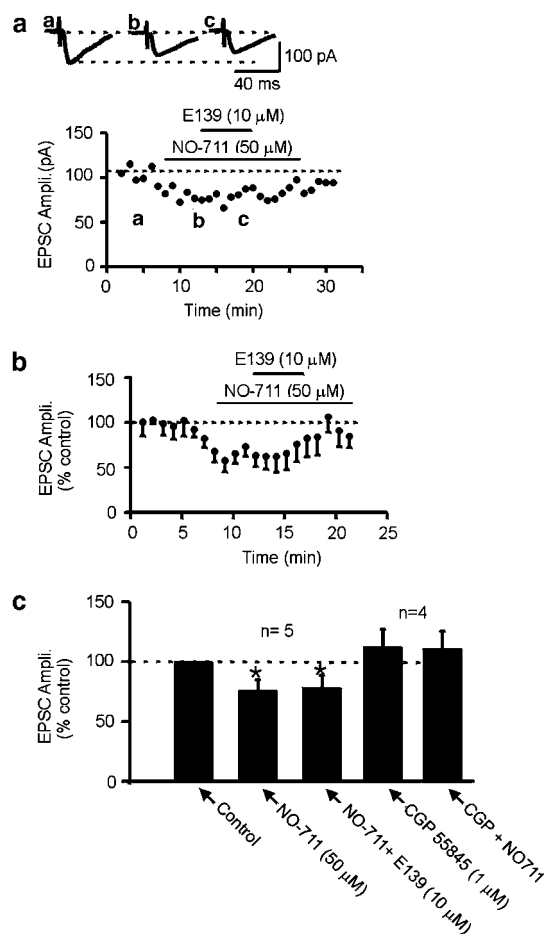


Figure 7 The EPSC depressing effect of E139 is occluded by pretreatment with NO-711, a GABA reuptake blocker. (a) A representative time-effect plot showing that 50 μM NO-711 reversibly depresses evoked EPSC amplitude. At the peak of the NO-711 effect, coapplication of E139 produces no additional EPSC inhibition. (b) A normalized and averaged time-effect plot generated from five cells that received the treatment above. (c) A summary bar graph of the effect of NO-711 (50 μM) and the lack of effect of E139 in the presence of NO-711. This graph also summarizes the blockade of the NO-711 effect in four additional cells that were pretreated with CGP55845 (1 μM) prior to NO-711.

NO-711 in control; $n=4$; unpaired *t*-test, Figure 7c). At the peak of the NO-711 effect, application of 10 μM E139 could no longer produce a further decrease in the evoked EPSC amplitude ($-23.6 \pm 10.8\%$, $P > 0.05$ compared to the effect of NO-711 alone; $n=5$, paired *t*-test, Figure 7). These results suggest that E139 may also inhibit GABA reuptake leading to the observed GABA-mediated effects.

Discussion

The results of this study show that some, but not all enaminones cause a concentration-dependent depression of glutamate-mediated excitatory synaptic transmission in the CNS. The concentrations of 0.1–100 μM used in this study (which are equivalent to 0.034–33.8 μg g⁻¹) are therapeutically relevant since quantities of approximately 1–10 μg g⁻¹ of enaminone have been reported in the brains of rats, 15–200 min

after intraperitoneal injection of an anticonvulsant dose (10 mg kg⁻¹) of E121 (Khurana *et al.*, 2003). In this study, we mainly perfused 10 μM, a molar concentration that works out to 3.38 μg ml⁻¹, a quantity close to that reported above. Concentrations $\leq 10 \mu\text{M}$ did not have a significant effect on resting membrane currents in the cells tested. As well, at or below this concentration, the anticonvulsant enaminones did not significantly alter the kinetics of the non-NMDA receptor-mediated currents. These findings indicate that a direct action of enaminones on the non-NMDA receptor did not contribute significantly to the observed synaptic depression and are not consistent with the modeling predictions (Abou-Zeid & Edafiogho, 2002). Our data, however, support a GABA-dependent action of enaminones to depress excitatory synaptic transmission as their synaptic depressing effect was blocked by an antagonist of GABA_B receptors and occluded by pharmacological manipulations that are known to enhance extracellular GABA concentration. The blockade of the E139 effect by a GABA_B receptor antagonist suggests that E139 interacts with this receptor directly to produce the synaptic depression. This is, however, unlikely as an enaminone, ADD 196022 which differs from E139 only in a *chloro*-substitution on the phenyl group, has been reported not to affect [³H]GABA binding (Mulzac & Scott, 1993). Our data are therefore consistent with an indirect action of the anticonvulsant enaminones to produce the GABA-dependent synaptic depression. Thus, the anticonvulsant enaminones may act to increase extracellular GABA concentration and GABA then acts on GABA_B receptors, located on presynaptic glutamate terminals, to decrease EPSC amplitude (Uchimura & North, 1991).

Mechanism(s) of action of anticonvulsant enaminones to depress excitatory synaptic transmission

Our data indicate that anticonvulsant enaminones caused a decrease in non-NMDA receptor-mediated currents at concentrations ranging from 0.1 to 100 μM. At concentrations of 10 μM and below, no significant changes in holding current or membrane potential was observed indicating that the active enaminones did not affect the excitability of the recorded postsynaptic cells at rest. However, at concentrations above 10 μM, we consistently observed an inward current or depolarization. The nature of this current and its effect on membrane excitability is currently being investigated.

A tonic presence of GABA in this region has been reported (Pennartz *et al.*, 1994; Kombian *et al.*, 2004), and altering the level of ambient GABA may be a mechanism by which enaminones work to cause the GABA effects observed in this study. After release, GABA is rapidly cleared from the extracellular space by two main mechanisms: reuptake into the presynaptic terminal and surrounding glial cells followed by enzymatic degradation by GABA-T (Löscher *et al.*, 1989; Qume & Fowler, 1997; Conti *et al.*, 1998) and diffusion (Isaacson *et al.*, 1993; Rossi & Hamann, 1998; Sipila *et al.*, 2004). Reuptake of GABA from the extracellular space back into the terminal and glial cells in the CNS by plasma membrane GABA transporters (Borden, 1996; Jensen *et al.*, 2003) is very important in terminating and/or limiting GABA actions (Overstreet & Westbrook, 2003) as well as playing a role in maintaining extracellular GABA level at equilibrium. Four types of GATs have so far been described in the mammalian CNS, GAT1–3 and betaine/GABA transporter,

with GAT1 being present in large amounts in regions that are rich in GABAergic neurons (Guastella *et al.*, 1990; Borden, 1996; Jensen *et al.*, 2003). Indeed, GAT1 has been found on GABAergic terminals as well as on glia using immunocytochemical and electron microscopic techniques (Minelli *et al.*, 1995; Conti *et al.*, 1998). As a large fraction (>90%) of neurons in the NAc are GABAergic (Pennartz *et al.*, 1994), GAT1 may play a very important function here to regulate the extracellular levels of GABA. Our finding that NO-711, a selective GABA reuptake inhibitor and a potent GAT1 inhibitor (Borden *et al.*, 1994; Soudijn & Wijngaarden, 2000), caused a GABA_B receptor-mediated depression of the evoked EPSC that occluded the actions of E139 suggests that this enaminone may utilize the same transporter and/or mechanism as NO-711 to enhance extracellular GABA levels. The exact mechanism by which the enaminones may alter the function of GAT1 to produce the observed effects is not yet known. Several possibilities however exist. For example, if an anticonvulsant enaminone works like NO-711, it may inhibit the transporter causing a pooling of GABA in the extracellular space. Alternatively, it may also engage this transporter at the expense of GABA with the same end result. Finally, it may cause the transporter to reverse and engage in nonvesicular GABA release (Wu *et al.*, 2001; 2003; Richerson & Wu, 2003).

In addition to a possible interaction with GAT1 to alter GABA levels, our results also indicate that anticonvulsant enaminones may inhibit GABA-T as the suicide GABA-T inhibitor, γ vG also occluded the ability of E139 to depress EPSCs. If E139 works by inhibiting GABA-T, its mechanism of action may be different from that of γ vG as its effects reversed quite rapidly whereas γ vG effect was irreversible. From our results, E139 and other anticonvulsant enaminones may enhance extracellular GABA levels by one or the other of the above mechanisms, or even both. It is possible that anticonvulsant enaminones may enter neurons to inhibit GABA-T resulting in an increase in cytosolic GABA concentration. This can then trigger a reversal of the GABA transporter leading to nonvesicular release (Wu *et al.*, 2001; 2003) that is thought to contribute to the anticonvulsant effects of established drugs such as γ vG.

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Structural requirements for anticonvulsant activity of enaminones

Our data on synaptic transmission is consistent with data obtained in *in vivo* studies that show that the enaminones E118 and E139 are potent (class 1) anticonvulsant agents, while E169 is inactive (Edafiogho *et al.*, 1994). The main structural difference in these enaminones is the hydroxyl (OH) group in the inactive E169 compared to the halide moieties (Cl and Br) in the anticonvulsant analogues. This is a classical example of pharmacologically active compounds whereby activity is abolished by hydroxylation at the *para* position of the phenyl ring. For example, carbamazepine, a well-known anticonvulsant agent is inactivated by hydroxylation at the *para* position of a phenyl ring *in vivo* (Edafiogho & Scott, 1996). *In vivo* metabolic studies of E121 in rats detected de-esterification and decarboxylation products as the major metabolites (Khurana *et al.*, 2003). Since no metabolites were found that had a hydroxyl group in place of the halide on the phenyl ring, these metabolites may also possess anticonvulsant activity. Further studies need to be conducted on these metabolites to verify if they retain anticonvulsant activity as the basic pharmacophoric structure is still maintained.

In conclusion, the effects of the anticonvulsant enaminones on fast excitatory synaptic transmission observed in this study may contribute to their anticonvulsant effects reported *in vivo*. Although our current data are consistent with an action of these enaminones to alter GABAergic transmission, these compounds may have additional actions on neurons that may also contribute to their anticonvulsant effects (Scott *et al.*, 1995). Further studies, including specific binding and biochemical experiments with these anticonvulsant enaminones, are required to determine the nature of their interaction with GABA_B receptors, GABA transporters and/or GABA-T enzyme.

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