

# Group II mGlu Receptor Activation Suppresses Norepinephrine Release in the Ventral Hippocampus and Locomotor Responses to Acute Ketamine Challenge

Daniel S Lorrain<sup>\*,1,2</sup>, Hervé Schaffhauser<sup>1,2</sup>, Una C Campbell<sup>1</sup>, Christopher S Baccell<sup>1</sup>, Lucia D Correa<sup>1</sup>, Blake Rowe<sup>1</sup>, Dana E Rodriguez<sup>1</sup>, Jeffery J Anderson<sup>1</sup>, Mark A Varney<sup>1</sup>, Anthony B Pinkerton<sup>1</sup>, Jean-Michel Vernier<sup>1</sup> and Linda J Bristow<sup>1</sup>

<sup>1</sup>Department of Neuropharmacology, Merck Research Laboratories, San Diego, CA, USA

Group II mGlu receptor agonists (eg LY379268 and LY354740) have been shown to reverse many of the behavioral responses to PCP as well as glutamate release elicited by PCP and ketamine. In the present set of experiments, we used *in vivo* microdialysis to show that, in addition to reversing PCP- and ketamine-evoked glutamate release, group II mGlu receptor stimulation also prevents ketamine-evoked norepinephrine (NE) release. Pretreating animals with the mixed 2/3 metabotropic glutamate (mGlu2/3) receptor agonist LY379268 (0.3–10 mg/kg) dose-dependently inhibited ketamine (25 mg/kg)-evoked NE release in the ventral hippocampus (VHipp). Ketamine hyperactivity was also reduced in a similar dose range. Following our initial observation on NE release, we conducted a series of microinjection experiments to reveal that the inhibitory effects of LY379268 on VHipp NE release may be linked to glutamate transmission within the medial prefrontal cortex. Finally, we were able to mimic the inhibitory effects of LY379268 on ketamine-evoked NE release by using a novel mGlu2 receptor selective positive modulator. (+/–) 2,2,2-Trifluoroethyl [3-(1-methyl-butoxy)-phenyl]-pyridin-3-ylmethyl-sulfonamide (2,2,2-TEMPS, characterized through *in vitro* GTP $\gamma$ S binding) at a dose of 100 mg/kg significantly reduced the NE response. Together, these results demonstrate a novel means to suppress noradrenergic neurotransmission (ie by activating mGlu2 receptors) and may, therefore, have important implications for neuropsychiatric disorders in which aberrant activation of the noradrenergic system is thought to be involved.

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## INTRODUCTION

Ketamine and phencyclidine (PCP) administered to humans produce symptoms common to schizophrenia (Javitt and Zukin, 1991) and have been used in rodents to model the behavioral and neurochemical abnormalities associated with schizophrenia. Upon acute challenge, ketamine and/or PCP activates limbic forebrain release of dopamine (DA) (Svensson, 2000), serotonin (5-HT) (Lindefors *et al*, 1997; Martin *et al*, 1998), norepinephrine (NE) (Kubota *et al*, 1999a,b), and glutamate (Moghaddam *et al*, 1997; Moghaddam and Adams, 1998; Adams and Moghaddam, 2001;

Lorrain *et al*, 2003) while decreasing the release of GABA (Yonezawa *et al*, 1998).

It is not clear which (if any) of these responses underlie the behavioral aberrations caused by these drugs. Recent findings, however, have highlighted the importance of glutamate neurotransmission. Pretreating animals with a mixed 2/3 metabotropic glutamate (mGlu2/3) receptor agonist (LY354740 or LY379268 (Monn *et al*, 1999)) prevented PCP and ketamine-evoked glutamate release, respectively, and LY354740 reversed the behavioral effects of PCP (Moghaddam and Adams, 1998; Cartmell *et al*, 1999; Lorrain *et al*, 2003).

In addition to blocking forebrain glutamate release, LY354740 has also been shown to inhibit the firing of neurons within the locus coeruleus (LC) (Vandergriff and Rasmussen, 1999) and thus may reduce NE release in terminal regions. Importantly, clinical evidence exists linking a hyperactive noradrenergic system to the pathophysiology of schizophrenia (Baldessarini *et al*, 1992). Firstly, atypical antipsychotics have a broad spectrum of activities including  $\alpha$ -adrenergic antagonistic properties

\*Correspondence: Dr DS Lorrain, Merck Research Laboratories, 3535 General Atomics Court, San Diego, CA 92121, USA, Tel: +1 858 202 5217, Fax: +1 858 202 5813, E-mail: Daniel.Lorrain@merck.com

<sup>2</sup>Both authors participated equally to the study.

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(Schotte *et al*, 1996). Secondly, NE levels have been shown to be elevated in cerebral spinal fluid and plasma of drug-free patients (Lake *et al*, 1980; Breier *et al*, 1990; van Kammen *et al*, 1990), while post-mortem evidence revealed an increase of NE in brain tissue taken from schizophrenic patients (Farley *et al*, 1978; Crow *et al*, 1979). Finally, blockade of  $\alpha$ -adrenergic receptors in rodents has been shown to inhibit the motor-stimulant effects of NMDA antagonists (Mathe *et al*, 1996) while also preventing the disruption of prepulse inhibition (Bakshi and Geyer, 1997, 1999). Together, these data suggest that decreasing norepinephrine neurotransmission may be beneficial for improving clinical outcome in schizophrenia.

In the present study, we used microdialysis to show that, in addition to reversing ketamine-evoked glutamate release, the mGlu2/3 receptor agonist LY379268 also reduces ketamine-evoked NE release. Similarly, ketamine hyperactivity was reduced by LY379268. As our earlier findings showed that LY379268 could inhibit glutamate release (Lorrain *et al*, 2003), we tested here whether ketamine-evoked NE release is indeed glutamate dependent. Through a series of microinjection experiments, we provide evidence that the group II effect on NE release may be a downstream consequence of reduced glutamate transmission within the medial prefrontal cortex. Finally, the mGlu2/3 receptor agonist effect on ventral hippocampus (VHipp) NE was mimicked by treating animals with an mGlu2 receptor selective potentiator (+/–) 2,2,2-Trifluoroethyl [3-(1-methyl-butoxy)-phenyl]-pyridin-3-ylmethyl-sulfonamide (2,2,2-TEMPS).

## MATERIALS AND METHODS

### Subjects

Male Sprague–Dawley rats (250–300 g) were purchased from Harlan (San Diego, CA) and were used for all microdialysis and locomotor experiments; Wistar rats (2–4 weeks old) were purchased from Harlan (Indianapolis, IN) and were used for *in vitro* characterization of 2,2,2-TEMPS. All animals were maintained under a 12 h light/dark cycle with lights on at 0630 and off at 1830. All microdialysis and behavioral experiments occurred during the light phase of the light/dark cycle. All procedures were performed in accordance with *The Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Before and after surgeries, rats were allowed free access to standard rat chow and water. Temperature and relative humidity were maintained at 22–24°C and 50–55%, respectively.

### Materials

Ketamine (in the form of Ketaset, 100 mg/ml) was obtained from Fort Dodge Animal Health (Fort Dodge, Iowa). LY379268 and 2,2,2-TEMPS were synthesized in house (Merck Research Laboratories, San Diego). LY341495 and CNQX were purchased from TOCRIS (Ellisville, MO). Glutamate, guanosine 5'-diphosphate (GDP), probenecid, and guanosine 5'-[ $\gamma$ -thio]triphosphate (GTP $\gamma$ S), were obtained from Sigma Chemical (St Louis, MO). [ $^{35}$ S]GTP $\gamma$ S (1250 Ci/mmol), and Unifilter GF/B plates and Microscint

20 were purchased from Perkin-Elmer Life Science (Boston, MA).

### Intracranial Implants

Under 3% isoflurane (in 1% O<sub>2</sub>) anesthesia, rats were implanted with a unilateral microdialysis probe CMA/11 with a 3 mm dialysis tip in the VHipp (anteroposterior, –5.3 mm; mediolateral, –4.6 mm; and dorsoventral, –7.5 mm) (Paxinos and Watson, 1998). The microdialysis probe was slowly lowered into position and was then fixed to the skull by means of three anchoring screws and application of dental acrylic. For microinjection experiments, in addition to a VHipp microdialysis probe, animals received a CMA/7 guide cannula aimed at either the mPFC (anteroposterior, +3.2 mm; mediolateral, –0.7 mm; and dorsoventral, –4.0 mm) or lateral ventricle (anteroposterior, –0.8 mm; mediolateral, +1.4 mm; and dorsoventral, –3.0 mm). Immediately following surgery, rats were placed in their testing arena and allowed to recover for a minimum of 14 h.

### In Vivo Microdialysis and Chromatography

For all microdialysis experiments, rats were tethered to a CMA awake animal system by means of a plastic collar (CMA Microdialysis, Acton, MA). All fluid connections were made using FEP tubing (internal volume of 1.2  $\mu$ l/100 mm, CMA). Following the appropriate postsurgery recovery period ( $\sim$ 0014 hours =  $\sim$ 0700 hours), perfusion through the dialysis probe with artificial cerebral spinal fluid (aCSF; 145 mM NaCl; 2.7 mM KCl; 1.0 mM MgCl<sub>2</sub>; and 1.2 mM CaCl<sub>2</sub>; pH 7.4) was increased and maintained at 3.0  $\mu$ l/min. A 2-h stabilization period was then allowed prior to sample collection. Fractions were collected at 20-min intervals (60  $\mu$ l) in 250  $\mu$ l glass vials via a BAS honeycomb fraction collector maintained at 4°C (BAS HoneyComb; BAS, West Lafayette, IA). Samples were subsequently analyzed for neurotransmitter content by HPLC (see details below) within a 24-h period.

NE, 5-HT, and HVA were assayed by HPLC coupled to electrochemical detection. Samples were loaded into an ESA autosampler (model 465), separated on a C18 reverse phase column (Phenomenex Luna, 150  $\times$  2.0 mm, 3  $\mu$ m particles) and detected by an ESA Coulochem controller unit using a model 5041 microdialysis analytical cell fitted with a glassy carbon electrode set to oxidize at +250 mV. The mobile phase used was a phosphate buffer purchased from ESA (MD-TM). The rate of mobile phase flow through the system was 0.2 ml/min. NE, HVA, and 5-HT eluted with a retention time of approximately 4.5, 10, and 16 min, respectively, under these isocratic conditions.

### Histology

At the completion of each experiment, rats were euthanized by CO<sub>2</sub> inhalation and their probes perfused with approximately 200  $\mu$ l of cresyl-violet dye. Animals implanted with a guide cannula received approximately 5–10  $\mu$ l of dye delivered to the brain via the microinjection cannula. Brains were then removed and either a single (probe-only animals) or double (probe + guide cannula animals) coronal

cut was made at the level where the probe (or guide cannula) penetrated the cortex. The resulting coronal section(s) was/were placed onto a glass slide and an image scanned into a personal computer using an HP ScanJet 7400L scanner. Only those animals demonstrating dye within the appropriate regions of the brain were included in the results.

### Inhibition of Stimulant-Induced Hyperactivity

In order to evaluate the effects of LY379268, rats were habituated to individual activity chambers (Med Associates Inc., Georgia, VT) for 60 min. Following habituation, subjects were injected with LY379268 (0.3–10 mg/kg) or vehicle and returned to the chambers. After a 30-min pretreatment period, rats were dosed with either vehicle or ketamine (25 mg/kg), and activity was recorded in 10 min intervals for 120 min. Antagonist experiments using the selective mGlu2/3 antagonist, LY341495, involved coinjecting LY341495 (1 mg/kg) with LY379268 (3 mg/kg) or vehicle.

### Administration of Drugs

For systemic administration, drugs were prepared in saline (pH adjusted to 7.0) with the exception of 2,2,2-TEMPS, which was dissolved in a 50% polyethylene glycol solution. All drugs were delivered subcutaneously (s.c.) in a volume of 1 ml/kg. All experiments had appropriate vehicle controls. For central administration, drugs were prepared in aCSF (pH adjusted to 7.4). Local delivery of LY379268 to the VHipp was accomplished via the microdialysis probe by manually switching the inlet tubing from a syringe containing aCSF to one containing a 1 mM concentration of drug. For the mPFC microinjection experiment, the drug was delivered via a CMA microinfusion pump in a volume of 1  $\mu$ l over a 2-min period. For the intracerebral ventricular (i.c.v.) experiment, the drug was delivered in a 10  $\mu$ l volume at a rate of 2  $\mu$ l/min. In both cases, this was accomplished by removing the obturator from the guide cannula and inserting a microinjection cannula without removing the animals from their testing environment.

### Membrane Preparation and [<sup>35</sup>S]GTP $\gamma$ S Binding

hmGlu2 and hmGlu3 receptor-expressing stable cell lines (Varney *et al.*, 1999) were grown to 80–90% confluency in T-225 cm<sup>2</sup> flasks and washed twice with ice-cold PBS. The cells were then scraped with a cell scraper in PBS and harvested by centrifugation (200g). The cell pellet was homogenized in buffer A containing HEPES (20 mM), EDTA (10 mM), pH = 7.4 using a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 40 000g for 20 min. The resulting pellet was washed once in the same buffer and once with buffer B containing HEPES (20 mM) and EDTA (0.1 mM) pH = 7.4. At the last centrifugation, the pellet was resuspended in buffer B and the homogenate was aliquoted and stored at –80°C at a protein concentration of approximately 1 mg/ml. Protein measurement was determined with the Bio-Rad DC kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Whole rat brain membranes were prepared. Rats (250–300 g) were decapitated and the whole brain removed on ice

and homogenized in 6 vol (w/v) of 10% sucrose at 4°C using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000g for 10 min, and the supernatant was centrifuged at 40 000g for 20 min. The pellet was resuspended in 25 ml water using a Polytron homogenizer and centrifuged at 8000g for 10 min. The supernatant was further centrifuged at 40 000g for 20 min at 4°C. The supernatant was removed and the pellet was resuspended in buffer C containing HEPES (5 mM)–KOH pH = 7.4). The homogenate was freeze-thawed twice before being centrifuged at 40 000g for 20 min. Finally, the resulting pellet was resuspended in buffer C, aliquoted, and stored at –70°C until use.

### [<sup>35</sup>S]GTP $\gamma$ S Binding Assay

Membranes from stably transfected cells or from rat brain were thawed and homogenized in 25 ml of buffer B, and centrifuged at 40 000g for 20 min. The pellet was resuspended in assay buffer containing HEPES (20 mM) (pH 7.4), NaCl (100 mM), MgCl<sub>2</sub> (3 mM) at a final protein concentration of 0.5 mg/ml (hmGlu2 and hmGlu3 receptors) or 0.1 mg/ml (rat brain). In a Beckmann 96-well plate, test compounds were added along with GDP (5  $\mu$ M), membrane (10  $\mu$ g/well for rat brain and 50  $\mu$ g for recombinant mGlu receptors), [<sup>35</sup>S]GTP $\gamma$ S (0.05 nM) to achieve a total volume of 0.5 ml in assay buffer. The plate was incubated at 30°C for 1 h and the assay was terminated by rapid filtration over Unifilter GF/B plate using a 96-well cell harvester (Brandel Gaithersburg, MD). The plate was rinsed three times with ice-cold assay buffer, dried and 50  $\mu$ l of Microscint 20 was added to each well. The plate was counted in a Topcount scintillation counter (Perkin-Elmer, Boston MA).

Nonspecific binding was determined by addition of 10  $\mu$ M unlabeled GTP $\gamma$ S. Each experiment was performed using triplicate samples per data point and then repeated on separate occasions to obtain a total of three determinations. Data were normalized to the maximal response obtained with 1 mM glutamate. The curves were fitted to a four-parameter logistic equation giving EC<sub>50</sub> values, Hill coefficient and maximal effect using Prism (GraphPad Software, San Diego, CA).

### Effect of 2,2,2-TEMPS on hmGlu5a and hmGlu7a Receptors

The activity of 2,2,2-TEMPS was examined against hmGlu5a or hmGlu7a receptors stably expressed in mouse fibroblast Ltk cells or CHO cells, respectively. To couple the activation of hmGlu7a receptors to changes in [Ca<sup>2+</sup>]<sub>i</sub>, hmGlu7a was stably expressed with G $\alpha$ 16. Receptor activity was detected by changes in [Ca<sup>2+</sup>]<sub>i</sub>, measured using the fluorescent Ca<sup>2+</sup>-sensitive dye Fura-2 as previously described by Varney *et al.* (1999) for mGlu5 activity or Fluo-3 on a VIPR-2 (Aurora Biosystems) for mGlu7 activity. For antagonist testing, a submaximal concentration of glutamate was used (10  $\mu$ M and 2 mM for hmGlu5 and hmGlu7 receptor, respectively).

### Data Analysis

Dialysis data collected and analyzed were not corrected for *in vitro* probe recovery. Time course data represent the

percent change from baseline (calculated by taking the average of two pretreatment samples). Summary data represent the raw area under each 120-min time course curve. Data were then subject to either a simple between-subjects *t*-test or to a one-factor ANOVA. *Post hoc* analyses were carried out using Dunnett's (for comparison to a single control group) or Student–Newman–Keuls tests (for all pairwise comparisons). Activity data (total distance traveled) represent the group mean ( $\pm$  SEM) recorded for the total duration of the 120-min test period. Data were analyzed by one-factor ANOVA followed by Dunnett's or Student–Newman–Keuls tests.

## RESULTS

### Group II mGlu Receptor Modulation of Ketamine-Evoked NE Release

Systemic administration of ketamine (12.5–50 mg/kg, s.c.) resulted in a dose-dependent increase in hippocampal NE release ( $F(3,13) = 42.3$ ,  $P < 0.001$ ; Figure 1a, b). Dunnett's *post hoc* comparisons revealed a significantly greater area under the curve in dialysates from animals treated with 25 and 50 mg/kg ketamine compared to vehicle controls ( $P < 0.05$ ). Pretreating animals with the mGlu2/3 receptor agonist LY379268 (0.3–10 mg/kg, s.c.) inhibited ketamine-evoked NE release in a dose-dependent fashion ( $F(3,21) = 15.9$ ,  $P < 0.001$ ; Figure 1c, d). Dunnett's *post hoc* comparisons revealed significant inhibition with 3 and 10 mg/kg LY379268 compared to vehicle controls ( $P < 0.05$ ). This effect of LY379268 (3 mg/kg) was reversed by cotreating animals with the mGlu2/3 receptor antagonist LY341495 (3 mg/kg) ( $F(3,19) = 7.6$ ,  $P < 0.01$ ; Figure 1e, f). Consistent with the locomotor data (see below), administration of the antagonist LY34149 (3 mg/kg) alone produced a marked increase in ketamine-evoked NE release ( $P < 0.05$ ). In a subset of animals, we tested whether the effect of LY379268 was selective for NE or whether it would generalize to all monoamine systems. Ketamine evoked a significant increase in both 5-HT and the DA metabolite HVA (DA itself was below detection limits in the dialysate collected). Pretreating animals with a high dose of LY379268 (10 mg/kg) failed to alter these responses. The ANOVA conducted on 5-HT revealed a significant overall effect of ketamine on 5-HT release ( $F(2,9) = 12.3$ ,  $P < 0.01$ ; Figure 2). Using Student–Newman–Keuls *post hoc* comparisons, we observed a significant effect of ketamine on 5-HT with and without LY379268 ( $P < 0.05$ ). No difference was observed between groups receiving ketamine alone vs ketamine + LY379268. Similar to 5-HT, the ANOVA conducted on HVA revealed a significant overall effect of ketamine on HVA release ( $F(2,11) = 11.8$ ,  $P < 0.01$ ; Figure 2). Again, like the 5-HT data, *post hoc* comparisons revealed a significant effect of ketamine on HVA regardless of pretreatment with LY379268 ( $P < 0.05$ ). No difference was observed between groups receiving ketamine alone vs ketamine + LY379268.

### Group II mGlu Receptor Modulation of Ketamine Hyperactivity

Ketamine (12.5–50 mg/kg, s.c.) dose-dependently increased locomotor activity in habituated rats as shown by an

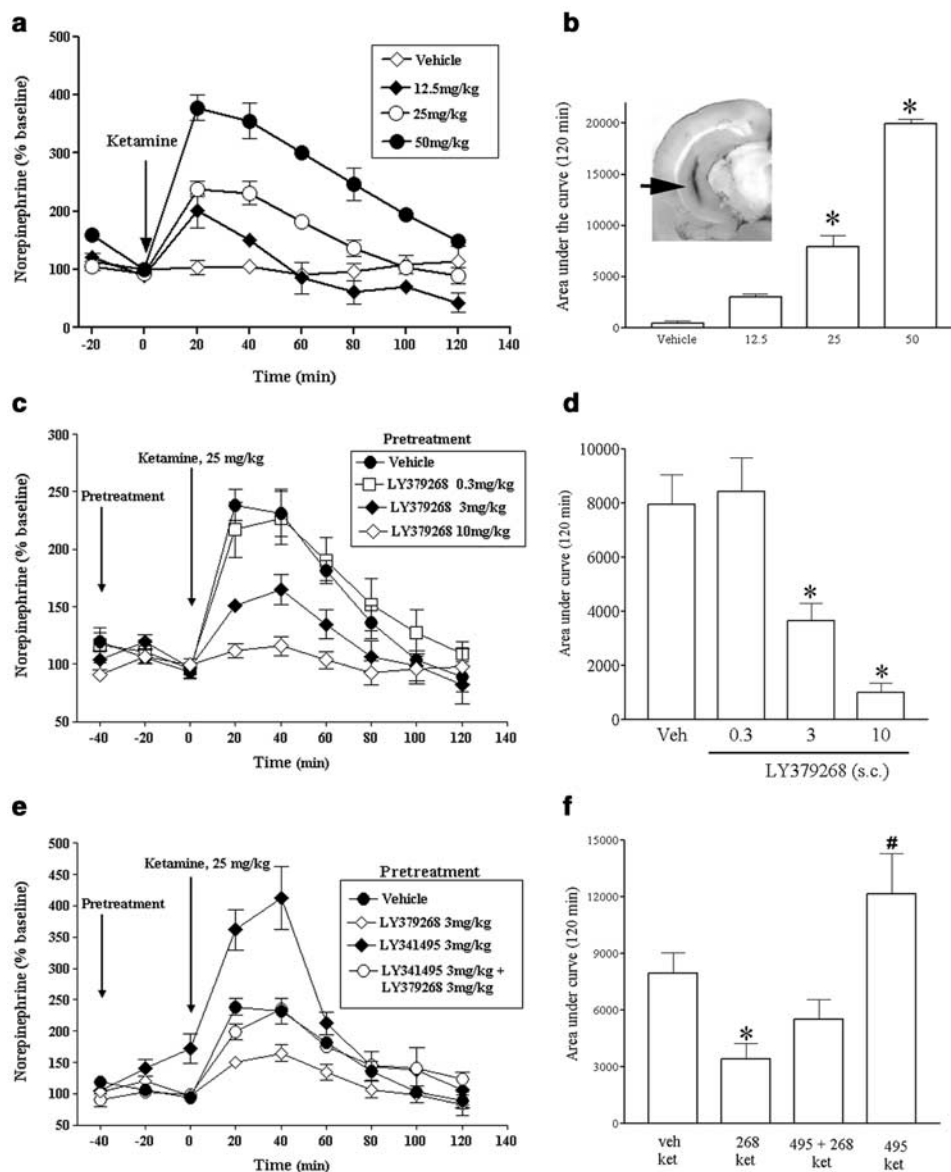
increase in the total distance traveled ( $F(3,28) = 14.15$ ,  $P < 0.001$ ; Figure 3a). Dunnett's *post hoc* analyses indicated a significant hyperactivity response following administration of 25 and 50 mg/kg of ketamine compared to vehicle-treated animals ( $P < 0.05$ ). Acute administration of LY379268 (0.3–10 mg/kg, s.c.) dose-dependently suppressed ketamine (25 mg/kg, s.c.)-induced hyperactivity ( $F(5,82) = 33.09$ ,  $P < 0.001$ ; Figure 3b). *Post hoc* testing revealed a significant inhibition of the ketamine hyperactivity response following administration of 1, 3, and 10 mg/kg of LY379268 ( $P < 0.05$ ). The inhibition of ketamine (25 mg/kg, s.c.) hyperactivity responses by LY379268 (3 mg/kg, s.c.) was reversed following the administration of LY341495 (1 mg/kg, s.c.). LY341495 (1 mg/kg) was also found to significantly potentiate the hyperactivity response induced by ketamine ( $F(5,42) = 25.84$ ,  $P < 0.001$ ; Figure 3c).

### Modulation of Ketamine-Evoked NE Release by Central Administration of LY379268 and CNQX

As our earlier findings convincingly showed that LY379268 could inhibit glutamate release (Lorrain *et al*, 2003), we questioned whether ketamine-evoked NE release is indeed glutamate dependent. To test this, we delivered the AMPA/kainate antagonist CNQX (15 nmol, i.c.v.) prior to systemic ketamine injection and compared this effect to that seen after i.c.v. LY379268 (1 nmol) pretreatment. The ANOVA conducted on the area under the curve data revealed a significant effect on NE release between treatment groups ( $F(2,13) = 9.38$ ,  $P < 0.01$ ; Table 1). Dunnett's *post hoc* comparisons showed that NE release in both LY379268- and CNQX-pretreated animals was significantly reduced compared to vehicle-treated controls ( $P < 0.05$ ). Next we focused on identifying the site of action of LY379268. To accomplish this, LY379268 was microinjected into discrete brain regions; (1) the VHipp (accomplished by reverse dialyzing 1 mM concentration of drug through the microdialysis probe) and (2) the mPFC (accomplished by microinjecting 0.1 nmol of LY379268 via a microinjection cannulae). We observed no significant effect of LY379268 on ketamine-evoked NE release when delivered to VHipp ( $t(9) = 0.57$ ,  $P = 0.58$ ; Table 1). When delivered directly to the mPFC, on the other hand, we observed a significant inhibition by LY379268 on ketamine-evoked NE release ( $t(8) = 5.0$ ,  $P < 0.001$ ; Table 1). Importantly, we showed in a previous experiment that ketamine increases extracellular glutamate levels in the mPFC and that local delivery of LY379268 could block this glutamate response (Lorrain *et al*, 2003). Together with our current findings, we hypothesized that LY379268 inhibition of ketamine-evoked NE release is a direct downstream consequence of LY379268 inhibition of mPFC glutamate release.

### Inhibition of Ketamine-Evoked NE Release by the mGlu2 Receptor Selective Positive Modulator 2,2,2-TEMPS

*In vitro characterization of 2,2,2-TEMPS.* In the current experiment, we tested whether the effects of the mixed mGlu2/3 receptor agonist LY379268 could be mimicked by an mGlu2 receptor selective glutamate positive modulator.

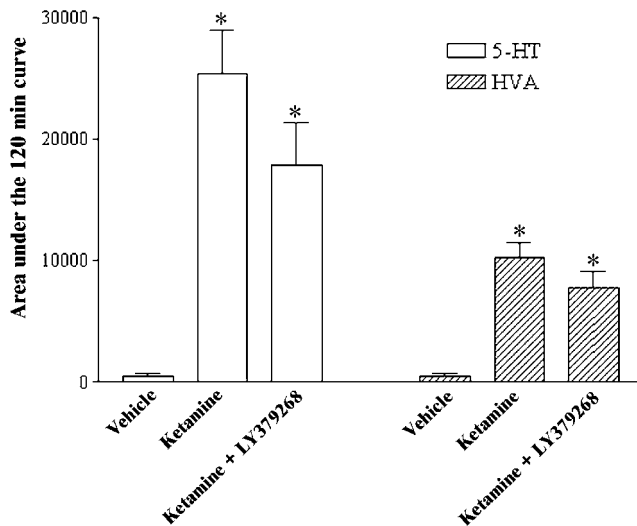


**Figure 1** Modulation of ketamine-evoked NE release by the mGlu2/3 receptor agonist LY379268. (a, c, and e) Microdialysis time course data. (b, d, and f) Summary data showing the area under each 120-min time course curve. (b, inset) Representative coronal section showing the spread of dye from the active portion of the dialysis membrane to the surrounding tissue in an individual subject with an appropriately placed microdialysis probe. (a, b) Ketamine (12.5–50 mg/kg, s.c.) produced a dose-dependent increase in VHipp NE release. (c, d) LY379268 (0.3–10 mg/kg, s.c.) given 30 min prior to ketamine (25 mg/kg, s.c.) dose-dependently inhibited ketamine-evoked NE release. (d, e) This effect was reversed by cotreatment with the group II mGlu receptor selective antagonist LY341495 (3 mg/kg LY379268 + 3 mg/kg LY341495). LY341495 alone significantly increased ketamine-evoked NE release. Data are expressed as mean  $\pm$  SEM. NE levels were estimated to be  $15 \pm 1$  fg/ $\mu$ l of sample;  $n = 3$ –7/group. Data were analyzed by a one-way ANOVA followed by Dunnett's or Student–Newman–Keuls tests. \* $P < 0.05$  compared to vehicle/ketamine-treated rats. # $P < 0.05$  compared to vehicle/ketamine, LY379268/ketamine, and LY341495 + LY379268/ketamine.

Prior to *in vivo* testing, we used [ $^{35}$ S]GTP $\gamma$ S binding to demonstrate that 2,2,2-TEMPS is indeed a selective mGlu2 receptor potentiator. 2,2,2-TEMPS on its own did not stimulate [ $^{35}$ S]GTP $\gamma$ S binding in membranes prepared from cells expressing hmGlu2 receptors, hmGlu3 receptors or rat brain (data not shown). However, in the presence of a low concentration of glutamate that evoked a small response (an approximate EC<sub>10</sub> of 1  $\mu$ M for rat brain and hmGlu2 and 10 nM for hmGlu3 receptors), 2,2,2-TEMPS increased the magnitude of the glutamate-induced [ $^{35}$ S]GTP $\gamma$ S binding in a concentration-dependent manner. Interestingly, while the EC<sub>50</sub> values for 2,2,2-TEMPS were similar under these

conditions, the magnitude of the potentiation was four times higher in rat brain compared to hmGlu2 membranes (Figure 4a). In contrast, 2,2,2-TEMPS was without effect on glutamate-induced [ $^{35}$ S]GTP $\gamma$ S binding in membranes prepared from cells expressing hmGlu3 receptors (Figure 4a).

Additionally, 2,2,2-TEMPS was tested for its ability to potentiate glutamate-stimulated responses in cell lines expressing human group I (hmGlu5) and group III (hmGlu7) mGlu receptors. 2,2,2-TEMPS was devoid of any agonist, antagonist, or allosteric modulator activities on these mGlu receptor subtypes (Table 2).



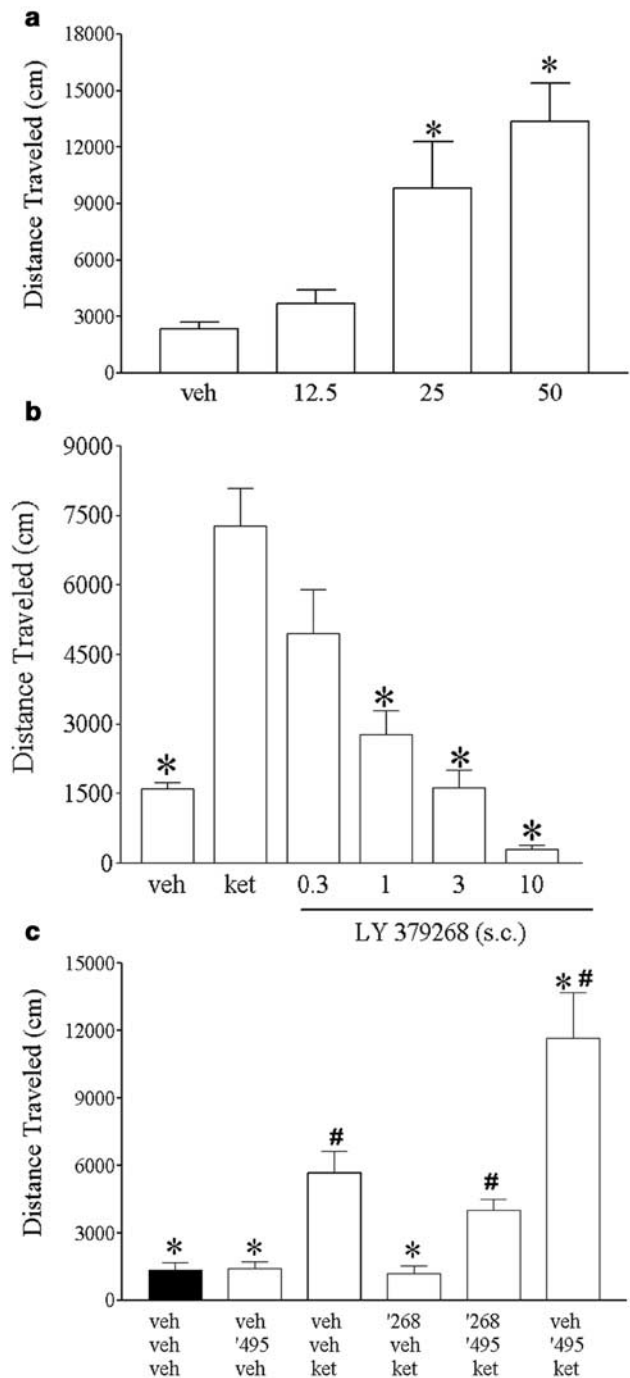
**Figure 2** Effects of the mGlu2/3 receptor agonist LY379268 on ketamine (25 mg/kg, s.c.)-evoked 5-HT and HVA release. Unlike the NE response, neither 5-HT nor HVA were significantly altered by pretreating animals with LY379268 (10 mg/kg, s.c.). Data (mean  $\pm$  SEM) are expressed as the area under each respective 120-min time course curve. 5-HT and HVA levels were estimated to be  $4.9 \pm 0.9$  fg/ $\mu$ l of sample and  $244.9 \pm 24.9$  fg/ $\mu$ l of sample, respectively;  $n = 3$ –6/group. \* $P < 0.05$  vs vehicle control as revealed by one-factor ANOVA followed by Dunnett's *post hoc* comparisons.

In order to characterize the effect of this positive modulator further on mGlu2 receptors, concentration-response curves to glutamate were performed using [ $^{35}$ S]GTP $\gamma$ S binding in the presence of a fixed concentration of 2,2,2-TEMPS (3  $\mu$ M). Under these conditions, 2,2,2-TEMPS produced a 2.5-fold increase in the apparent potency ( $EC_{50}$ ) of glutamate and did not further increase the maximum response induced by glutamate on mGlu2 receptors (Table 3). Similar studies carried out in recombinant cell lines expressing hmGluR3 showed no change in either the  $EC_{50}$  or the  $E_{max}$  for glutamate-induced stimulation of [ $^{35}$ S]GTP $\gamma$ S binding. These results suggest that 2,2,2-TEMPS is a selective mGlu2 receptor positive modulator that increases both the affinity and the efficacy of an agonist ligand.

**Effect of 2,2,2-TEMPS on ketamine-evoked NE release.** Systemic administration of the mGlu2 receptor selective potentiator 2,2,2-TEMPS at a dose of 100 mg/kg, s.c. significantly inhibited ketamine-evoked hippocampal NE release ( $F(2,24) = 5.65$ ,  $P < 0.01$ ; Figure 4b, c). This effect of 2,2,2-TEMPS was reversed by the selective mGlu2/3 receptor antagonist LY341495 (3 mg/kg, s.c.).

## DISCUSSION

The present study demonstrates an important role for group II mGlu receptors in the modulation of ketamine-evoked NE release and ketamine hyperactivity. Upon acute challenge, ketamine increased extracellular levels of NE (as well as 5-HT and HVA) in the VHipp. We chose this region because of earlier work showing strong activation of 2-deoxyglucose uptake in the hippocampus following systemic ketamine



**Figure 3** Modulation of ketamine hyperactivity by the mGlu2/3 receptor agonist LY379268. (a) Ketamine (12.5–50 mg/kg, s.c.) produced a dose-dependent increase in locomotor activity. (b) LY379268 (0.3–10 mg/kg, s.c.) produced a dose-dependent suppression of ketamine (25 mg/kg, s.c.)-induced hyperactivity. (c) The inhibition of ketamine hyperactivity by LY379268 (3 mg/kg) was reversed by the mGlu2/3 receptor antagonist LY341495 (1 mg/kg). LY341495 (1 mg/kg) significantly potentiated ketamine-evoked hyperactivity. Subjects were dosed with LY379268 and/or LY341495 or vehicle 30 min before receiving s.c. injections of ketamine. Activity data (total distance traveled) are presented as the group mean ( $\pm$  SEM) recorded for the total duration of the 2 h test period. Data were analyzed by a one-way ANOVA followed by Dunnett's or Student–Newman–Keuls tests; \* $P < 0.05$  compared to (a) vehicle or (b) vehicle/ketamine-treated animals; # $P < 0.05$  compared to LY379268/ketamine-treated rats.

**Table 1** Effects of LY379268 and CNQX on Ketamine (25 mg/kg, s.c.)-Evoked NE Release when Administered in Discrete Brain Regions

Brain region	Drug	Dose	AUC (n)	% Inhibition
i.c.v.	Vehicle	0 nmol/10 $\mu$ l	7951 $\pm$ 1103 (8)	0
	CNQX	15 nmol/10 $\mu$ l	2633 $\pm$ 1072* (3)	-67
	LY379268	1 nmol/10 $\mu$ l	2045 $\pm$ 692* (4)	-75
VHipp	Vehicle	0 mM	8073 $\pm$ 1266 (7)	0
	LY379268	1 mM	7034 $\pm$ 802 (4)	-13
mPFC	Vehicle	0 nmol/1 $\mu$ l	6127 $\pm$ 922 (5)	0
	LY379268	0.1 nmol/1 $\mu$ l	1141 $\pm$ 342* (5)	-81

Drugs were either microinjected into the lateral ventricle (i.c.v.), ipsilateral medial prefrontal cortex (mPFC) or perfused directly into the ventral hippocampus (VHipp, via the microdialysis probe). i.c.v. Administration of both LY379268 and the AMPA/kainate antagonist CNQX inhibited ketamine-evoked NE (drug was given 30 min prior to ketamine). No effect was observed when LY379268 was applied directly to the VHipp via the microdialysis probe (drug perfusion was initiated 30 min prior to ketamine and continued throughout the 120 min collection period). Direct injection of LY379268 into the ipsilateral mPFC significantly inhibited ketamine-evoked NE (LY379268 was delivered 10 min prior to ketamine). Data (mean  $\pm$  SEM) are expressed as area under each respective 120-min time course (AUC). \* $P$  < 0.05: Significant effect on AUC compared to vehicle control group as revealed by Dunnett's *post hoc* analysis following ANOVA or *t*-test.

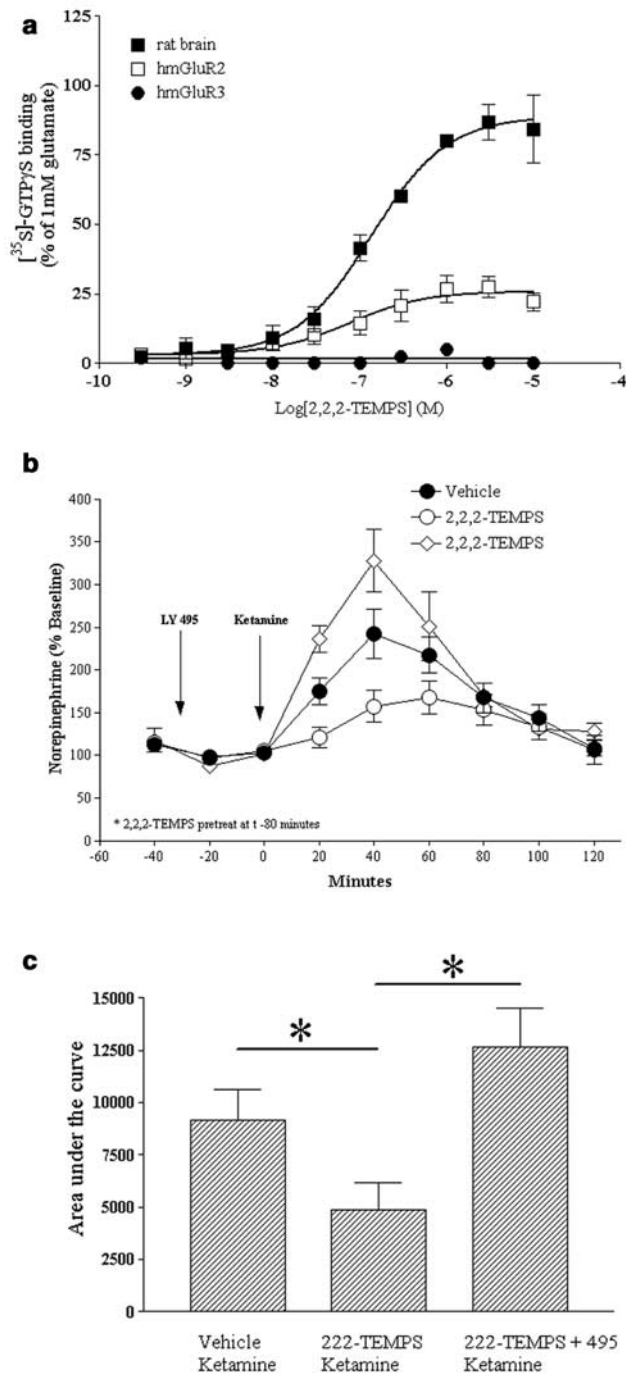
injection (Duncan *et al*, 1999). Pretreating animals with a group II mGlu receptor agonist LY379268 prevented the increase in NE release while having no effect on either 5-HT or HVA. Coadministration of the selective mGlu2/3 receptor antagonist, LY341495, reversed the inhibitory effects of LY379268. LY341495 on its own enhanced ketamine-evoked NE release, an effect also noted in the ketamine hyperactivity tests. This is an important observation and suggests that endogenous glutamate acting on mGlu2/3 receptors partly suppresses NE release and locomotion under conditions of ketamine challenge. To our knowledge, these are the first *in vivo* microdialysis data providing direct evidence that group II mGlu receptors can selectively modulate NE release. These results are consistent with one earlier finding by Vandergriff and Rasmussen (1999), showing that LY354740, a selective but less potent mGlu2/3 receptor agonist (Schoepp *et al*, 1997; Monn *et al*, 1999), can attenuate morphine-withdrawal-induced activation of LC neurons.

We also demonstrate that the inhibition of ketamine-evoked NE release following activation of group II mGlu receptors may be linked to reduced glutamate transmission within the mPFC. First we found that ketamine-evoked NE release was inhibited by i.c.v. administration of the AMPA/kainate receptor antagonist CNQX, suggesting that the ketamine response is dependent on glutamate transmission. In addition, i.c.v. administration of LY379268 diminished ketamine-evoked NE release, thus, establishing that the effect of LY379268 is centrally mediated. Next we showed that LY379268 blocked VHipp NE release when microinjected directly into ipsilateral mPFC, but not when delivered directly to the VHipp. The mPFC was chosen for two reasons. Firstly, studies using [ $^3$ H]-LY354740 revealed a high density of specific binding throughout the cerebral cortex (Schaffhauser *et al*, 1998) and specifically within the mPFC (Marek *et al*, 2000, 2001). Secondly, we have recently shown that ketamine increases the release of glutamate within the mPFC and that perfusing LY379268 directly to the mPFC reduced local glutamate release elicited by systemic ketamine, presumably through presynaptic inhibi-

tion (Lorrain *et al*, 2003). As neuronal pathways exist linking the mPFC to the LC (Takagishi and Chiba, 1991; Buchanan *et al*, 1994), it is likely that prefrontal glutamate may drive LC activity and hence NE release. Therefore, preventing glutamate release in the prefrontal cortex, as in the case with LY379268, may prevent efferent activation of LC and subsequent NE release. This would explain our current findings. If true, we should expect to see an effect of a group II mGlu receptor agonist on LC firing and in fact this has been observed (Dube and Marshall, 1997; Vandergriff and Rasmussen, 1999). In addition, if LY379268 is mediating its effects on NE release through inhibition of LC activity, then we predict a global effect on NE release throughout the forebrain that is not limited to the VHipp. Preliminary experiments from our laboratory support such a model. We cannot, however, rule out a direct connection from the mPFC to the VHipp in mediating the LY379268 effect.

In a manner consistent with the microdialysis data, LY379268 inhibited ketamine-evoked locomotion; an effect that was also reversed by LY341495. Although we cannot convincingly conclude a causal link between NE release and locomotion from the current study, previous reports do support such a relationship. For example, intraventricular injection of NE produced an increase in locomotor activity of rats (Segal and Mandell, 1970). More importantly, locomotor activity increased when NE was microinjected directly into the hippocampus (Flicker and Geyer, 1982). Finally, blockade of  $\alpha$ -adrenergic receptors in rodents has been shown to inhibit the motor-stimulant effects of NMDA antagonists (Mathe *et al*, 1996). It will be important to know whether ketamine hyperactivity can similarly be inhibited by direct infusion of an  $\alpha$ -adrenergic antagonist to the hippocampus.

Finally, we tested whether our effects of LY379268 on NE release and locomotion could be reproduced using an mGlu2 receptor selective ligand. For this purpose we selected 2,2,2-TEMPS, a positive allosteric modulator of the mGlu2 receptor previously reported to produce *in vivo* behavioral effects similar to those produced by mGlu2/3



**Figure 4** *In vitro* and *in vivo* effects of the mGlu2 selective modulator 2,2,2-TEMPS. (a) Stimulation of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding by 2,2,2-TEMPS in the presence of glutamate ( $\text{EC}_{10}$ ) in membranes from rat brain or cell lines stably expressing hmGlu2 or hmGlu3 receptors. Results are expressed as the percentage of the maximal stimulation induced by glutamate (1 mM) alone and are the means of three individual experiments performed in triplicate. Rat brain cortex  $\text{EC}_{50}$  (nM) = 135 (range: 125–144),  $E_{\text{max}}$  =  $88 \pm 8$ ; hmGluR2  $\text{EC}_{50}$  (nM) = 80 (range: 50–114),  $E_{\text{max}}$  =  $23 \pm 2$ ; no effects observed at hmGluR3. (b, c) Systemic administration of 2,2,2-TEMPS (100 mg/kg, s.c.) reduced ketamine-evoked NE release in the rat Vhipp and this effect was reversed by the group II antagonist LY341495 (3 mg/kg, s.c.);  $n = 5$ –11/group. (b) Time course of the effect. (c) Summary data illustrating the area under each 120-min time course curve. The summary microdialysis data were analyzed by ANOVA followed by Student–Newman–Keuls *post hoc* comparisons. \* $P < 0.05$  compared to vehicle/ketamine and LY341495 + 2,2,2-TEMPS/ketamine-treated rats.

**Table 2** Test of any Potentiator, Agonist, or Antagonist Activities of 2,2,2-TEMPS on hmGlu3, hmGlu5, and hmGlu7 Receptor Using Functional Assays

Receptor	Assay	Assay mode	Effect ( $\mu\text{M}$ )
hmGuR3	$[^{35}\text{S}]\text{GTP}\gamma\text{S}$	Potentiator	n.e. at 10
		Agonist	n.e. at 10
		Antagonist	n.e. at 10
hmGuR5	$\text{Ca}^{2+}$	Potentiator	n.e. at 3
		Agonist	n.e. at 3
		Antagonist	n.e. at 3
hmGuR7	$\text{Ca}^{2+}$	Potentiator	n.e. at 3
		Agonist	n.e. at 3
		Antagonist	n.e. at 3

The antagonist effect of 2,2,2-TEMPS was tested against a submaximal concentration of glutamate as described in Materials and methods. Summary data are calculated from three individual experiments performed in triplicate. n.e. = no effect at concentrations up to 3 or 10  $\mu\text{M}$ .

receptor agonists (Johnson *et al*, 2002). Using a functional binding assay, we demonstrated that 2,2,2-TEMPS alone does not alter  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding in membranes prepared from rat brain or from cells stably expressing hmGlu2 receptors, although 2,2,2-TEMPS did markedly enhance glutamate-induced  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding. These effects are similar to those reported on mGlu1 and  $\text{GABA}_{\text{B}(1\text{b}/2\text{A})}$  receptors with Ro 67-7476 (Knoflach *et al*, 2001) and CGP7930 (Urwylar *et al*, 2001), respectively. This positive modulator effect observed with 2,2,2-TEMPS is highly selective for hmGlu2 receptors, since there was a complete absence of any modulatory activity on glutamate-induced  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding or  $\text{Ca}^{2+}$  response in cells stably expressing hmGlu3, hmGlu5, and hmGlu7 receptors. In the presence of 2,2,2-TEMPS, the potency of glutamate was increased approximately two-fold with no change in the maximal response. This change in the potency might reflect a change in the affinity of the receptor for glutamate. A similar effect was observed with Ro 67-7476, which increases the affinity of  $[^3\text{H}]\text{quisqualate}$  for recombinant mGlu1 receptors without having any effect on the  $B_{\text{max}}$ . Also, the  $\text{GABA}_{\text{B}}$  positive allosteric modulator CGP7930 enhances the binding of the  $\text{GABA}_{\text{B}}$  agonist  $[^3\text{H}]\text{APPA}$  in rat brain membranes via an increase in the affinity (three-fold decrease of the  $K_d$ ) without affecting the number of binding sites ( $B_{\text{max}}$ ) (Urwylar *et al*, 2001). 2,2,2-TEMPS was not only a positive modulator at recombinant human mGlu2 receptors but was also effective at increasing  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding to native rat brain mGlu2 receptors. When tested *in vivo*, 2,2,2-TEMPS reduced ketamine-evoked NE release by approximately 50%. This effect was reversed by the selective mGlu2/3 receptor antagonist LY341495. It seems, therefore, that mGlu2 receptor stimulation is sufficient to reduce the NE response to a ketamine challenge. From these and previous experiments (Lorrain *et al*, 2003), we suggest that mGlu2 receptor modulation may be occurring at the level of the mPFC.

### Clinical Implications

Our primary interest for conducting the current set of experiments was to further establish a potential therapeutic



**Table 3** Effect of 2,2,2-TEMPS on Glutamate-Evoked [ $^{35}$ S]GTP $\gamma$ S Stimulation at Human mGlu2 and mGlu3 Receptors

Agonist	hmGlu2		hmGlu3	
	EC <sub>50</sub> (nM)	E <sub>max</sub>	EC <sub>50</sub> (nM)	E <sub>max</sub>
Glutamate	7700 (7200–81 000)	98.14 $\pm$ 1.5	162 (144–178)	100.95 $\pm$ 1.7
+2,2,2-TEMPS	3000 (2950–3100)	109.67 $\pm$ 0.65	200 (155–263)	91 $\pm$ 3

Potency (EC<sub>50</sub>) and efficacy (E<sub>max</sub>) for the stimulation of [ $^{35}$ S]GTP $\gamma$ S binding on recombinant human mGlu2 or mGlu3 receptor preparation in the absence or presence of 3  $\mu$ M 2,2,2-TEMPS. Summary data represent the geometric mean EC<sub>50</sub> values (lower-upper SD) and mean efficacy  $\pm$  SEM (compared to glutamate alone) calculated from three individual experiments performed in triplicate.

link between mGlu2 receptor ligands and schizophrenia. As ketamine (in humans) causes symptoms similar to schizophrenia (Javitt and Zukin, 1991; Tamminga *et al*, 1995), we reason that identifying its CNS effects and then reversing these effects may prove useful in the development of novel antipsychotic drugs. Although much focus has been ascribed to DA regarding the pathophysiology of schizophrenia, noradrenergic systems may also contribute to this illness. As outlined in the introduction, high NE levels have been observed in the cerebral spinal fluid and plasma of drug-free patients (Lake *et al*, 1980; Breier *et al*, 1990; van Kammen *et al*, 1990). Likewise, post-mortem analysis revealed elevated NE in brain tissue taken from schizophrenic patients (Farley *et al*, 1978; Crow *et al*, 1979). Together with our current data, it seems reasonable to suggest that a mixed mGlu2/3 receptor agonist or a selective mGlu2 receptor agonist/potentiator may reduce NE neurotransmission and thus provide some benefit in treating the symptoms associated with schizophrenia.

Modulating NE release may also have important implications in anxiety-related behaviors as well as in the expression of opiate withdrawal. It is well established that novel and stressful stimuli to an awake rat activate the LC and increase the release of NE in forebrain regions (Bremner *et al*, 1996b). Additionally, administration of  $\alpha$ 2 antagonists increases LC firing (Rasmussen and Jacobs, 1986) and increases anxiety-like behaviors (Redmond and Huang, 1979). Agents which decrease LC firing, such as opiates, benzodiazepines, (Drugan *et al*, 1984; Rasmussen and Jacobs, 1986; Tanaka *et al*, 1990), and clonidine (Aghajanian, 1978; Aghajanian and VanderMaelen, 1982), on the other hand, decrease anxiety behaviors. In humans trials, clonidine has been shown to be effective in some, but not all, patients with panic disorder (Bremner *et al*, 1996a). Likewise, Levine *et al* (2002) recently reported anxiolytic effects of LY354740 in human panic patients undergoing CO<sub>2</sub>-induced panic. In a manner consistent with anxiety, behaviors associated with morphine withdrawal appear to be, at least in part, a reflection of LC activity (Aghajanian, 1978; Rasmussen *et al*, 1990; Maldonado and Koob, 1993). As mentioned above, previous experimentation has revealed an effect of the group II mGlu receptor agonist LY354740 on reducing morphine-withdrawal-induced activation of LC neurons and many of morphine withdrawal symptoms (Vandergriff and Rasmussen, 1999).

In conclusion, the mixed mGlu2/3 receptor agonist LY379268 and the mGluR2 selective positive modulator 2,2,2-TEMPS suppressed ketamine-evoked VHipp NE release. This effect appears to be mediated, at least in part, through activation of mGlu receptors located within the mPFC. These results demonstrate a novel means to suppress

noradrenergic neurotransmission (ie by activating mGlu2 receptors) and may, therefore, have important implications for neuropsychiatric disorders in which aberrant activation of the noradrenergic system is thought to be involved (eg schizophrenia, anxiety/panic, morphine/heroin drug withdrawal).

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