Cellular/Molecular

Ketamine, But Not Phencyclidine, Selectively Modulates Cerebellar $GABA_A$ Receptors Containing $\alpha6$ and δ Subunits

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Phencyclidine (PCP) and ketamine are dissociative anesthetics capable of inducing analgesia, psychomimetic behavior, and a catatonic state of unconsciousness. Despite broad similarities, there are notable differences between the clinical actions of ketamine and PCP. Ketamine has a lower incidence of adverse effects and generally produces greater CNS depression than PCP. Both noncompetitively inhibit NMDA receptors, yet there is little evidence that these drugs affect $GABA_A$ receptors, the primary target of most anesthetics. $\alpha6\beta2/3\delta$ receptors are subtypes of the $GABA_A$ receptor family and are abundantly expressed in granular neurons within the adult cerebellum. Here, using an oocyte expression system, we show that at anesthetically relevant concentrations, ketamine, but not PCP, modulates $\alpha6\beta2\delta$ and $\alpha6\beta3\delta$ receptors. Additionally, at higher concentrations, ketamine directly activates these $GABA_A$ receptors. Comparatively, dizocilpine (MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate]), a potent noncompetitive antagonist of NMDA receptors that is structurally unrelated to PCP, did not produce any effect on $\alpha6\beta2\delta$ receptors. Of the recombinant $GABA_A$ receptor subtypes examined ($\alpha1\beta2$, $\alpha1\beta2\gamma2$, $\alpha1\beta2\delta$, $\alpha4\beta2\gamma2$, $\alpha4\beta2\delta$, $\alpha6\beta2\gamma2$, $\alpha6\beta2\delta$, and $\alpha6\beta3\delta$), the actions of ketamine were unique to $\alpha6\beta2\delta$ and $\alpha6\beta3\delta$ receptors. In dissociated granule neurons and cerebellar slice recordings, ketamine potentiated the GABAergic conductance arising from $\alpha6$-containing $GABA_A$ receptors, whereas PCP showed no effect. Furthermore, ketamine potentiation was absent in cerebellar granule neurons from transgenic functionally null $\boldsymbol{\alpha6}^{-/-}$ and $\boldsymbol{\delta}^{-/-}$ mice. These findings suggest that the higher CNS depressant level achieved by ketamine may be the result of its selective actions on $\alpha6\beta2/3\delta$ receptors.

Key words: **ketamine; $\alpha6\beta2/3\delta$ $GABA_A$ receptors; granule neurons; slice recording; cerebellum; transgenic mice; tonic conductance**

Introduction

Ketamine and phencyclidine (PCP) are chemical congeners belonging to a class of pharmacological agents known as dissociative anesthetics. These drugs produce similar clinical actions, although the impact of ketamine on CNS functioning differs from that of PCP and its analogues (Greifenstein et al., 1958; Johnstone et al., 1959; Collins et al., 1960; Chen, 1965; Domino et al., 1965; Corssen and Domino, 1966). Ketamine has a lower potency, a shorter duration of action, a faster rate of induction, results in a lower incidence of adverse emergence reactions, and is an effective anesthetic across different animal species (PCP shows species selectivity). Furthermore, ketamine lacks the convulsive side effects that PCP produces at higher doses. Collectively, these studies suggest that ketamine possesses a higher CNS depressant activity and produces a better quality of anesthesia than PCP (McCarthy et al., 1965).

Received Dec. 10, 2007; revised April 10, 2008; accepted April 10, 2008.

This work was supported by grants from the German Science Foundation (Deutsche Forschungsgemeinschaft Lu 319/2–1) (H.L.), the MAIFOR program of the University of Mainz (W.H.), and American Heart Established Investigator Awards (J.A.). We thank Blake Singletary for help with editing, Stacey Kolar for help with Figures, and Ingo Bohme for genotyping the mouse lines.

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DOI:10.1523/JNEUROSCI.5443-07.2008

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It has been established that dissociative anesthetics are noncompetitive inhibitors of NMDA excitatory ligand-gated ion channels (Lodge and Anis, 1982; Anis et al., 1983; MacDonald et al., 1987, 1991; ffrench-Mullen and Rogawski, 1989; Rogawski and Wenk, 2003). These drugs also modulate the activity of nicotinic acetylcholine, muscarinic, and opioid receptors, and voltage-gated ion channels, but at significantly higher concentrations than needed to block NMDA receptors (Finck and Ngai, 1982; Ramoa et al., 1990; Hustveit et al., 1995; Hirota and Lambert, 1996; Scheller et al., 1996; Brau et al., 1997; Furuya et al., 1999; Flood and Krasowski, 2000; Yamakura et al., 2000; Schnoebel et al., 2005). Studies conducted at low doses of ketamine suggest that the inhibition of NMDA receptors results in its psychomimetic and analgesic properties (Tricklebank et al., 1987; Klepstad et al., 1990; Oye et al., 1992). However, the mechanism by which ketamine produces a higher CNS depression than PCP at high doses has remained an enigma (Kress, 1997).

$GABA_A$ receptors are the prime target for the hypnotic class of anesthetic compounds (Franks and Lieb, 1994; Macdonald and Olsen, 1994). A number of studies also implicated $GABA_A$ receptors in ketamine anesthesia (Scholfield, 1980; Gage and Robertson, 1985; Bennett et al., 1988; Irifune et al., 1992, 2000; Lin et al., 1992; Wakasugi et al., 1999), although these provided either indirect evidence for the action of ketamine on $GABA_A$ receptors,

or the concentration of ketamine used was significantly higher than the anesthetically relevant concentration.

The $GABA_A$ receptor subtypes $\alpha6\beta2/3\delta$ are expressed at high levels exclusively within mature cerebellar granule neurons (Laurie et al., 1992; Wisden et al., 1996) and show high sensitivity to GABA (Saxena and Macdonald, 1996; Storustovu and Ebert, 2006; Hadley and Amin, 2007).

We investigated the effects of ketamine, PCP, and MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate] (a potent noncompetitive inhibitor of NMDA receptor) on $\alpha6$- and δ-containing $GABA_A$ receptors. We show that ketamine, but not PCP or MK-801, within an anesthetically relevant concentration range, potentiated the GABA current arising from $\alpha6$- and δ-containing $GABA_A$ receptors in oocytes, dissociated granule neurons, and cerebellar slices isolated from rodents. The potential role of $GABA_A$ $\alpha6\beta2/3\delta$ receptors in ketamine anesthesia is discussed.

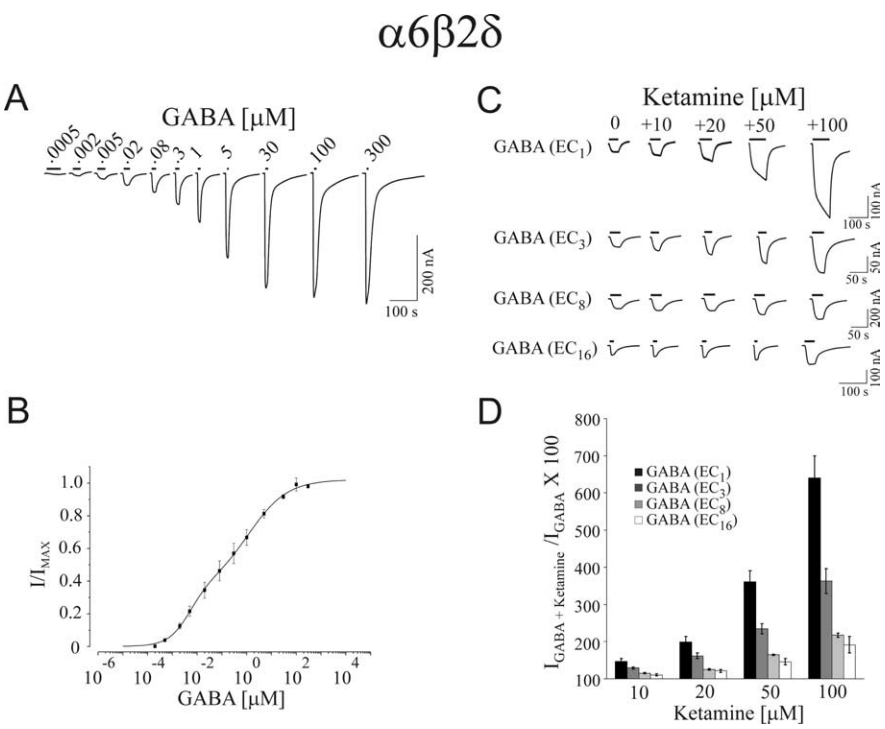

Figure 1. Ketamine potentiation of GABA-induced currents in $\alpha6\beta2\delta$ receptors. ***A***, ***B***, GABA concentration–response relationship of $\alpha6\beta2\delta$ receptors. ***A***, GABA-induced current traces for $\alpha6\beta2\delta$ receptors. ***B***, Data points for GABA concentration–response relationship. The $\alpha6\beta2\delta$ receptors show two distinct and separable states of agonist affinity, one exhibiting μM and the other nM affinities for GABA. The high-affinity state is associated with a significant level of constitutive channel activity. ***C***, Representative current traces for ketamine-dependent potentiation of $EC_{1,GABA}$, $EC_{3,GABA}$, $EC_{8,GABA}$, and $EC_{16,GABA}$ for $\alpha6\beta2\delta$ receptors. ***D***, Bar graph depicting the average of 10, 20, 50, and 100 μM ketamine potentiation at $EC_{1,GABA}$, $EC_{3,GABA}$, $EC_{8,GABA}$, and $EC_{16,GABA}$. Error bars indicate SEM.

Materials and Methods

cRNA quantitation and oocyte injection. Xenopus laevis frogs were anesthetized by bathing in a solution containing 0.1% MS-222 (tricaine methane sulfonate; Sigma-Aldrich). Before ovariectomy, the toe of the frog was pinched to assess the state of anesthesia. After surgery, the frog was killed by decapitation according to the protocol approved by the Institutional Animal Care and Use Committee. Oocyte preparation, *in vitro* transcription of complementary rat RNA (cRNA), and the drug perfusion system have been described previously (Walters et al., 2000). The quality of cRNA was determined by electrophoresis of the cRNA on a 1% formaldehyde-containing agarose gel and then quantified spectrophotometrically. Two to four preparations of cRNAs were tested for each subunit. The cRNA mixture (in diethylpyrocarbonate-treated water) was injected into *Xenopus laevis* oocytes using a Picospritzer II (General Valve Corporation). The ratio of the coinjected subunits α:β:γ/δ was1:1:1.8 except for expression of $\alpha6\beta2\delta$ receptors in the low-affinity state where the amount of $\beta2$ was relatively decreased to one-tenth (1:0.1:1.8) (Hadley and Amin, 2007). Five to ten nanograms of $\alpha6$, $\beta2$, and δ cRNA mixture per oocyte was used for the ketamine studies.

Oocytes electrophysiology. Recording microelectrodes were fabricated with a Narishige PP-83 puller filled with 3 M KCl, and had input resistances of 0.7–1.6 MΩ. A Turbo TEC-05 (NPI Electronic) two-electrode voltage-clamp amplifier (Adams and List) was used to record the currents. In all oocyte recordings, membrane potential was clamped to −70 mV. Data were visualized on a Gould TA-240 chart recorder during the experiments and stored online using Pulse Fit (HEKA).

Cerebellar preparation. Sprague Dawley rats, C57BL/6 mice, $\delta^{-/-}$ mice (provided by G. Homanics, University of Pittsburgh, Pittsburgh, PA) (Mihalek et al., 1999), and $\alpha6^{-/-}$ mice [provided by W. Wisden (University of Aberdeen, Aberdeen, UK) and E. Korpi (University of Turku, Turku, Finland)] (Jones et al., 1997) were reared in the central animal facilities of the University of Mainz and the University of Leipzig and transferred to the local adaptation facilities several days before experiments. All procedures followed the German guidelines for animal care. Male animals between postnatal day 20 (P20) and P48 were decapitated and their brains rapidly removed into ice-cold carboxygenated (5% CO_2/95% O_2) cutting solution (see below, Solutions and drugs). The cerebellar vermis was trimmed and then glued to the stage of an IntegraSlice 7550MM (cyanoacrylate glue; Campden Instruments). Sagittal slices of ~200 or ~500 μm thickness were prepared and maintained in a continuously carboxygenated perfusion chamber at room temperature (21°C) containing artificial CSF (ACSF) (see below, Solutions and drugs) for 1 h. Single slices (~200 μm) were transferred to a recording chamber (RC-26GLP; Warner Instruments), placed under an upright fixed stage microscope (Axioskop FS; Zeiss), and perfused at ~4 ml/min with carboxygenated ACSF. Using Nomarski optics and an enhanced infrared video system (Luigs and Neumann), whole-cell patch-clamp recordings of individual cells within the second or third layer of the inner granule cell area were obtained. To account for possible regional differences in the expression of the $\alpha6$-subunit (Mellor et al., 1998), we restricted slice studies to lobes VIII to X of the cerebellar cortex.

Preparation of dissociated granule neurons. To obtain dissociated cells, slices of ~500 μm thickness were transferred to preheated cutting solution (35°C, bubbled with carbogen) supplemented with 0.5–1 mg/ml pronase. After 20–40 min, slices were transferred to ice-cold HEPES-buffered cutting solution and carefully triturated with fire-polished glass pipettes of decreasing tip diameter. An aliquot (~ 20 μl) of dissociated-cell suspension was plated onto poly-L-lysine-coated coverslips and allowed to settle in ACSF for ~20 min before recordings.

Granule neurons electrophysiology. Whole-cell recordings were obtained with thick-walled borosilicate electrodes (1.5 mm outer diameter, 0.5 mm inner diameter; 8–12 MΩ resistances; Vitrex 11394; Science Products) using standard procedures (Hamill et al., 1981). Recordings were routinely filtered at 1 kHz, amplified and recorded on a personal computer using an EPC-9 amplifier and Pulse 8 software (HEKA). Current responses were recorded under voltage-clamp conditions at a holding potential of −60 mV and series resistances within 20–35 MΩ were compensated (70–80%; Pulse software; HEKA). Adequate clamp conditions (error <5 mV) (Rossi et al., 1994) were verified by the fast activa-

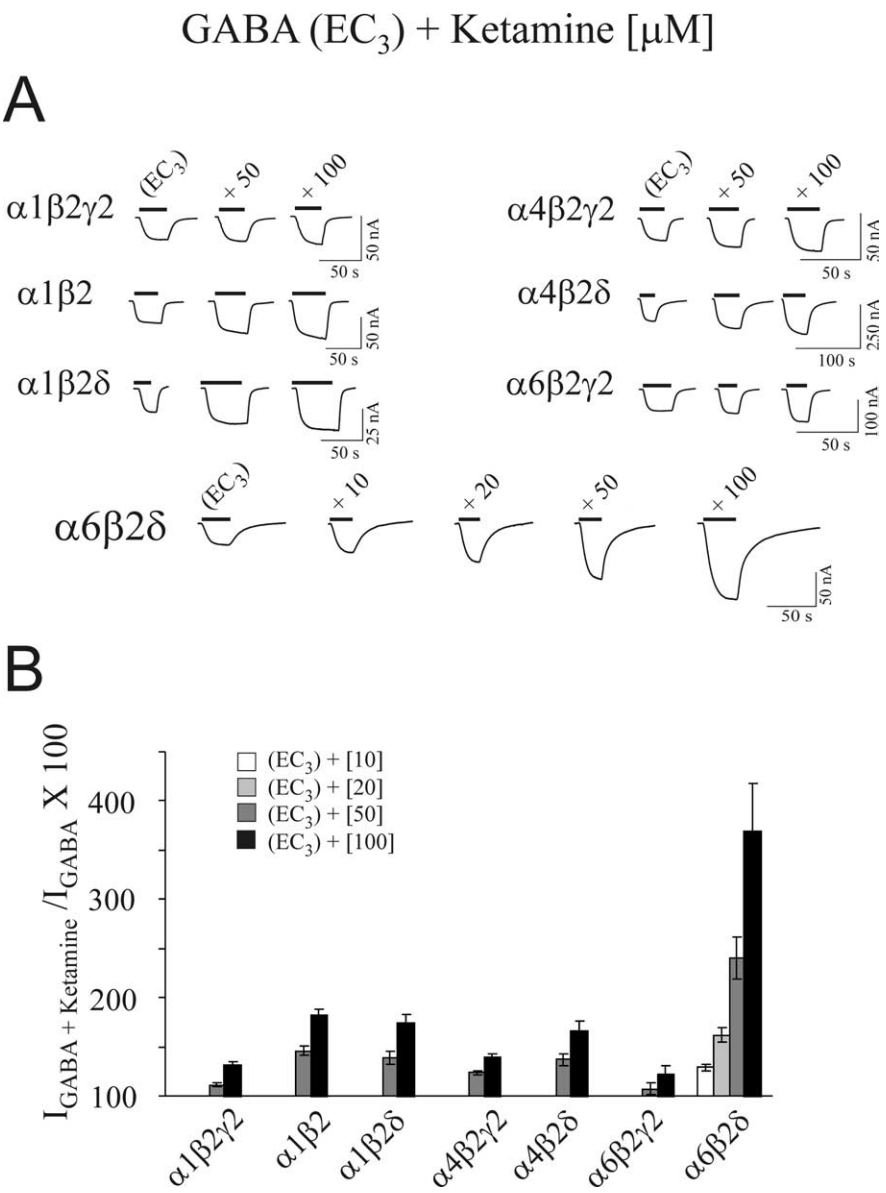

Figure 2. High sensitivity of $\alpha 6\beta 2\delta$ receptors to ketamine is unique among different $GABA_A$ receptor subtypes. ***A***, Representative current traces for ketamine-dependent potentiation of GABA currents arising from $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$, $\alpha 1\beta 2\delta$, $\alpha 4\beta 2\gamma 2$, $\alpha 4\beta 2\delta$, $\alpha 6\beta 2\gamma 2$, and $\alpha 6\beta 2\delta$ $GABA_A$ receptors. ***B***, Bar graph representing the average of ketamine-dependent potentiation of the respective $EC_{3,GABA}$ current for the above $GABA_A$ receptor subtypes. Ketamine produced a low level of potentiation for the three GABA receptor subtypes containing $\gamma 2$ subunit. In comparison, ketamine induces a marked potentiation of GABA currents arising from $\alpha 6\beta 2\delta$ receptors. Error bars indicate SEM.

tion of routinely recorded voltage-gated Na^+ currents (300 μs time to peak).

Solutions and drugs. For oocyte recording, the extracellular oocyte ringer (OR2) solution contained (in mM) 83.5 NaCl, 2.5 KCl, 10 HEPES, 1 $CaCl_2$, and 1 $MgCl_2$, pH 7.5. For cerebellar recording, the ACSF was bubbled with carbogen and contained (in mM) 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 30 Na_2HCO_3, and 1.5 $NaHPO_4$, pH 7.4. For tissue preparation and cutting, the solution contained (in mM) 60 NaCl, 140 sucrose, 5 KCl, 0.3 $CaCl_2$, 6 $MgCl_2$, 10 glucose, 30 Na_2HCO_3, and 1.5 $NaHPO_4$, pH 7.4. For cell dissociation, Na_2HCO_3 and $NaHPO_4$ were replaced by HEPES. For dissociated granule neurons or slice recordings, drugs were diluted in (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, with pH 7.4 (NaOH). All solutions were adjusted to ~305 mOsmol with sucrose or H_2O. Patch electrodes were filled with (in mM) 125 CsCl, 2 $MgCl_2$, 10 EGTA, 10 tetra-ethyl-ammonium chloride (TEA), 4–5 Na_2-ATP, 0.5 Na-GTP, and 10 HEPES, pH 7.3 (CsOH), adjusted to ~295 mOsmol with sucrose or H_2O.

All solutions were made from analytical grade salts. TEA, picrotoxinin, furosemide, ketamine, and bicuculline were purchased from Sigma-Aldrich. For some experiments, a premade ketamine solution (ketalar, 100 mg/ml) was used without noticeable differences. GABA was diluted from a 100 mM fresh or frozen stock solution to the final concentrations, furosemide from a 100 mM stock solution in 200 mM NaOH, picrotoxinin from a 10 mM stock solution in ethanol, bicucculine from a 100 mM in DMSO, and ketamine from a 100 mM in water. DMSO or ethanol at their highest concentrations used (0.1 and 0.5%, respectively, for oocytes) did not alter the responses. For oocyte recordings, stock solutions were diluted in the recording OR2 solution.

For slice recordings, drugs were applied focally using a gravity-fed Y-tube system described previously (Hevers and Luddens, 2002). In short, a 5-mm-long fused-silica tube (Micro-Fil; World Precision Instruments) was inserted into a sharply bent Teflon tube connected on one side to a vacuum source and on the other to the drug reservoirs. Focal drug application was initiated by interrupting the vacuum line with the tip, positioned in close proximity (<50 μm) to the cell, allowing a solution exchange within ~150 ms. For dissociated cells, a parallel three-barrel system was used in which the middle barrel contained buffer and the outside barrels contained different drugs. The middle barrel outlet was positioned in close proximity to the cells and switched to one of the drug lines with a stepping motor (Warner SF-77B; Warner Instruments) or a piezo translator (Burleigh LSS-3200; NPI Electronic).

Data analysis. We estimated the EC_{50} value for GABA and ketamine direct action by fitting the data from concentration–response relationships to the logistic equation according to the following formula (Sigmaplot 2000 or origin 6.0; OriginLab): $I = I_{max}/(1+ (EC_{50}/[A])^n)$.

The data for the GABA concentration response relationship of $\alpha 6\beta 2\delta$ receptors was fitted with a sum of two logistic equations: $I = I_{max1}/(1+ (EC_{501}/[A])^{n1} + I_{max2}/(1+ (EC_{502}/[A])^{n2})$, where I is the peak current at a given concentration of agonist A, I_{max}, I_{max1} and I_{max2} are the maximum currents, EC_{50}, EC_{501}, and EC_{502} are the concentrations of agonist yielding the half maximal currents, and n, n_1, and n_2 are the Hill coefficients of the curves.

Determinations of the statistical significant were performed either using the student t test or ANOVA as appropriate, requiring a p value of <0.05 for statistical significance. All statistical calculations are presented as means ± SE.

Results

$\alpha 6\beta 2\delta$ receptors show high sensitivity to ketamine

The degree a drug inhibits or potentiates the activity of a $GABA_A$ receptor depends markedly on the level of receptor channel activation by GABA (Morris et al., 1999; Walters et al., 2000). To test the modulatory activity of ketamine on $\alpha 6\beta 2\delta$ receptors, we determined the GABA concentration–response relationship for $\alpha 6\beta 2\delta$ receptors to establish the concentrations eliciting 1, 3, 8, and 16% of the maximal current ($EC_{1,GABA}$ $EC_{3,GABA}$, $EC_{8,GABA}$, and $EC_{16,GABA}$, respectively). Three to four days after cRNA in-

jection, GABA-activated currents were recorded (Fig. 1*A*,*B*). The GABA data for $\alpha 6\beta 2\delta$ receptors were fitted with a logistic equation (see Materials and Methods) and the extrapolated EC values were tested and adjusted empirically. These experiments revealed both a high-affinity and a low-affinity state of the $\alpha 6\beta 2\delta$ receptors [the detailed characterization of GABA concentration–response relationship for these receptors has recently been described further (Hadley and Amin, 2007)]. A significant level of spontaneous channel activity accompanies the high-affinity state.

The sensitivity of $\alpha 6\beta 2\delta$ receptors to ketamine was examined next at the presence of $EC_{1,GABA}$ $EC_{3,GABA}$, $EC_{8,GABA}$, and $EC_{16,GABA}$ values (Fig. 1*C*,*D*). Ketamine at 10 μM increased the $EC_{1,GABA}$ current by 46 ± 8%, whereas at 20, 50, and 100 μM the increases in GABA currents by ketamine were 100 ± 14%, 261 ± 30%, and 540 ± 60%, respectively. Ketamine at 10 to 100 μM augmented the $EC_{3,GABA}$ currents from ~30–260% (Fig. 1). At $EC_{8,GABA}$ and $EC_{16,GABA}$, ketamine produced a moderate increase in the GABA currents; i.e., for 10 and 100 μM ketamine, the increase of the $EC_{16,GABA}$ currents ranged from 10 to 91%, respectively. After ketamine application, the subsequent GABA currents were similar in magnitude to the preceding GABA control. Therefore ketamine exhibited little to no residual effects (data not shown).

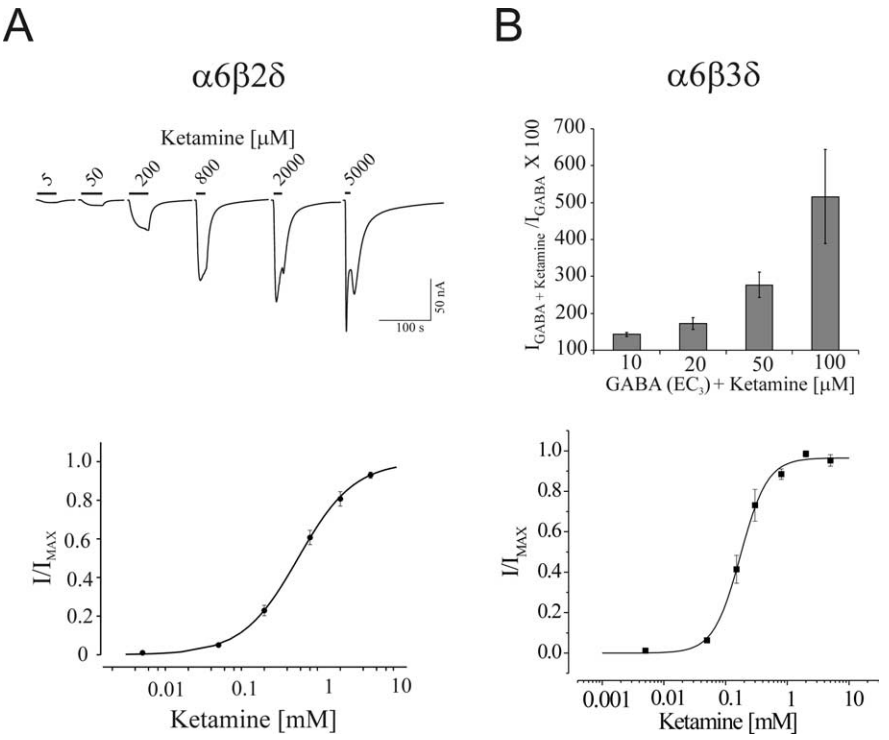

Figure 3. Ketamine modulates and can directly activate $\alpha 6\beta 2\delta$ or $\alpha 6\beta 3\delta$ receptors. ***A***, Ketamine-induced current traces for $\alpha 6\beta 2\delta$ receptors and ketamine concentration–response relationship. Ketamine directly activates the $\alpha 6\beta 2\delta$ receptors with an EC_{50} of ~570 μM. ***B***, Bar graph represents the average ketamine potentiation at $EC_{3,GABA}$ current and ketamine concentration–response relationship for $\alpha 6\beta 3\delta$ receptors. Similar to $\alpha 6\beta 2\delta$ receptors, ketamine produces a marked potentiation of GABA current arising from $\alpha 6\beta 3\delta$ receptors. The EC_{50} for ketamine direct activation was ~245 μM for $\alpha 6\beta 3\delta$ receptors. Error bars indicate SEM.

The high sensitivity of ketamine is unique to $\alpha 6\beta 2\delta$ receptors

We compared the sensitivity of ketamine to $\alpha 1\beta 2\gamma 2$, $\alpha 6\beta 2\gamma 2$, $\alpha 1\beta 2\delta$, $\alpha 4\beta 2\delta$, $\alpha 4\beta 2\gamma 2$, $\alpha 1\beta 2$, and $\alpha 6\beta 2\delta$ $GABA_A$ receptors at their respective $EC_{3,GABA}$ value (Fig. 2). Ketamine produced diminutive effects on $\alpha 1\beta 2\gamma 2$, $\alpha 4\beta 2\gamma 2$, and $\alpha 6\beta 2\gamma 2$ receptors. Ketamine (100 μM) increased the respective $EC_{3,GABA}$ currents between 22 and 39%, the values being significantly below those induced by 20 μM ketamine on $\alpha 6\beta 2\delta$ receptors. The increases by 50 and 100 μM ketamine for $\alpha 4\beta 2\delta$ receptors were 37 ± 6% and 66 ± 10%, respectively, whereas the corresponding values for $\alpha 1\beta 2\delta$ receptors were 39 ± 7% and 74 ± 9%. Thus, ketamine produced a modest potentiation at the $EC_{3,GABA}$ currents for $\alpha 1\beta 2\delta$ and $\alpha 4\beta 2\delta$ receptors. The $\alpha 1\beta 2$ GABA current (EC_3) increased by 46 ± 5% and 81 ± 7% at 50 and 100 μM ketamine, respectively.

In summary, the presence of the $\gamma 2$ subunit within $GABA_A$ receptors reduced the effects of ketamine, whereas those receptors containing the δ subunits (or $\alpha 1\beta 2$ alone) exhibited relatively higher sensitivity to ketamine. However, both $\alpha 6$ and δ subunits were needed to confer the highest sensitivity to ketamine. Thus, among the tested $GABA_A$ receptor subtypes, the high sensitivity to ketamine is unique to $\alpha 6\beta 2\delta$ receptors.

Ketamine directly activates $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors

Intravenous anesthetics such as pentobarbital modulate and directly activate most, if not all, $GABA_A$ receptor subtypes. To examine whether ketamine can also act as an agonist, we bath applied ketamine in concentrations ranging from 5 to 5000 μM on oocytes expressing $\alpha 6\beta 2\delta$ receptors (Fig. 3*A*). Ketamine directly activated $\alpha 6\beta 2\delta$ receptors at concentrations as low as 50 μM, with an EC_{50} of 577 ± 63 μM and a Hill slope of 1.20 ± 0.09 ($n = 4$). When higher concentrations of ketamine were removed, the residual current increased before returning to the baseline, suggesting that ketamine possessed a low-affinity blocking action (Fig. 3*A*). This apparent block by ketamine at higher concentrations resembled the direct action of pentobarbital on $GABA_A$ receptors.

For $\alpha 6\beta 2\delta$ receptors, the relative maximal current of ketamine (1.8 mM) to GABA (50 μM) was 0.51 ± 0.06 ($n = 10$), indicating that ketamine is a partial agonist on $\alpha 6\beta 2\delta$ receptors. In contrast, ketamine did not directly activate $\alpha 1\beta 2\delta$, $\alpha 6\beta 2\gamma 2$, $\alpha 4\beta 2\delta$, and $\alpha 4\beta 2\gamma 2$ receptors, with the relative efficacies ranging from 0.01 to 0.02 for ketamine (1.8 mM) to GABA (1.2 mM).

In addition to the $\beta 2$ subunit, the $\beta 3$ subunit is abundantly expressed in adult cerebellar granule neurons. Thus, we examined the modulatory and direct actions of ketamine on $\alpha 6\beta 3\delta$ receptors as well (Fig. 3*B*). At 10, 20, 50, and 100 μM ketamine potentiated the $EC_{3,GABA}$ by 43 ± 6%, 72 ± 16%, 177 ± 35%, and 416 ± 128%, respectively, similar to those of $\alpha 6\beta 2\delta$ receptors. Ketamine also directly activated $\alpha 6\beta 3\delta$ receptors with an EC_{50} of 245 ± 26 μM and slope of 1.9 ± 0.1 ($n = 11$). Thus, ketamine at low concentrations (10–20 μM) significantly potentiated the GABA current of $\alpha 6\beta 3\delta$ receptors and at higher concentration directly activated these receptors.

In summary, ketamine directly activated $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors. The direct and modulatory actions of ketamine resemble those of the classical intravenous anesthetic pentobarbital. However, direct activation of ketamine appeared to be selective

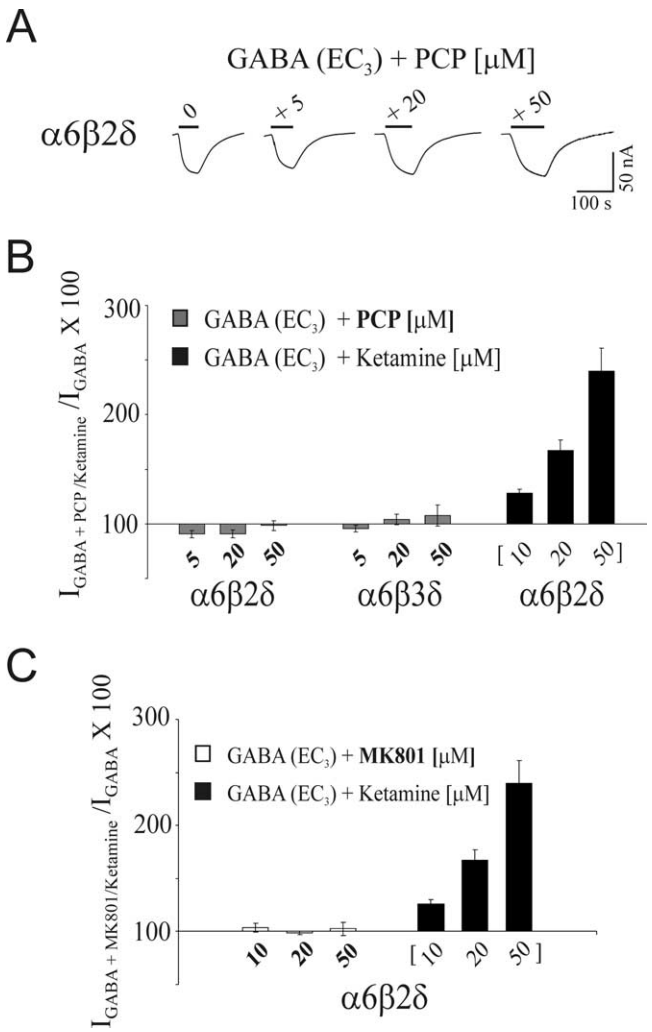

Figure 4. Comparison of PCP, MK-801, and ketamine action on $\alpha 6\beta 2\delta$ receptors. ***A***, Representative current traces depicting the effect of coapplication of 5, 20, and 50 μM PCP with GABA (EC_3) on $\alpha 6\beta 2\delta$ receptors. ***B***, Bar graph depicting the effect of 5, 20, and 50 μM PCP on GABA (EC_3) current arising from $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors. The ketamine potentiation of the $EC_{3,GABA}$ current from $\alpha 6\beta 2\delta$ is included. PCP shows no significant effect on $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors. ***C***, The bar graph representing the effect of 10, 20, and 50 μM MK-801 on GABA (EC_3) current arising from $\alpha 6\beta 2\delta$ receptors. The ketamine potentiation of the $EC_{3,GABA}$ current from $\alpha 6\beta 2\delta$ is included. Similar to PCP, MK-801 produced no significant change in the magnitude of GABA-evoked currents of the $\alpha 6\beta 2\delta$ receptors. Error bars indicate SEM.

to $\alpha 6\beta 2/3\delta$ receptors, whereas pentobarbital directly activates most $GABA_A$ receptors, including $\alpha 1\beta 2/3\gamma 2$ receptors.

MK-801 or PCP do not modulate $\alpha 6\beta 2/3\delta$ receptors

PCP blocks NMDA receptors by an order of magnitude higher than that of ketamine. MK-801, a dissociative anesthetic chemically unrelated to PCP, possesses an even greater affinity to, and inhibits NMDA receptors with higher potency than PCP (Wong et al., 1986; Huettner and Bean, 1988; MacDonald and Nowak, 1990; MacDonald et al., 1991). Furthermore, the blocking action of MK-801 on the NMDA receptor is less dependent on the membrane potential than ketamine or PCP (Halliwell et al., 1989; Dravid et al., 2007). We examined whether the sensitivity of $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors to ketamine extends to PCP or MK-801. First, the modulatory effect of PCP at the $EC_{3,GABA}$ value for $\alpha 6\beta 2\delta$ and $\alpha 6\beta 3\delta$ receptors was examined (Fig. 4*A*,*B*). PCP produced no significant potentiation of the GABA current arising from $\alpha 6\beta 2\delta$ receptors even at 50 μM, a concentration significantly higher than the anesthetically relevant concentrations. Second, the effect of MK-801 up to 50 μM was tested on $\alpha 6\beta 2\delta$ receptors (Fig. 4*C*). As with PCP, MK-801 did not affect the $EC_{3,GABA}$ currents arising from $\alpha 6\beta 2\delta$ receptors. Thus, among the three dissociative anesthetics tested, $\alpha 6\beta 2\delta$ receptors are uniquely sensitive to ketamine.

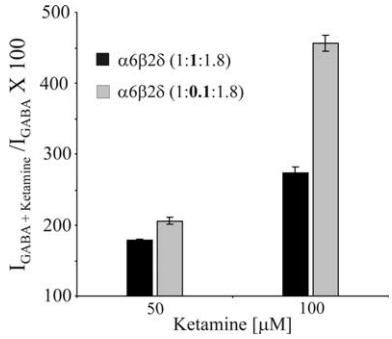

Figure 5. Ketamine modulation of the high-affinity versus low-affinity state of $\alpha 6\beta 2\delta$ receptors. Oocytes were injected with either 10–15 ng of cRNA per injection of the standard ratio $\alpha 6$:$\beta 2$:δ (1:1:1.8) or with a low $\beta 2$ subunit ratio of 1:0.1:δ1.8 to express the $\alpha 6\beta 2\delta$ receptors in predominantly the high- or low-affinity state, respectively. Bar graphs show ketamine potentiation (50 and 100 μM) of $EC_{3,GABA}$ current arising from $\alpha 6\beta 2\delta$ receptors in the predominantly low versus high-affinity state. Ketamine produced a marked potentiation of $\alpha 6\beta 2\delta$ receptors GABA current in either low or high-affinity states. Error bars indicate SEM.

Action of ketamine on the $\alpha 6\beta 2\delta$ receptors within a low- or high-affinity state

The affinity state of the $\alpha 6\beta 2\delta$ receptors toward GABA expressed in *Xenopus* oocytes can be controlled by (1) the expression level and (2) the alteration of the ratio of $\beta 2$ to $\alpha 6$ and δ subunits (Hadley and Amin, 2007). The ketamine data for $\alpha 6\beta 2\delta$ receptors shown up to here was obtained using a cRNA injection ratio for $\alpha 6$:$\beta 2$:δ of 1:1:1.8 (5–10 ng of cRNA per injection), where both the high- and low-affinity states of the receptor coexist. The high-affinity state exhibits constitutive activity. Previous studies have shown that injection using a lower ratio of β subunit cRNA, i.e., 1:0.1:1.8, results in a preponderance of $\alpha 6\beta 2\delta$ receptors in the low-affinity state (Hadley and Amin, 2007). To investigate the sensitivity of $\alpha 6\beta 2\delta$ receptors to ketamine in the high- versus low-affinity state, we injected oocytes with either the standard ratio of 1:1:1.8 with 10–15 ng of cRNA per injection (see Materials and Methods) or with a low $\beta 2$ subunit ratio of 1:0.1:1.8 to express the $\alpha 6\beta 2\delta$ receptors in predominantly the high- or low-affinity state, respectively. For the $\alpha 6\beta 2\delta$ receptor in the low-affinity state, the percentage increases of the GABA current at $EC_{3,GABA}$ by 50 and 100 μM ketamine were 106 $\pm$ 5% and 356 $\pm$ 11%, respectively (Fig. 5), compared with the $\alpha 6\beta 2\delta$ receptor in the high-affinity state, whereas the increase was 79 $\pm$ 1% and 173 $\pm$ 8% for 50 and 100 μM ketamine, respectively. Furthermore, ketamine on its own showed low efficacy (I_{max} ketamine/I_{max} GABA $\sim$ 0.1) for $\alpha 6\beta 2\delta$ receptors in the low-affinity state. Collectively, ketamine can significantly potentiate the GABA currents arising from $\alpha 6\beta 2\delta$ receptors in either the low or the high-affinity states yet the direct activation by ketamine appears to be selective for $\alpha 6\beta 2\delta$ receptors in the high-affinity state.

Action of ketamine on cerebellar granule neurons

We investigated the action of ketamine on granule neurons in rat cerebellar slices from P20–P48 when the expression of $\alpha 6$ and δ subunit reaches high levels (Laurie et al., 1992). Individual gran-

ule neurons from the inner granule layer were voltage clamped (−60 mV) in whole-cell recording configurations using equimolar Cl^- concentrations. In these recordings, granule neurons showed a tonic current that could be blocked by adding the $GABA_A$ receptor antagonist bicuculline (20 μM), or the specific α6-containing $GABA_A$ receptor antagonist furosemide (300 μM) (Korpi et al., 1995; Korpi and Luddens, 1997) (Fig. 6*A*,*B*). Ketamine (1 mM) evoked currents that ranged in amplitude from 15 to over 100 pA (58.5 ± 8.6 pA; $n = 11$). Bicuculline (20 μM) and furosemide (300 μM) blocked the ketamine-induced currents within the granule neurons, indicating that these currents arose from α6-containing $GABA_A$ receptors (Fig. 6*C*).

We next examined the potentiation of the tonic current by 10, 30, 100, 300, and 1000 μM ketamine (Fig. 7). The level of bicuculline-dependent block of the tonic current was used as the denominator to determine the ketamine potentiation. Ketamine, at a concentration as low as 30 μM, produced a significant increase in the tonic current, resulting in 20 ± 15% potentiation ($p = 0.0003$; $n = 10$). Furthermore, 100, 300, and 1000 μM ketamine increased the tonic current by 50, 130, and 280%, respectively. Thus, ketamine at clinically relevant concentrations (see Discussion) of 30 or 100 μM can increase the tonic current significantly.

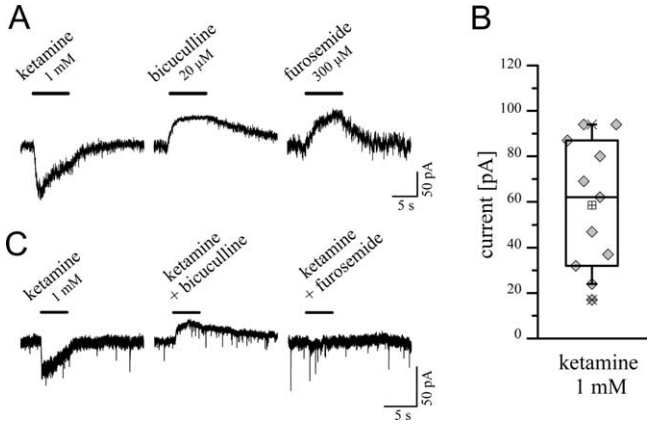

Figure 6. Ketamine-induced currents in rat cerebellar granule cells. ***A***, Whole-cell patch-clamp recordings of granule cells were obtained in adult cerebellar slices (here P30). At a holding potential of −60 mV, using equimolar Cl^- concentrations, ketamine evoked currents with a rapid time course. Granule neurons showed a tonic current that could be blocked by adding $GABA_A$ receptor antagonists, i.e., bicuculline or the specific α6-containing $GABA_A$ receptor antagonist furosemide. ***B***, Ketamine (1 mM) induced currents that ranged from ~15–100 pA (mean 58.5 ± 8.6 pA; $n = 11$; P21–P34). ***C***, Ketamine-induced currents were blocked by bicuculline or furosemide coapplication indicating that the ketamine effects were mediated through α6-containing $GABA_A$ receptors. Error bars indicate SEM.

Ketamine does not induce currents in $\alpha6^{-/-}$ or $\delta^{-/-}$ transgenic mice

To further elucidate the involvement of α6 and test whether δ subunits play a role in the ketamine-induced currents recorded in cerebellar slices, we used mice in which the functional expression of α6- and δ- ($\alpha6^{-/-}$) or δ-subunits ($\delta^{-/-}$) alone was abolished (Jones et al., 1997). The GABAergic tonic background conductance was found to be absent or nearly absent within the cerebellar slice recordings from granule neurons of $\alpha6^{-/-}$ or $\delta^{-/-}$ mice, respectively (Fig. 8). In contrast to experiments from wild-type animals, ketamine (1 mM) did not induce a current in granule cells from either $\alpha6^{-/-}$ or $\delta^{-/-}$ mice. These experiments demonstrate that the δ subunit and, likely, the α6 subunit are needed to confer ketamine sensitivity to cerebellar granule neurons.

Ketamine's modulation of dissociated granule neurons

The tonic activation of extrasynaptically localized α6β2/3δ receptors may indicate the omnipresence of low concentrations of GABA at these receptor sites. To differentiate the modulatory effects in the presence of GABA versus a direct effect of ketamine on granule neurons, we developed a preparation of dissociated granule neurons from mice and rats up to an age of P28. In this procedure, neurons resembled granule neurons with small globular shape with several offshoots and dendrites (Fig. 9*A*). The cell capacitance was not significantly ($p = 0.29$) altered a few hours after dissociation (2.76 ± 0.27 pF; $n = 6$) when compared with granule neurons within the slice (2.89 ± 0.16 pF; $n = 15$). We verified presence of α6- and δ-containing $GABA_A$ receptors within the dissociated neuron using furosemide, 3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline (DMCM), and 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP). Furosemide inhibited, whereas DMCM potentiated, the GABA-induced current in these cells (Fig. 9*B*). Furthermore, 1 μM THIP, a concentration that can selectively activate α6- and δ-containing $GABA_A$ receptors (Storustovu and Ebert, 2006; Saarelainen et al., 2008), evoked 37.5 ± 4.8 pA compared with 38.3 ± 5.2 pA ($n = 11$) of current for 1 μM GABA within these dissociated granule neurons. These data suggest the presence of α6- and δ-containing $GABA_A$ receptors within the dissociated granule neurons. Ketamine produced a marked potentiation of the GABA current (1 μM), whereas, in contrast to slice recordings, bicuculline did not change the control current (Fig. 9*C*).

We tested the ketamine concentration response relationship in the presence of a low concentration of GABA (1 μM) for the dissociated granule neurons isolated from rats or mice (Fig. 9*D*,*E*). The half maximal potentiation was ~300 μM for the two species (mice: 260 ± 16 μM, maximal potentiation 310 ± 50%; for rats: 198 ± 32 μM, maximal potentiation 240 ± 80%). Within these experiments, 100 μM ketamine induced a significant potentiation of the GABA current (Fig. 9*F*). In the presence of 3 μM GABA, 100 μM ketamine produced a 140 ± 5% potentiation ($n = 26$, rats and mice pooled because there were little species-dependent difference between the mice and rats in terms of ketamine modulation). Under equivalent conditions, PCP at 1 and 3 mM was ineffective in modulating the GABA current (Fig. 9*F*) (1 mM PCP, 106 ± 2.7%, $n = 8$, $p = 0.78$; 3 mM PCP, 112 ± 3.5%, $n = 8$, $p = 0.67$). Thus, similar to the oocyte experiments, ketamine, but not PCP, modulated the α6- and δ-containing $GABA_A$ receptors.

Ketamine evoked a relatively small current on its own with a mean value of 4.2 pA (Fig. 9*C*), which could be blocked by bicuculline (data not shown). These experiments suggest that α6β2/3δ receptors may be present predominately in a low-affinity state and the α6β2/3δ receptors in the high-affinity state could constitute a small fraction of the total receptor population in these dissociated granule neurons from preadolescent p28 or younger animals.

The effect of increasing concentrations of GABA on the ketamine response (1 mM) was also examined within wild-type and $\alpha6^{-/-}$ or $\delta^{-/-}$ mice dissociated granule cells (Fig. 10). For wild-type mice, the effect of ketamine was most pronounced at concentrations of GABA <100 μM with an EC_{50} of 57 ± 18 μM (slope = 0.98; $n = 6$). In contrast, ketamine potentiation was absent in the mutant $\alpha6^{-/-}$ or $\delta^{-/-}$ mice regardless of the GABA concentration, indicating the importance of α6 or δ subunits in the action of ketamine.

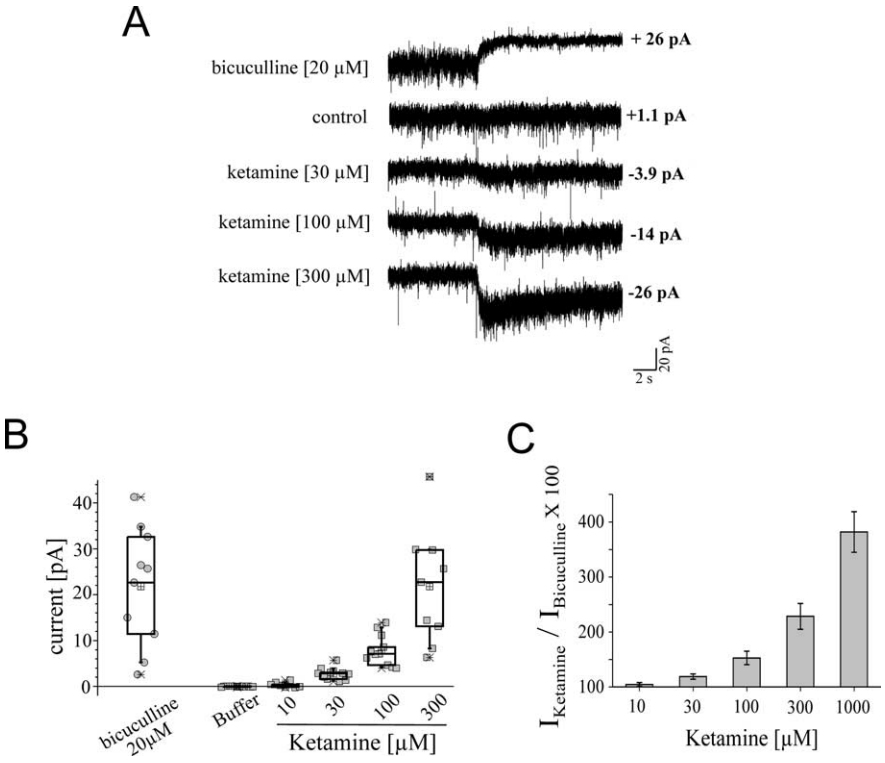

Figure 7. Potentiation of ketamine at 30, 100, 300, and 1000 μM tonic current in cerebellar slice recordings. ***A***, Current traces representing block of the tonic current by bicuculline and potentiation of the tonic current by 30, 100, and 300 μM ketamine. The drugs were applied for 20 s. The current amplitudes shown were determined from all point histographs of the current traces. ***B***, The range of current amplitudes for bicuculline-dependent block, and ketamine (10, 30, 100, and 300 μM)-induced potentiation, of the tonic current. ***C***, Ketamine, at a concentration as low as 30 μM, produced a significant increase in the tonic current ($p = 0.0003$). The level of bicuculline-dependent block of the tonic current was used as the denominator to determine the ketamine potentiation. Error bars indicate SEM.

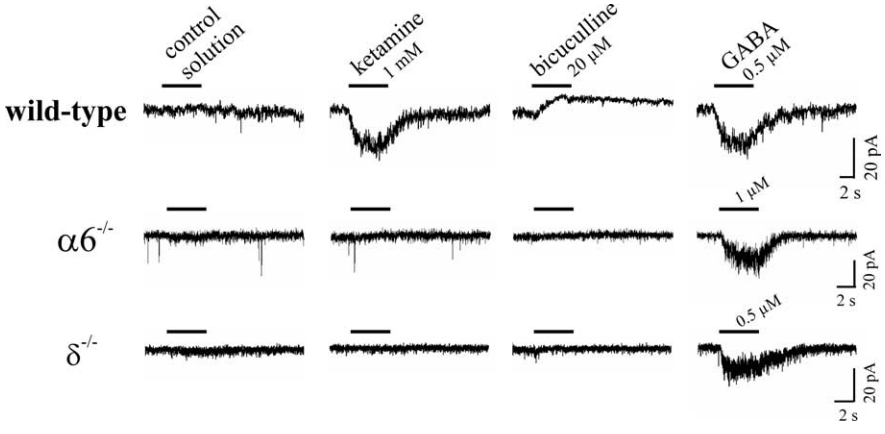

Figure 8. Absence of the effect of ketamine in cerebellar slice recordings from $\alpha 6^{-/-}$ or $\delta^{-/-}$ mice. Ketamine-induced currents in cerebellar granule cells from wild-type mice, similar to the experiments using rats. Bicuculline verified the presence of a tonic background conductance in these experiments (here, P31). In $\alpha 6^{-/-}$ mice, where there is also a lack of expression of the δ subunit, bicuculline failed to reveal any background conductance. Ketamine (1 mM) produced no effect in cerebellar slice recording from granule neurons of $\alpha 6^{-/-}$ mice (here, P32). In $\delta^{-/-}$ mice, in which only the δ subunits are missing, ketamine modulation was also absent indicating the importance of the δ subunit in ketamine-dependent modulation of granule neurons (shown for P36).

Collectively, these data support the findings from the oocyte and slice recording experiments, demonstrating the unique action of ketamine on the α6- and δ-containing $GABA_A$ receptors, whereas PCP had no effect.

Discussion

We demonstrated that ketamine selectively modulates rat $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors within the oocyte expression system. These receptors were highly sensitive to ketamine, but showed no response to PCP or MK-801. Ketamine-induced currents were observed in the recordings of cerebellar granule neurons from rodents, where they were confirmed to arise from α6- and δ-containing $GABA_A$ receptors. Furthermore, the ketamine-evoked currents were absent within cerebellar granule neurons of transgenic functionally null $\alpha 6^{-/-}$ or $\delta^{-/-}$ mice.

The cerebellum has a primary function in motor control and learning. Previous anatomical, neuroimaging, and pyschological studies suggest that the cerebellum may also participate in higher cognitive functions. Patients with physical-induced lesions of the cerebellum exhibit both motor and cognitive disabilities. Cerebellum anomalies have been noted in patients suffering from schizophrenia, dyslexia, and autism (Martin and Albers, 1995; Timmann and Daum, 2007). Furthermore, neuroimaging recordings of volunteers performing cognitive tasks suggest that the cerebellum is involved in memory, learning, language and attention (Bellebaum and Daum, 2007; Haarmeier and Thier, 2007; Sultan and Glickstein, 2007; Thach, 2007; Timmann and Daum, 2007). Collectively, the cerebellum appears to coordinate not only motor, but also cognitive activity.

Cerebellar granule cells are the most abundant neurons in the CNS and play a pivotal role in storing motor commands (Tyrrell and Willshaw, 1992). A key characteristic of these neurons is the postnatal development of a tonic chloride current, known as tonic inhibition, which arises from $GABA_A$ receptors containing α6 subunits (Kaneda et al., 1995; Brickley et al., 1996; Tia et al., 1996; Wall and Usowicz, 1997; Hamann et al., 2002). The tonic inhibition plays an important role in tuning the indirect transmission of information from mossy fibers to the Purkinje neurons (via granule neurons) by reducing the overall gain of information transmitted through the cerebellar cortex. It is calculated that at the adult stage, contribution of the tonic inhibition to overall inhibitory conductance is at least threefold larger than phasic inhibition. Moreover, >90% of the tonic inhibition of granule neurons is mediated by α6- and δ-containing $GABA_A$ receptors (Hamann et al., 2002). Thus, the high sensitivity of $\alpha 6\beta 2/3\delta$ receptors to ketamine has the potential to impact the informational transfer from mossy fibers to the Purkinje neurons. Ketamine/xylazine

anesthesia has been used to study cerebellar circuitry *in vivo*. In these studies, ketamine/xylazine significantly suppresses the activity within the parallel fiber synapses, and the states of the membrane potential of the Purkinje neurons are significantly affected as well (Schonewille et al., 2006; Bengtsson and Jorntell, 2007). In addition to the $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors, there are also NMDA and AMPA receptors expressed within the granule neurons (Garthwaite and Brodbelt, 1990). Compared with the action of ketamine on the $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors, however, block of these receptors by ketamine occurs at markedly higher concentrations (Arenz et al., 2006). Given the relatively higher sensitivity of $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors to ketamine and the abundance of these receptors at the adult stage, the ketamine potentiation of the tonic current arising from $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors may have a profoundly greater impact on the overall suppression of the parallel fiber synapses than the blocking action of ketamine on the excitatory pathway during ketamine anesthesia.

The action of ketamine on cerebellar $\alpha 6\beta 2/3\delta$ receptors occurred at anesthetically relevant concentrations. The steady-state ketamine concentration in the plasma of anesthetized rats is ~100 μM after the distribution phase. In the brain, the ketamine concentration has been shown to exceed 300 μM (Cohen and Trevor, 1974). Here, we demonstrate that 10–100 μM ketamine significantly potentiated GABA currents arising from $\alpha 6\beta 2/3\delta$ receptors. Thus, the concentrations of ketamine effective at $\alpha 6\beta 2/3\delta$ receptors are within the realm of anesthetically relevant concentrations.

The anesthetic efficacies of ketamine, PCP, and MK-801 measured by CNS depressive action do not show a strong correlation to their actions on NMDA receptors. Binding and electrophysiological studies have demonstrated that PCP and MK-801 are superior noncompetitive inhibitors of, and have markedly higher affinity for, NMDA receptors than ketamine (Chen et al., 1959; Johnstone et al., 1959; Chen, 1965; McCarthy et al., 1965; Wong et al., 1986; MacDonald et al., 1991; Rogawski and Wenk, 2003). In addition, MK-801 block can occur across different membrane potentials, whereas PCP and ketamine inhibit NMDA receptors predominately at depolarized potentials (Halliwell et al., 1989; Dravid et al., 2007). However, a number of studies have shown that PCP and MK-801 are not effective anesthetics in rats, mice or pigeons, even at near lethal doses (Chen et al., 1959; Koek et al., 1987a,b, 1988; Kelland et al., 1993; Irifune et al., 2007). Therefore, contrary to the order of their impact on NMDA receptors, ketamine is a more effective anesthetic across different animal species and appears to possess a higher CNS depressant activity than PCP and MK-801 (Chen et al., 1959; Johnstone et al., 1959; Chen, 1965; Domino et al., 1965; McCarthy et al., 1965).

In human studies, a number of patients placed under cata-

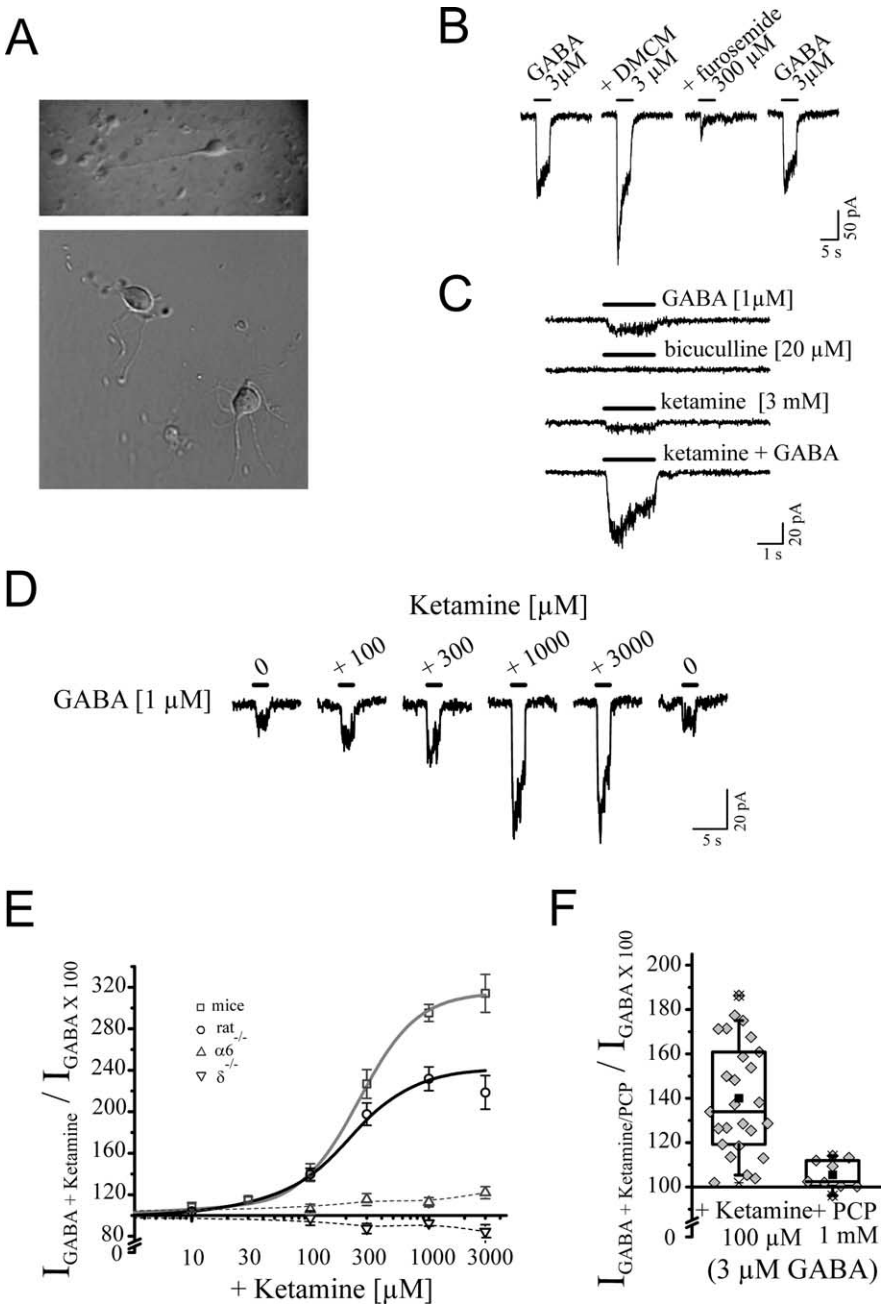

Figure 9. Ketamine action on acutely dissociated granule cells. ***A***, These neurons (P20 to P30) resembled granule neurons with their small globular shape and several offshoot dendrites. ***B***, The pharmacological characteristics of these dissociated cells, such as the inhibitory effect by furosemide or potentiation by DMCM, were similar to granule neuron recording within slices. ***C***, Bicuculline (20 μM) alone did not produce an effect indicating the absence of tonic background conductance within these dissociated neurons. Ketamine (1 mM) produced a pronounced potentiation of GABA (3 μM) currents, whereas ketamine (3 mM) alone induced a small current in the absence of GABA. ***D***, Current traces for ketamine-dependent modulation of GABA-evoked currents (1 μM) for mice. ***E***, Concentration–response relationship of the ketamine modulatory action for wild-type mice yielded EC_{50} values of ~260 and 200 μM ketamine and a maximal potentiation of 310 and 240% for mice and rat, respectively. Ketamine did not produce a significant effect on GABA current (1 μM) from dissociated neurons isolated from $\alpha 6^{-/-}$ or $\delta^{-/-}$ mice, when compared with rats or wild-type mice. ***F***, Ketamine at 100 μM showed pronounced potentiation of GABA currents (3 μM) for mice and rats (140 ± 5%; $n = 26$, rat and mice pooled) within these neurons. PCP did not modulate GABA (3 μM) currents arising from dissociated granule neurons isolated from rat or mice (mean 105 ± 3%; $n = 8$). Error bars indicate SEM.

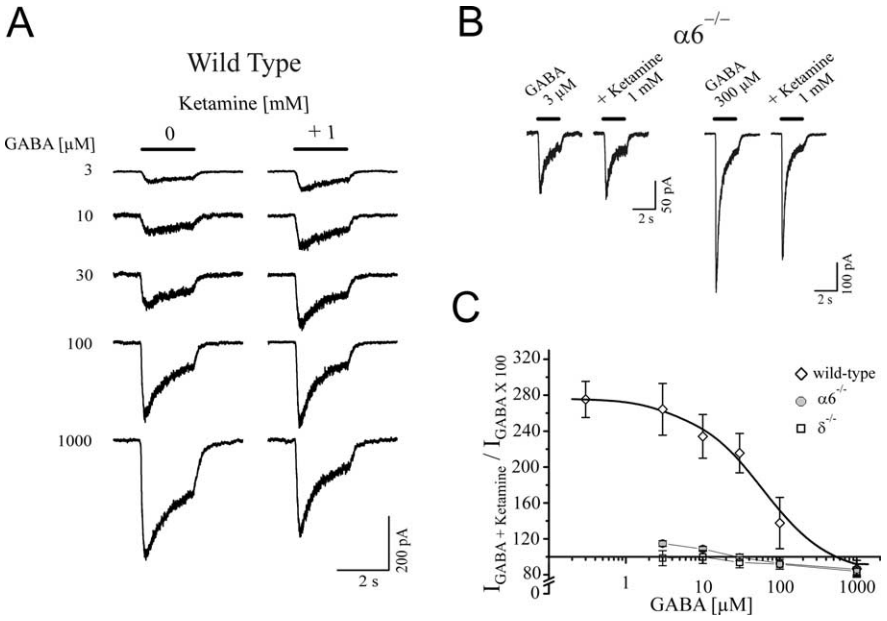

Figure 10. The degree of Ketamine potentiation depends on the concentration of GABA for granule neurons isolated from mice. ***A***, ***B***, Current traces and GABA-concentration response in the presence of ketamine (1 mM) for wild-type and $\alpha 6^{-/-}$ mice. The ketamine potentiation is most pronounced at concentrations of GABA <100 μM. ***B***, Current traces representing GABA (3 or 300 μM) and GABA plus 1 mM ketamine within $\alpha 6^{-/-}$ mice dissociated granule neuron. Ketamine did not produce a significant change in the current induced by 3 μM GABA and at higher concentration of GABA (300 μM) caused a moderate inhibition. ***C***, Potentiation by ketamine (1 mM) was dependent on GABA concentration. Half-maximal potentiation by ketamine occurred at $\sim$60 μM GABA. Ketamine did not produce a significant potentiation at any GABA concentration in dissociated granule neuron derived from $\alpha 6^{-/-}$ and $\delta^{-/-}$ mice. Furthermore, at 30 μM GABA or higher, ketamine produced a moderate inhibition in granule cells of $\alpha 6^{-/-}$ and $\delta^{-/-}$ mice. Error bars indicate SEM.

tonic state using PCP are arousable (Johnstone et al., 1959), indicating suboptimal CNS depression, whereas patients under ketamine anesthesia do not respond to verbal commands. Furthermore, with increasing doses, PCP can induce surgically unrelated excitation (Greifenstein et al., 1958), an adverse effect absent with ketamine administration. Ketamine also produces a lower rate and a shorter duration of emergence reactions, such as vivid dreams and hallucinations, when compared with PCP.

Collectively, these observations have led to the withdrawal of PCP from further clinical trails and veterinary practice despite the higher potency of PCP in producing dissociation and having a significantly higher affinity to NMDA receptors. Although MK-801 has been extensively characterized for two decades and possesses a nearly 1000 times greater affinity for the NMDA receptors than ketamine, MK-801 has not been approved for clinical or veterinary practice. Our findings suggest the distinguishing factor is the alternative action of ketamine on the inhibitory pathway via $GABA_A$ $\alpha 6\beta 2/3\delta$ receptors.

We have shown that ketamine, but not PCP, can increase the activity of $GABA_A$ receptors containing $\alpha 6$ and δ subunits within an anesthetically relevant concentration range. This is the first demonstration of two anesthetics, highly analogous in chemical structure, molecular action, and clinical effect, in which each exhibits differentiating all or none action on cerebellar $\alpha 6\beta 2/3\delta$ $GABA_A$ receptors. The actions of ketamine are disproportionate regarding their impact on excitatory (NMDA) versus inhibitory ($GABA_A$) pathways, with the former showing higher potency. These findings have the potential to lead to the development of PCP analogues that exhibit more balanced actions on the inhibitory versus excitatory pathways. Such anesthetics would offer a broader spectrum for desired effects, lower the incidence of adverse effects, and provide a higher margin of safety.

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