Rat $\alpha_6\beta_2\delta$ $GABA_A$ receptors exhibit two distinct and separable agonist affinities

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The onset of motor learning in rats coincides with exclusive expression of $GABA_A$ receptors containing α_6 and δ subunits in the granule neurons of the cerebellum. This development temporally correlates with the presence of a spontaneously active chloride current through α_6-containing $GABA_A$ receptors, known as tonic inhibition. Here we report that the coexpression of α_6, β_2, and δ subunits produced receptor–channels which possessed two distinct and separable states of agonist affinity, one exhibiting micromolar and the other nanomolar affinities for GABA. The high-affinity state was associated with a significant level of spontaneous channel activity. Increasing the level of expression or the ratio of β_2 to α_6 and δ subunits increased the prevalence of the high-affinity state. Comparative studies of $\alpha_6\beta_2\delta$, $\alpha_1\beta_2\delta$, $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$ receptors under equivalent levels of expression demonstrated that the significant level of spontaneous channel activity is uniquely attributable to $\alpha_6\beta_2\delta$ receptors. The pharmacology of spontaneous channel activity arising from $\alpha_6\beta_2\delta$ receptor expression corresponded to that of tonic inhibition. For example, $GABA_A$ receptor antagonists, including furosemide, blocked the spontaneous current. Further, the neuroactive steroid 5α-THDOC and classical glycine receptor agonists β-alanine and taurine directly activated $\alpha_6\beta_2\delta$ receptors with high potency. Specific mutation within the GABA-dependent activation domain (β^{Y157F}) impaired both low- and high-affinity components of GABA agonist activity in $\alpha_6\beta^{Y157F}\delta$ receptors, but did not attenuate the spontaneous current. In comparison, a mutation located between the second and third transmembrane segments of the δ subunit (δ^{R287M}) significantly diminished the nanomolar component and the spontaneous activity. The possibility that the high affinity state of the $\alpha_6\beta_2\delta$ receptor modulates the granule neuron activity as well as potential mechanisms affecting its expression are discussed.

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Ligand-gated ion channels comprise a large family of membrane-embedded receptors which play a central role in neuronal transmission. The binding of a neurotransmitter to the receptor domain of this class of membrane proteins opens an integrated ion channel, allowing selected ions to permeate. Such receptor–channels are either excitatory or inhibitory in nature, modulating the frequency of action potential initiation. The most widely expressed inhibitory class of ligand-gated ion channels are γ-aminobutyric acid type A ($GABA_A$) receptors. Assembled from a diverse range of subunits termed $\alpha_{(1\text{-}6)}$,$\beta_{(1\text{-}3)}$, $\gamma_{(1\text{-}3)}$, δ, π and ε, each subtype of $GABA_A$ receptor exhibits unique kinetics, agonist affinity and pharmacology (Hevers & Luddens, 1998; Sieghart & Sperk, 2002; Wallner *et al.* 2003; Hanchar *et al.* 2005).

Cerebellar granule neurons are central to the control of information flow through the cerebellar cortex and are postulated to play a fundamental role in motor learning activity (Marr, 1969; Tyrrell & Willshaw, 1992; Thompson & Stephenson, 1994; Mellor *et al.* 1998). These neurons evince unique anatomical characteristics and $GABA_A$ subunit expression that temporally coincide with the learning and development of motor skills. During the first postnatal week in rats, granule cells start migrating towards the inner granule layer, where they progressively begin to express both α_6 and δ $GABA_A$ subunits (Laurie *et al.* 1992; Persohn *et al.* 1992; Wisden *et al.* 1996). The α_6 subunit mRNA becomes detectable approximately 1 week after birth and is followed by the α_6-dependent expression of the δ subunit (Laurie *et al.* 1992; Jones *et al.* 1997; Nusser *et al.* 1999). The expression of α_6

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and δ subunits gradually increases throughout postnatal development, reaching their highest levels in adulthood (Laurie *et al.* 1992; Persohn *et al.* 1992; Jechlinger *et al.* 1998). This exclusive expression paradigm makes receptors containing α_6 and δ subunits the predominant $GABA_A$ receptor subtype expressed within cerebellar granule neurons in adulthood (Quirk *et al.* 1994; Nusser *et al.* 1999; Tretter *et al.* 2001). The temporal expression of α_6 and δ subunits within the granule neurons correlates with the development of a spontaneous chloride current known as tonic inhibition (Kaneda *et al.* 1995; Brickley *et al.* 1996; Wall & Usowicz, 1997; Hamann *et al.* 2002). The spillover or diffusion of GABA from synaptic events is thought to activate the α_6-containing $GABA_A$ receptors resulting in a tonic inhibition (Isaacson *et al.* 1993; Rossi & Hamann, 1998; Hamann *et al.* 2002; Mody & Pearce, 2004; Semyanov *et al.* 2004; Farrant & Nusser, 2005). Studies in animal models are gradually establishing the importance of tonic inhibition in the regulation of motor activity (Thompson *et al.* 1998; Chiu *et al.* 2005). For example, GABA transporter type 1 (GAT1) knockout mice display various neuronal deficits, including tremor and ataxia, that may arise due to a significant increase in the level of tonic chloride conductance within the cerebellar granule neurons (Chiu *et al.* 2005).

To simulate the temporal coexpression of α_6 and δ subunits within granule neurons, we investigated the characteristics of $\alpha_6\beta\delta$ receptors under different levels and conditions of expression. The structure–function relationship of $\alpha_6\beta_2\delta$ receptors was further examined using mutations of conserved residues within the β_2 or δ subunit.

Methods

Oocyte preparation

The *Xenopus laevis* frogs were anaesthetized by bathing in a solution containing 0.1% MS222 (Tricaine methane sulphonate, Sigma-Alderich, St Louis, MO, USA). Before ovariectomy, the state of anaesthesia was assessed by pinching the toe of the frog. After surgery, the frog was killed by decapitation according to a protocol approved by the Institutional Animal Care and Use Committee. Oocytes were placed in a calcium-free oocyte Ringer solution (calcium-free OR2; 83.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4 and 5 mM Hepes, pH 7.5) plus 0.3% collagenase A (Roche Applied Science, Indianapolis, IN, USA) for approximately 1 h. Stage V and VI oocytes were isolated and maintained by incubating in OR2 (82.5 mM NaCl, 1 mM $CaCl_2$, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM sodium pyruvate, 1 mM Na_2HPO_4, 50 U ml^{-1} penicillin, 50 U ml^{-1} streptomycin and 5 mM Hepes, pH 7.5) with 2% horse serum at 18°C.

Quantification of complementary RNAs (cRNA) and oocyte injections

The procedure for *in vitro* transcription of cRNA have been previously described (Walters *et al.* 2000). The quality of cRNA was determined by electrophoresis on a 1% formaldehyde-containing agarose gel. cRNA concentrations were measured spectrophotometrically. For most experiments, we tested two preparations of cRNAs for each subunit.

Micropipettes for injecting cRNA were fabricated using a Sutter P87 horizontal puller (Sutter Instruments Co., Novato, CA, USA) and, to ensure uniformity of size, the tip of each micropipette was cut with microscissors under 45× magnification next to a control-cut needle. Using a Picospritzer II (General Valve Corporation, Fairfield, NJ, USA), cRNA subunits reconstituted in diethylpyrocarbonate-treated water were injected into *Xenopus laevis* oocytes at a ratio of $1\alpha : 1\beta_2 : 1.8(\gamma_2$ or $\delta)$. The cRNA combinations were injected in amounts of 1.5–3 ng, 5–7 ng, and 8–12 ng per oocyte to produce, respectively, low, intermediate and high levels of expression. For comparison of the different $GABA_A$ subtypes, we coinjected 5–7 ng of cRNA (intermediate expression level) for each combination ($\alpha_6\beta_2\delta$, $\alpha_1\beta_2\delta$, $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$), using two sets of cRNA-mixture preparations and two batches of oocytes.

Drug preparations

Forusemide, bicuculline and picrotoxinin were purchased from Sigma-Alderich Corp. (St Louis, MO, USA). Allotetrahydrodeoxycorticosterone (5α-THODC) was obtained from Steraloids, Inc (Newport, RI, USA). Forusemide, bicuculline, picrotoxinin and 5α-THODC were dissolved in dimethylsulfoxide at their respective stock solution concentrations of 100, 40, 100 and 20 mM. The test solutions were made by diluting the stock solutions in the recording OR2 solution (mM: NaCl, 82.5; KCl, 2.5; Hepes, 5; $CaCl_2$, 1; $MgCl_2$, 1; pH 7.5). The highest concentration of the vehicle solution (0.5% of DMSO) did not significantly alter the level of $\alpha_6\beta_2\delta$ recptors activity.

Electrophysiology

Three to four days after injection, oocytes were placed on a mesh within a small perfusing volume chamber (~75 μl), with $t_{1/2}$ and clearance times of approximately 3 and 10 s, respectively. For complete description of the drug application system see Walters *et al.* (2000).

We used a two-electrode voltage-clamp amplifier (Turbo TEC-05 npi, Adams and List, Westbury, NY, USA) to record currents in response to the application of drugs. Recording microelectrodes were fabricated with a Narishige PP-83 puller (Narishige, Japan) and filled

with 3 M KCl. We used electrodes with input resistances of 0.7–1.6 MΩ. Membrane potential was clamped to −70 mV. Data were visualized on a TA-240 chart recorder (Gould Instrument System, Valley View, CA, USA) during the experiments and stored online using Pulse Fit.

Measurement of the spontaneous current and statistical analysis

High concentrations of GABA (mM) do not evoke a current in mock-injected oocytes indicating an absence of endogenous $GABA_A$ receptors. Upon impaling an oocyte with a pair of electrodes, the oocyte initially displayed a leak current (holding potential = −70 mV). This leak current did not reverse at −30 mV (the predicted reversal potential for chloride in oocytes under these conditions) and within 4–5 min reduced to < 30 nA. If waiting time following the impalement was longer (∼10–15 min), the leak current would have gradually decreased to a value of a few nanoamps, suggesting that the initial random leak may result from an incomplete sealing of the membrane around the electrode. In experiments where the time allocated for recording from each oocyte were short (∼4–5 min, due to large number of oocytes tested in one day), the averaged control leak current measured from the mock-injected oocytes was subtracted from the data and such corrections are noted in Results. However, in most experiments, the wait-time was more than 10 min and thus the mock-injected oocytes did not show any significant leak current. It is also important to note that spontaneous currents arising from $\alpha_6\beta_2\delta$ receptors (in most experiments) are at least an order of magnitude higher than any control leak current recorded (the range of averages of leak current in control cells in different experiments was 14–23 nA where the wait time before measurement was < 5 min).

The analysis of variance (ANOVA one-way) and Fisher's LSD multiple comparison test were used for statistical investigation. All statistical calculations are presented as means ± standard error of the mean.

Data analysis

The EC_{50} and Hill coefficients for the agonists were estimated by fitting the data from concentration–response relationships to the Hill equation according to the following formula (Sigma plot 2000 or Origin 6.0):

$$I = I_{max} / \left(1 + [EC_{50}/A]^{n_H}\right)$$

Alternatively, the data were fitted with a sum of two Hill equations (Origin, 6.0)

$$I = I_{max,1} / \left(1 + \left[EC_{50,1}/A\right]^{n_{H,1}}\right) + I_{max,2} / \left(1 + \left[EC_{50,2}/A\right]^{n_{H,2}}\right)$$

where I is the peak current at a given concentration of agonist (A), I_{max} is the maximum current, EC_{50} is the concentration of agonist yielding a half-maximal current, and n_H is the Hill coefficient.

In the high or low expression condition for the wild-type $\alpha_6\beta_2\delta$ receptors, where one component dominates, the fit of the data points to the sum of two Hill equations using Origin software does not give a satisfactory result (confidence > 0.95). Even in cases when the fitting was successful, the obtained double fit for most low and high expression experiments did not closely follow the experimental data point obtained at high or low concentration ranges, respectively. We postulate that under low or high expression conditions, the apparent desensitization/inactivation of the high affinity component may hinder reliable fitting with the sum of two Hill equations.

To quantify the inhibitory effect of antagonists, the data were fitted to the following equation

$$I = I_{max} / \left(1 + [An/IC_{50}]^{n_H}\right)$$

where I is the peak current at a given concentration of antagonist (An), I_{max} is the level of spontaneous current in the absence of antagonist, IC_{50} is the concentration of antagonist inhibiting half of the spontaneous current, and n_H is the slope.

Results

$\alpha_6\beta_2\delta$ receptors exhibit distinct agonist affinity states

cRNA of rat wild-type α_6, β_2 and δ subunits (Bernard *et al.* 1998) was injected into *Xenopus laevis* oocytes in increments of 1.5–3, 5–7 and 8–12 ng per oocyte to produce low, intermediate and high levels of expression, respectively (at a ratio of $1\alpha_6 : 1\beta_2 : 1.8\delta$). Three to four days after injection, GABA-activated currents were recorded using a GABA concentration range from 0.0002 to 300 μM. Figure 1 shows the current traces and GABA concentration–response relationships for three representative oocytes with low, intermediate and high levels of expression of $\alpha_6\beta_2\delta$ receptors. At low expression levels (Fig. 1, filled circles; holding potential −70 mV), the GABA concentration–response relationship yielded an EC_{50} (a concentration eliciting half-maximal current) of 1.77 μM and a Hill coefficient (n_H) of 0.49 (GABA maximal current; GABA I_{max} = 206 nA). At intermediate expression levels (Fig. 1, open circles), a spontaneous current became apparent (shaded area; 80 nA; GABA I_{max} = 328 nA) which reversed at −33 mV (reversal potential = −28.70 ± 1.64 mV, range −22 to −35 mV; n = 15) in accordance with the predicted chloride reversal potential (Taleb & Betz, 1994). Fitting of the data points from this oocyte with a single Hill equation yielded an EC_{50} of 0.28 μM and an n_H of 0.41, representing a more

than 5-fold increase in GABA sensitivity as compared to the low expression data set. At high expression levels, the spontaneous chloride current increased to over 200 nA (Fig. 1, filled triangles; 269 ± 61 nA, $n = 15$). The presence of a larger spontaneous current (200 nA) was concomitant with a further increase in overall GABA sensitivity ($EC_{50} = 0.01\ \mu$M, $n_H = 0.53$) and desensitization at GABA concentrations greater than the EC_{50} value (the group data for GABA EC_{50}, n_H and maximum values for the low, intermediate and high expression are given in Table 1). Collectively, the range of GABA EC_{50} values derived from different levels of expression ranged from 0.01 to 4.86 μM, with an n_H of ~0.5–0.7 ($n = 24$).

The shallow slope of these concentration–response relationships suggests the presence of a mixture of receptor–channels with differing agonist sensitivities (Kuhse *et al.* 1993; Amin & Weiss, 1996). Refitting the data from the oocyte from the intermediate expression group (open circles) with the sum of two Hill equations suggested two distinct affinity components within the nanomolar and micromolar ranges ($EC_{50} = 0.0052\ \mu$M, $n_H = 0.87$, and GABA $I_{max} = 124$ nA for the high-affinity component, and $EC_{50} = 1.54\ \mu$M, $n_H = 0.76$, GABA $I_{max} = 202$ nA for the low-affinity component; Fig. 1*C*). The mean EC_{50} and n_H parameters derived for the two components at the intermediate expression level are presented in Table 1

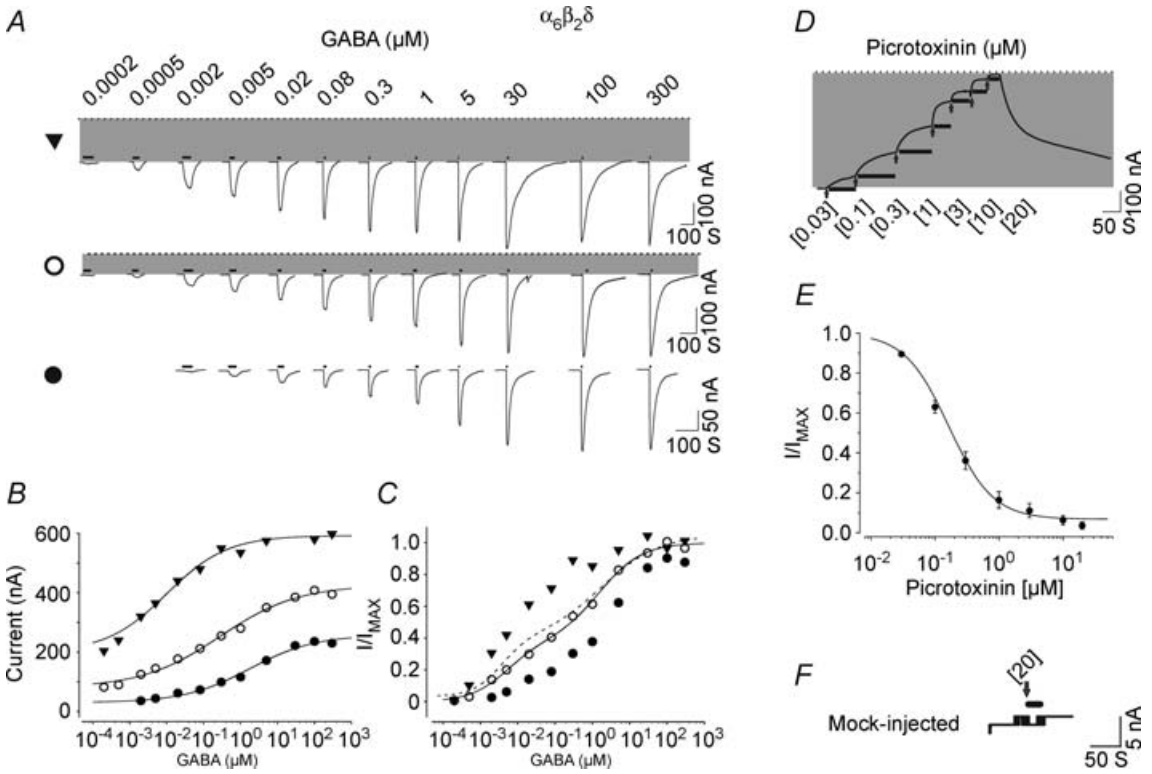

Figure 1. $\alpha_6\beta_2\delta$ receptors exhibit spontaneous activity and two separable state of agonist affinities
A, representative GABA current traces for high (top), intermediate (middle) and low (bottom) expression conditions with oocytes clamped at −70 mV. The dotted line indicates the zero-current level; the shaded area represents the spontaneous activity. The thick lines above the current traces represent the duration of GABA application. The duration of agonist application decreased with increasing concentration of agonist since the currents reached steady state (peaked) more rapidly at higher concentrations of agonist. *B*, plot of GABA concentration–response relationships including the spontaneous currents. All data were fitted with a single Hill equation. *C*, concentration–response relationship of the normalized GABA currents. The EC_{50}, n_H and maxima parameters for the group data for high, intermediate and low expression conditions are shown in Table 1. The continuous line shows the plot of the fit of a sum of two Hill equations to the data points from an oocyte with intermediate expression level demonstrating the presence of two components with different sensitivities to GABA. The dashed line shows the overall plot of the fit (group data) of sum of two Hill equations to the GABA concentration–response data points for the $\alpha_6\beta_2\delta$ receptor at median expression. *D*, a representative current trace for consecutive application of 0.03–20 μM picrotoxinin on an oocyte with high expression of $\alpha_6\beta_2\delta$ recptor. The thick lines below the current traces represent the duration of antagonist application. The arrow indicates the start of the picrotoxinin application at the concentration shown below it. *E*, the concentration–response relationship for picrotoxinin block of the spontaneous current form $\alpha_6\beta_2\delta$ receptor. Picrotoxinin inhibited greater than 90% of the spontaneous current with an IC_{50} of approximately 0.2 μM. *F*, the effect of 20 μM picrotoxinin application on the leak current (20 nA) from a mock-injected oocyte (see methods).

Table 1. Parameters obtained from fitting the Hill equation to GABA, β-alanine, taurine, I4AA, and 5α-THDOC data

$GABA_AR$ subtype	EC_{50} (μM)	n_H	I_{max} (nA)	n
GABA-dependent activation				
$\alpha_6\beta_2(1)\delta$				7
High-affinity component	0.005 ± 0.001	1.16 ± 0.11	259.50 ± 73.96	
Low-affinity component	2.29 ± 0.85	0.56 ± 0.05	479.10 ± 123.94	
Low expression	2.62 ± 0.39	0.63 ± 0.02	129.06 ± 10.72	9
Intermediate expression	0.46 ± 0.12	0.54 ± 0.02	317.39 ± 56.43	7
High expression	0.04 ± 0.009	0.65 ± 0.04	690.60 ± 80.23	11
$\alpha_6\beta_2(0.3)\delta$	1.52 ± 0.38	0.63 ± 0.03	362.80 ± 97.95	6
$\alpha_6\beta_2(0.1)\delta$	1.25 ± 0.21	0.74 ± 0.02	321.67 ± 50.62	10
$\alpha_1\beta_2(1)\gamma_{2S}$	2.87 ± 1.20	1.33 ± 0.10	2287.43 ± 237.76	7
$\alpha_1\beta_2(0.4)\gamma_{2S}$	10.61 ± 3.27	1.22 ± 0.10	2270.33 ± 354.79	6
$\alpha_1\beta_2(0.08)\gamma_{2S}$	37.20 ± 4.58	1.26 ± 0.14	974.33 ± 497.32	3
$\alpha_1\beta_2\delta$	6.45 ± 0.85	1.14 ± 0.05	510.02 ± 80.43	9
$\alpha_6\beta_2\gamma_{2S}$	22.30 ± 1.98	0.88 ± 0.14	484.00 ± 122.45	4
$\alpha_4\beta_2\delta$	1.53 ± 0.14	0.85 ± 0.03	338.09 ± 52.61	11
$\alpha_6\beta_2\delta^{R287M}$	3.71 ± 0.94	0.69 ± 0.18	44.67 ± 2.33	3
$\alpha_6\beta^{Y157F}{}_\delta$				13
High-affinity component	0.37 ± 0.05	0.97 ± 0.04	355.54 ± 80.52	
Low-affinity component	104.65 ± 28.04	0.85 ± 0.14	389.23 ± 63.43	
$\alpha_6\beta^{Y157F}$ (0.1)δ	53.27 ± 13.99	0.94 ± 0.14	161.33 ± 36.99	3
I4AA-dependent activation				
$\alpha_6\beta_2\delta$				4
High-affinity component	0.06 ± 0.01	0.82 ± 0.04	254.68 ± 52.02	
Low-affinity component	202.25 ± 17.71	2.61 ± 0.44	183.41 ± 16.77	
β-Alanine-dependent activation				
$\alpha_6\beta_2\delta$				5
High-affinity component	1.16 ± 0.72	0.89 ± 0.12	160.61 ± 69.75	
Low-affinity component	594.66 ± 142.86	0.67 ± 0.05	277.94 ± 64.02	
Low expression	412.14 ± 68.76	0.71 ± 0.03	302.74 ± 58.79	10
Intermediate expression	111.86 ± 13.72	0.55 ± 0.15	426.33 ± 166.71	3
High expression	2.56 ± 1.52	0.59 ± 0.16	945.00 ± 76.51	3
Taurine-dependent activation				
$\alpha_6\beta_2\delta$				3
High-affinity component	5.80 ± 0.84	1.03 ± 0.05	225.33 ± 43.42	
Low-affinity component	806.67 ± 193.50	0.91 ± 0.29	205.00 ± 40.31	
Low expression	1817.58 ± 865.99	0.69 ± 0.06	145.40 ± 13.31	4
Intermediate expression	45.16 ± 25.57	0.55 ± 0.05	398.30 ± 63.92	3
High expression	6.03 ± 1.62	0.90 ± 0.10	607.63 ± 160.86	4
5α-THDOC-dependent activation				
$\alpha_6\beta_2\delta$	0.89 ± 0.49	0.97 ± 0.06	550.67 ± 67.25	3

(see also dashed line in Fig. 1*C*). The high-affinity component displayed a 400-fold greater apparent affinity for GABA than the low-affinity component (EC_{50} values of 0.005 *versus* 2.29 μM). The relative magnitude of the two components (high to low affinity) was 0.54 (259.50 to 479.10 nA, see Table 1).

Reversal potential measurements indicate that a chloride conductance underlies the spontaneous current but do not demonstrate that the current is mediated by $\alpha_6\beta_2\delta$ receptors (since there are also endogenous chloride channels present within oocytes). Picrotoxinin, a specific pore blocker of $GABA_A$ receptors, was used to determine whether the spontaneous chloride current originates from $\alpha_6\beta_2\delta$ receptor expression. Figure 1*D* and *E* shows the picrotoxinin-induced current traces and concentration–response relationship from an oocyte expressing high levels of $\alpha_6\beta_2\delta$ receptor. Seven incremental concentrations of picrotoxinin (from 0.03 to 20 μM) were applied to oocytes expressing $\alpha_6\beta_2\delta$ subunits to construct a concentration–response relationship. Concentrations were applied incrementally because picrotoxinin possesses a high-affinity binding component for the $\alpha_6\beta_2\delta$ receptor

Table 2. Parameters obtained from fitting the Hill equation to data from Picrotoxinin-, Furosemide- and Zn^{2+}-dependent block of the spontaneous current

$GABA_A$R subtype	IC_{50} (μM)	n_H	Maximal inhibition	*n*
$\alpha_6\beta_2\delta$				
Picrotoxinin	0.16 ± 0.02	1.26 ± 0.06	93.68 ± 2.29%	6
Furosemide	12.31 ± 0.51	1.03 ± 0.05	75.82 ± 4.59%	9
Zn^{2+}	1.42 ± 0.21	0.99 ± 0.08	56.77 ± 4.18%	5
$\alpha_6\beta_1\delta$				
Picrotoxinin	0.28 ± 0.05	0.95 ± 0.06	86.06 ± 2.68%	5
$\alpha_6\beta_3\delta$				
Picrotoxinin	0.28 ± 0.05	1.18 ± 0.09	93.04 ± 2.04%	6

that is resilient to complete wash out. Picrotoxinin blocked the spontaneous current arising from $\alpha_6\beta_2\delta$ receptors with both high efficacy and potency. The IC_{50} value for the picrotoxinin action was approximately 0.2 μM, with picrotoxinin, at 20 μM, blocking more than 90% of the spontaneous current (for all parameters, see Table 2). Figure 1*F* shows a control in which picrotoxinin is added to a mock-injected oocyte displaying 20 nA of leak current. Picrotoxinin (20 μM) did not attenuate the control leak current. These experiments demonstrate that the spontaneous current observed following expression of α_6, β_2 and δ cRNAs originates from $GABA_A$ receptors.

$GABA_A$ antagonists block the spontaneous activity arising from $\alpha_6\beta_2\delta$ receptors

We examined the effects of several specific $GABA_A$ antagonists on the spontaneous current arising from $\alpha_6\beta_2\delta$ receptors. Among the antagonists tested, furosemide proved to be a specific antagonist for $GABA_A$ receptors containing the α_6 subunit (Korpi *et al.* 1995; Korpi & Luddens, 1997). Furosemide was added at 2, 8, 20, 50, 200 and 500 μM concentrations to oocytes expressing $\alpha_6\beta_2\delta$ receptors. Figure 2*A* shows the corresponding current traces and the concentration–response relationship for inhibition of the spontaneous current. Furosemide

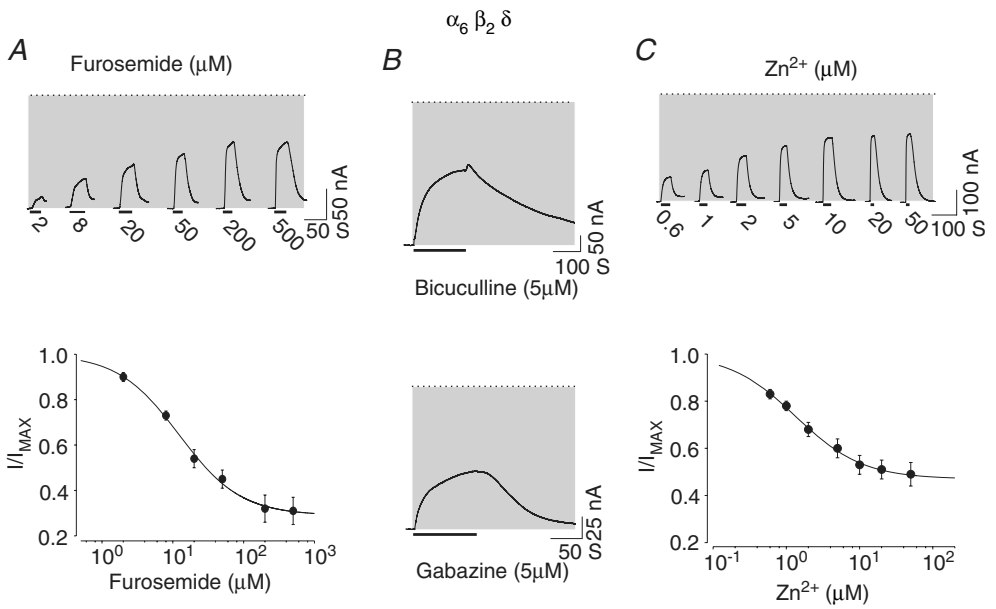

Figure 2. Furosemide, bicuculline, gabazine and Zn^{2+} inhibit the spontaneous current arising from $\alpha_6\beta_2\delta$ receptors

A, furosemide blocked the spontaneous activity of $\alpha_6\beta_2\delta$ receptors. Current traces and the concentration–response relationship for furosemide-dependent inhibition of the spontaneous current arising from an oocyte with a high level of expression of $\alpha_6\beta_2\delta$ receptors. The dotted line indicates the zero-current level; the shaded area represents the spontaneous activity. The thick lines below the current traces represent the duration of antagonist application. *B*, bicuculline and gabazine inhibited the spontaneous activity of $\alpha_6\beta_2\delta$ receptors. Current traces representing bicuculline (5 μM) and gabazine (5 μM) inhibitory action on the spontaneous activity. *C*, the representative current traces and concentration–response relationship for Zn^{2+} block of the spontaneous current arising from $\alpha_6\beta_2\delta$ receptors.

inhibited 76% of the spontaneous current with an IC_{50} of 12.3 μM (see Table 2).

Bicuculline and gabazine (SR95531) are specific competitive antagonists for $GABA_A$ receptors and 5 μM bicuculline or 5 μM gabazine blocked the GABA-independent component of the $\alpha_6\beta_2\delta$ receptors current by $48 \pm 5\%$ and $29 \pm 5\%$, respectively ($n = 4$; Fig. 2*B*), demonstrating that competitive antagonists of $GABA_A$ receptors attenuate the spontaneous activity arising within $\alpha_6\beta_2\delta$ receptors.

Zinc inhibits GABAergic responses within neurons and is postulated to function as an endogenous modulator of ion channels in the CNS (Legendre & Westbrook, 1991; Smart, 1992; Dunne *et al.* 2002; Smart *et al.* 2004). We tested the effect of Zn^{2+} on the spontaneous current arising from $\alpha_6\beta_2\delta$ receptor expression. Figure 2*C* shows representative current traces and the concentration–response relationship of the Zn^{2+}-mediated inhibition of the spontaneous current (IC_{50} of ~1.42 μM; Table 2).

Thus a range of established $GABA_A$ antagonists, including furosemide, bicuculline, gabazine, picrotoxinin and Zn^{2+} all inhibit spontaneous activity arising from the expression of $\alpha_6\beta_2\delta$ receptors.

Expression of a functional receptor–channel requires α_6, β_2, and δ subunits

The presence of two components in the $\alpha_6\beta_2\delta$ GABA concentration–response relationship suggests the coexistence of at least two distinct and separable populations of ion channels. We tested the capacity of α_6 and β_2, β_2 and δ, and α_6 and δ, as well as that of β_2 alone to express ligand-gated ion channels by injecting these cRNA combinations into oocytes at quantities that yield a high level of expression for the $\alpha_6\beta_2\delta$ receptor (8–12 ng per oocyte). Four days post-injection, oocytes were tested for the presence of spontaneous activity and GABA-dependent activity using GABA concentrations of up to 500 μM. These subunit combinations yielded neither functional receptor–channels, nor a spontaneous current ($n = 46$). At significantly greater quantities of cRNA (20–30 ng per oocyte), β_2 or β_2 and α_6 yielded receptor–channels which displayed spontaneous channel activity. However, the resulting β_2 or $\alpha_6\beta_2$ receptors exhibited a markedly reduced GABA maximal current (< 30 nA for β_2 $n = 15$ and < 150 nA for $\alpha_6\beta_2$ $n = 30$). Moreover, even at expression levels of 20–30 ng of cRNA per oocyte (~4-fold the quantities used for intermediate expression of $\alpha_6\beta_2\delta$), neither β_2 nor $\alpha_6\beta_2$ receptors produced the magnitude of spontaneous current activity observed in $\alpha_6\beta_2\delta$ receptors (data not shown). Together these results suggest that neither the β_2 nor the $\alpha_6\beta_2$ receptors contribute significantly to the observed channel activity arising from the expression of $\alpha_6\beta_2\delta$ receptors.

Spontaneous channel activity is a unique property of the $\alpha_6\beta_2\delta$ receptor

Using the intermediate expression protocol (5–7 ng of cRNA), we tested expression of $\alpha_1\beta_2\gamma_{2S(Short)}$, $\alpha_1\beta_2\delta$, $\alpha_4\beta_2\delta$, $\alpha_6\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$ subunit combinations to determine if these receptors produced spontaneous channel activity. For each oocyte expressing a given subtype of $GABA_A$ receptor, we measured the magnitude of the spontaneous current as well as the GABA- and pentobarbital-induced maximal current 4 days post-injection. Figure 3*A* shows the background leak-subtracted magnitudes of spontaneous current for the aforementioned $GABA_A$ subunit combinations (see Methods and Table 3). The spontaneous current activity recorded from $\alpha_6\beta_2\delta$ receptors was significantly higher than that for any other $GABA_A$ receptors (ANOVA one-way analysis F ratio $= 15.76$; $P < 0.001$). Fisher's LSD multiple comparison test also showed that the magnitudes of spontaneous currents arising from $\alpha_6\beta_2\delta$ receptors were different from those of other $GABA_A$ receptors ($P < 0.05$). These experiments demonstrated that the significant level of spontaneous channel activity is a property unique to $\alpha_6\beta_2\delta$ receptors.

GABA exhibits a low efficacy for $\alpha_6\beta_2\delta$ receptors

The maximal GABA-induced current for each subunit combination tested ($\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_2\delta$, $\alpha_4\beta_2\delta$, $\alpha_6\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\delta$) was determined in the preceding experiments (where the spontaneous currents at intermediate expression levels were compared). GABA concentrations were used at 20–50 times the respective EC_{50} values (for EC_{50} values, see Table 1). A comparison of the maximal GABA-evoked currents for the five $GABA_A$ receptor subtypes is shown in Fig. 3*B* (for current values see Table 3). The maximal GABA-evoked current for $\alpha_6\beta_2\delta$ receptors was only 16–28% of that of the other subunit combinations tested. These data reveal that the GABA-sensitive component of $\alpha_6\beta_2\delta$ receptors is significantly smaller than that seen for other $GABA_A$ receptor subunit combinations. A comparison of the spontaneous current relative to the total current (maximal GABA-induced plus the spontaneous current) demonstrates that for $\alpha_6\beta_2\delta$ receptors the spontaneous activity represented approximately 23% of the total attainable current.

Pentobarbital, an intravenous anaesthetic, is a potent modulator of $GABA_A$ receptors and at high concentrations can directly activate them. Previous studies have shown that the GABA-dependent and pentobarbital-dependent activation domains are distinct, given that pentobarbital can activate a mutated $GABA_A$ receptor whose GABA-dependent activation domain is impaired (Amin & Weiss, 1993; Amin, 1999). We also determined the

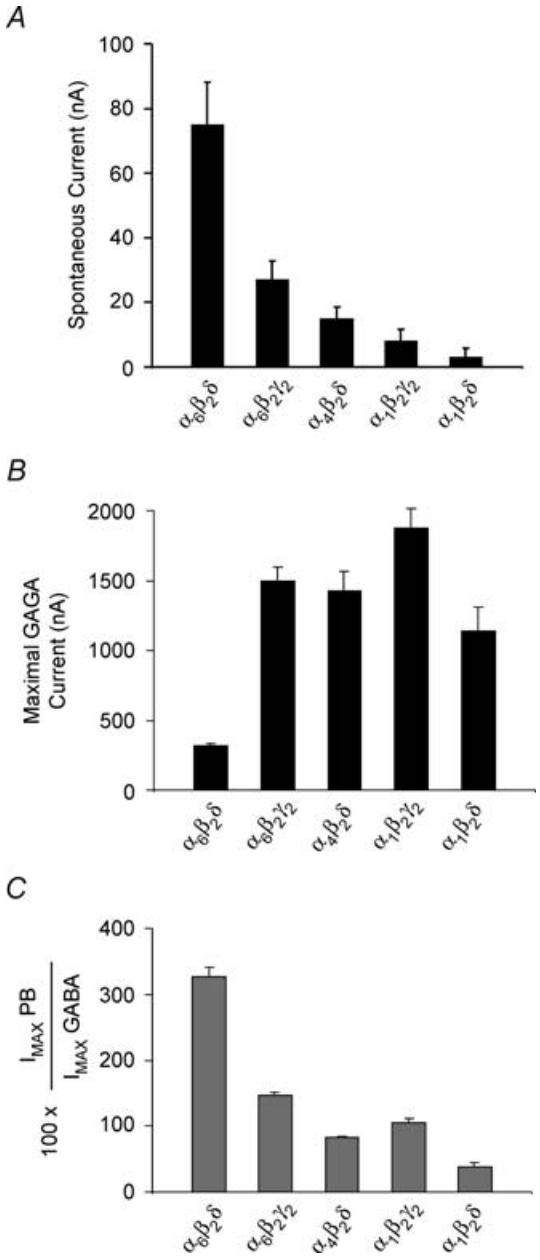

Figure 3. Comparison of the spontaneous currents, GABA-induced maxima and pentobarbital relative maxima to GABA for $\alpha_6\beta_2\delta$, $\alpha_6\beta_2\gamma$, $\alpha_4\beta_2\delta$, $\alpha_1\beta_2\gamma$, and $\alpha_1\beta_2\delta$ receptors under equivalent expression conditions
A, comparison of the spontaneous currents for $\alpha_6\beta_2\delta$, $\alpha_6\beta_2\gamma_2$, $\alpha_4\beta_2\delta$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\delta$ receptors under equivalent expression conditions. The $\alpha_6\beta_2\delta$ receptor exhibited a significantly higher spontaneous current than did the other $GABA_A$ receptors. *B*, comparison of the GABA-induced maximal current for $\alpha_6\beta_2\delta$, $\alpha_6\beta_2\gamma_2$, $\alpha_4\beta_2\delta$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\delta$ receptors under equivalent expression conditions. GABA maximal current was determined from experiments in *A* using GABA concentrations 20–50 times the respective EC_{50} value. GABA had the lowest efficacy (maximal) for $\alpha_6\beta_2\delta$ receptors. *C*, comparison of the relative maximal current of pentobarbital to GABA for the $GABA_A$ receptor subtypes. Pentobarbital maximal current was determined from experiments in *A* using 1 mM concentration. Pentobarbital exhibited a significantly higher efficacy than did GABA for $\alpha_6\beta_2\delta$ receptors.

maximal pentobarbital-induced current for each $GABA_A$ receptor subtype within the preceding experiments at intermediate expression levels (on the same oocytes where the spontaneous activity and the GABA maxima were determined). Figure 3*C* shows the maximal current induced by 1 mM pentobarbital relative to that induced by GABA (pentobarbital I_{max}/GABA $I_{max} \times 100$) for the $GABA_A$ receptor subtypes tested (see also Table 3). For all $GABA_A$ receptors tested, excepting $\alpha_6\beta_2\delta$ receptors, pentobarbital produced similar or lower maximal current than GABA. The pentobarbital-evoked maximal current was more than three times greater than that induced by GABA for $\alpha_6\beta_2\delta$ receptors and was similar in magnitude to the GABA maximal current for other $GABA_A$ receptors. Thus, pentobarbital is markedly more efficacious than GABA and acts as a full agonist for the $\alpha_6\beta_2\delta$ receptor when compared to GABA.

$\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ receptors also exhibit the high-affinity state

Cerebellar granule neurons express high levels of α_1, γ_2, α_6, δ, β_2 and β_3 subunits in the adult rats (Laurie *et al.* 1992; Persohn *et al.* 1992; Wisden *et al.* 1996; Jechlinger *et al.* 1998). The α_6 and δ subunits in combination with either β_1, β_2 or β_3 cRNAs (at intermediate expression levels) were coinjected into oocytes and the maximal GABA-induced current (100 μM) and the extent of the spontaneous activity for each receptor subtype was measured to determine whether the high-affinity state and the spontaneous activity of the $\alpha_6\beta_2\delta$ receptor depend upon the subtype of β subunit (Fig. 4*A*). GABA induced a similar maximal current for $\alpha_6\beta_1\delta$, $\alpha_6\beta_2\delta$ and $\alpha_6\beta_3\delta$ receptors. Further, all three $\alpha_6\beta_{1\text{-}3}\delta$ receptors displayed high levels of spontaneous activity, indicating the presence of the high-affinity state. The level of spontaneous activity was greater for $\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ than for $\alpha_6\beta_2\delta$ receptors, suggesting that the $\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ receptors may exhibit a higher propensity to assemble into the high-affinity state. Next, we determined a picrotoxinin concentration–response relationship (0.03–20 μM) to establish whether the spontaneous activity indeed arises from $\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ receptors (Fig. 4*B* and *C*). Similar to the $\alpha_6\beta_2\delta$ receptor, picrotoxinin blocked the spontaneous current arising from $\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ receptors with high potency (IC_{50} of ~0.3, see Table 2) and efficacy (~90% block at 20 μM). These experiments demonstrated that either the β_1, β_2 or β_3 subunit may coassemble with α_6 and δ subunits to express spontaneously active receptor–channels in the high-affinity state.

Potency and efficacy of different GABA and glycine agonists upon $\alpha_6\beta_2\delta$ receptors

We compared the maximal induced current evoked by GABA with that of two other established GABA

Table 3. Spontaneous, GABA-induced (20–50× their relative EC_{50} values) and pentobarbital-evoked (1 mM) maximum currents (nA) for different $GABA_A$ receptor subtypes

$GABA_AR$ subtype	Spontaneous	GABA	Pentobarbital	$I_{maxPB}/I_{maxGABA}$	*n*
$\alpha_6\beta_2\delta$	98 ± 13.2	318 ± 14	1027 ± 53	326 ± 15%	30
$\alpha_6\beta_2\gamma_{2S}$	50 ± 5.9	1499 ± 98	2156 ± 122	147 ± 4%	25
$\alpha_4\beta_2\delta$	38 ± 4	1440 ± 110	1185 ± 127	83 ± 2%	24
$\alpha_1\beta_2\gamma_{2S}$	31 ± 3.6	1874 ± 138	1974 ± 210	106 ± 6%	25
$\alpha_1\beta_2\delta$	26 ± 2.9	1136 ± 173	570 ± 144	39 ± 6%	27
Control	Leak				
Mock-injected	23 ± 2.5	—	—	—	16

agonists, *trans*-4-aminocrotonic acid (TACA) and imidazole-4-acetic acid (I4AA), in the $\alpha_6\beta_2\delta$ receptor (at intermediate expression levels). Previous studies have established that TACA is a full agonist and I4AA as a partial agonist in $GABA_A$ receptors (Woodward *et al.* 1993; Chebib & Johnston, 1999; Mortensen *et al.* 2004). Figure 5*A* shows the maximal current induced by I4AA (1100 μM ~200 × EC_{50}) and TACA (500 μM ~200 × EC_{50}) relative to that of saturating concentrations of GABA (300 μM). For I4AA, the relative maximal current to that of evoked by GABA was 0.59 ± 0.06 ($n = 9$). In comparison, maximal currents evoked by TACA were consistently larger than that those evoked by GABA (1.22 ± 0.03, $n = 5$) demonstrating that for $\alpha_6\beta_2\delta$ receptors both GABA and I4AA behave as partial agonists relative to TACA.

A concentration–response relationship was constructed with 10 concentrations of I4AA, ranging from 0.001 to 4000 μM. Figure 5*B* depicts the fit of a sum of two Hill equations to these data points (under intermediate expression conditions). The response of $\alpha_6\beta_2\delta$ receptors to I4AA concentrations exhibited two components with a marked difference in their apparent affinity. For I4AA, the high- and the low-affinity components had EC_{50} values of 0.06 and 202.25 μM, respectively, translating into a 3400-fold difference in apparent affinity between the two components (Table 1). The relative maxima of the high- to the low-affinity components of the responses to I4AA was 1.39, as compared to 0.54 for GABA, suggesting that I4AA may have a significantly greater efficacy for the high-affinity state than for the low affinity state.

Both β-alanine and taurine are classical glycine receptor agonists (Kuhse *et al.* 1993) and may act as neurotransmitters within the CNS. Using a range of β-alanine concentrations from 0.05 to 10 000 μM, we determined the efficacy and potency of this agonist in oocytes expressing low, intermediate and high levels of $\alpha_6\beta_2\delta$ receptors (displaying 0, 70 and 190 nA of spontaneous current, respectively; Fig. 5*C*). For an oocyte with a low level of expression (filled circles), the EC_{50} for β-alanine agonist was ~0.5 mM (the group data for β-alanine are shown in Table 1). With increasing expression, the sensitivity of the $\alpha_6\beta_2\delta$ receptor to β-alanine increased concomitantly with the high spontaneous activity (see open circles and filled triangles), reducing the EC_{50} value to approximately 1 μM (single fit to the Hill equation). The fit of the β-alanine data from the $\alpha_6\beta_2\delta$ intermediate expression to the sum of two Hill equations yielded EC_{50} values of 1.16 and 594.66 μM, respectively, for the high and the low affinity components (see Table 1). A comparison of the overall GABA and β-alanine maximal currents demonstrated that β-alanine was as efficacious as GABA for $\alpha_6\beta_2\delta$ receptors (I_{max} β-alanine at 10 000 μM/I_{max} GABA at 300 μM = 1.08 ± 0.03, $n = 7$).

Figure 5*D* shows taurine concentration–response relationships for three sets of oocytes with low (filled circles), intermediate (open circles) and high (filled triangles) levels of $\alpha_6\beta_2\delta$ expression (dispalying 40, 80 and 140 nA of spontaneous current, respectively; the group data for taurine are shown in Table 1). Fitting of the data points to the sum of two Hill equations yielded EC_{50} values of 5 and 807 μM for the high- and low-affinity components, respectively (Table 1). Taurine behaved as a partial agonist for $\alpha_6\beta_2\delta$ receptors as compared to GABA or β-alanine. The relative efficacy of taurine (50 000 μM) to β-alanine (10 000 μM) at near saturating concentrations was 0.69 ± 0.04 ($n = 7$).

The level of β_2 subunit expression determines the apparent affinity of $\alpha_6\beta_2\delta$ receptors

We injected the α_6, β_2 and δ cRNA into oocytes in the ratios of $1\alpha_6 : 0.1\beta_2 : 1.8\delta$, $1\alpha_6 : 0.3\beta_2 : 1.8\delta$, or in the control $1\alpha_6 : 1\beta_2 : 1.8\delta$. The amount of injected cRNA was 5–7 ng of cRNA (intermediate expression) except for $1\alpha_6 : 0.1\beta_2 : 1.8\delta$ where 8–12 ng (high expression) of cRNA was injected. A GABA concentration–response relationship was constructed as shown in Fig. 6*A* and *B*. At the $0.1\beta_2$ ratio ($1\alpha_6 : 0.1\beta_2 : 1.8\delta$; filled squares), the $\alpha_6\beta_2\delta$ receptors were predominantly present in the low-affinity state and displayed three notable properties: (1) the resulting $\alpha_6\beta_2\delta$ receptors were insensitive to GABA concentrations below 0.02 μM (EC_{50} of 1.3 μM; Table 1); (2) after removal of GABA, the current's return to baseline was satisfied by a fit to a single exponential; and (3) these receptor–channels showed no

discernible spontaneous activity. With an increase in the β_2 ratio ($0.3\beta_2$, filled circles), GABA-induced currents were detected at concentrations as low as 0.002 μM (EC_{50} of ~1.5 μM; single fit, see Table 1). Moreover, the higher sensitivity to GABA was concomitant with an appearance of spontaneous channel activity (shaded area) and a shallower Hill coefficient than for the $0.1\ \beta_2$ ratio. Further, current decay, following agonist washout at higher concentrations followed a multiexponential decay (analysis not shown). At the control ratio of $1\alpha_6 : 1\beta_2 : 1.8\delta$ (filled triangles), both high and low affinity components were readily discernible. The appearance of the high-affinity component thus coincided with a significant level of spontaneous channel activity with the current decay following removal of the agonist exhibiting a multiexponential paradigm. In experiments with the β_2 cRNA at a ratio 2–4 times higher (e.g. $1\alpha_6 : 2$ or $4\beta_2 : 1.8\delta$; data not shown), the resulting $\alpha_6\beta_2\delta$ receptors were predominantly present in the high-affinity state, similar to that observed under condition of high expression (see also Fig. 1).

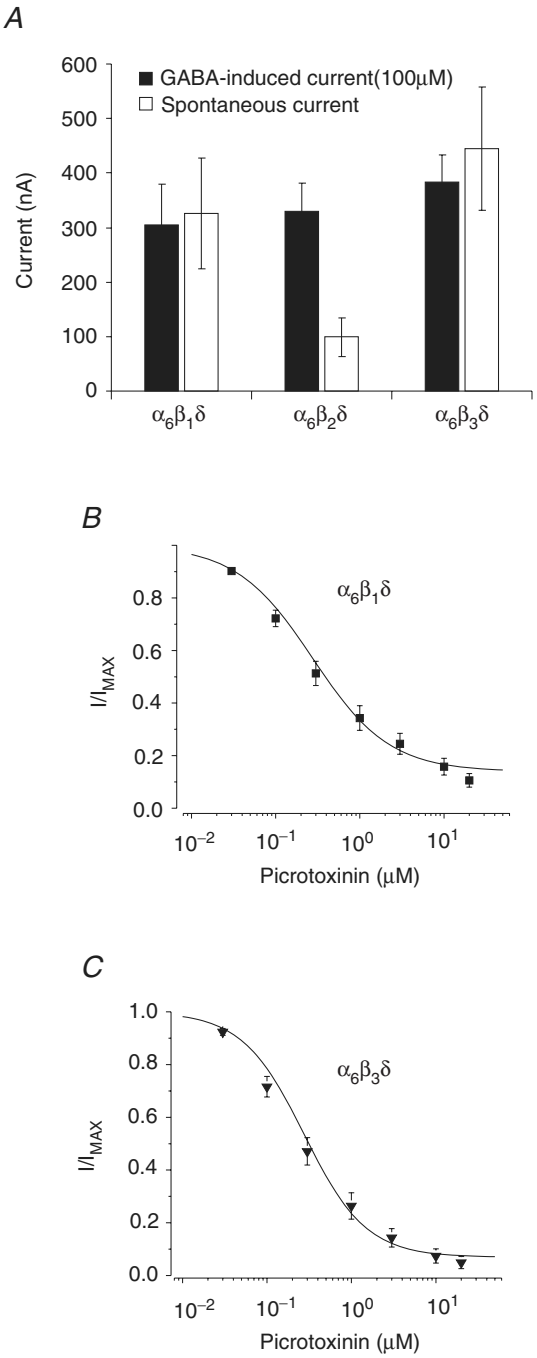

Figure 4. Expression of the high-affinity state of $\alpha_6\beta_2\delta$ receptors is independent of the isoform of the β subunit
GABA induced similar maximal current for $\alpha_6\beta_1\delta$, $\alpha_6\beta_2\delta$, and $\alpha_6\beta_3\delta$ receptors (filled bars) with high levels of spontaneous activity indicating the presence of the high-affinity state (open bars). *B* and *C*, the concentration–response relationship for picrotoxinin block of the spontaneous current from $\alpha_6\beta_1\delta$ and $\alpha_6\beta_3\delta$ receptors.

The $\alpha_1\beta_2\gamma_{2S}$ receptor is one of the most abundantly expressed $GABA_A$ receptor subtypes present within the CNS. To assess whether the marked change in GABA sensitivity brought on by altering the β_2 ratio is unique to $\alpha_6\beta_2\delta$ receptors, we repeated the preceding experiments, but varying the ratio of β_2 to α_1 and γ_{2S} cRNA. We injected these cRNAs, in ratios of either $1\alpha_1 : 0.08\beta_2 : 1.8\gamma_{2S}$, $1\alpha_1 : 0.4\beta_2 : 1.8\gamma_{2S}$ or the control ratio of $1\alpha_1 : 1\beta_2 : 1.8\gamma_{2S}$ into oocytes (intermediate expression condition) and determined GABA concentration–response relationships (Fig. 6*C*). For the $0.08\beta_2$ ($1\alpha_1 : 0.08\beta_2 : 1.8\gamma_{2S}$; open squares), the EC_{50} and Hill coefficient parameters from a fit to a single Hill equation were 37.2 μM and 1.26, respectively (Table 1). These values were similar to those obtained from previous experiments with $\alpha_1\beta_2\gamma_{2S}$ receptors in which the maximal GABA current was limited to ~1000 nA (for the range of maximal currents and EC_{50} values, see Table 1). At $0.4\ \beta_2$ (open circles) and at the control ratio (open triangles), the sensitivity of the resulting $\alpha_1\beta_2\gamma_{2S}$ receptors increased by approximately 4- and 12-fold, respectively, in comparison to $0.08\ \beta_2$ with GABA maxima greater than 2 μA in magnitude (Table 1). Previous studies have shown that coinjection of cRNA for α_1, β_2, and γ_{2S} also yields $\alpha_1\beta_2$ receptors which show an approximately 10-fold higher sensitivity to GABA than the $\alpha_1\beta_2\gamma_{2S}$ receptor (Walters *et al.* 2000). The presence of $\alpha_1\beta_2$ receptors may contribute, in part, to the observed increase in GABA sensitivity of $\alpha_1\beta_2\gamma_{2S}$ receptors. Nevertheless neither expression conditions for $\alpha_1\beta_2\gamma_{2S}$ receptors resulted in the appearance of spontaneous channel activity.

With the increase in the ratio of β_2 cRNA, both $\alpha_6\beta_2\delta$ and $\alpha_1\beta_2\gamma_{2S}$ receptors showed increases in their apparent affinity for GABA. However, the magnitude of the shift in GABA sensitivity to lower concentrations was 12-fold for the $\alpha_1\beta_2\gamma_{2S}$ receptor in comparison to more than 400-fold for the $\alpha_6\beta_2\delta$ receptor. The increase in GABA sensitivity was concomitant with the appearance of spontaneous channel activity within the $\alpha_6\beta_2\delta$ receptor, but not for the $\alpha_1\beta_2\gamma_{2S}$ receptor.

5α-THDOC directly activates $\alpha_6\beta_2\delta$ receptors

Neuroactive steroids are metabolites of the principal sex and stress steroid hormones and represent a large class of endogenous compounds active within the CNS (Belelli & Lambert, 2005). One such metabolite, 5α-THDOC, is a potent modulator of $GABA_A$ receptors (Puia *et al.* 1994), and is a highly hydrophobic compound that mediates its actions through a mechanism that is distinct from that of GABA binding (Morris & Amin, 2004). We examined the direct action of 5α-THDOC upon $\alpha_6\beta_2\delta$ receptors at a range of concentrations. Figure 7 shows 5α-THDOC-induced current traces and the concentration–response relationship for $\alpha_6\beta_2\delta$ receptors (at intermediate- and high-expression conditions). Concentrations as low as 30 nM of 5α-THDOC augmented the spontaneous current. For direct activation by 5α-THDOC, EC_{50} and n_H values of 0.89 μM and 0.97 were derived, respectively (see Table 1). The 5α-THDOC-induced maximal current at 20 μM was similar in magnitude to that of GABA. Thus, 5α-THDOC, previously known for its modulatory action on $GABA_A$ receptors, can directly activate $\alpha_6\beta_2\delta$ receptors with high potency.

Mutation of the β^{Y157F} impairs both the high and the low affinity states of $\alpha_6\beta_2\delta$ receptors

Previous studies have demonstrated that the mutation of Tyr157 to Phe within the β_2 subunit (β^{Y157F}) impairs the GABA-dependent activation of $\alpha_1\beta^{Y157F}\gamma_{2S}$ receptors (~50-fold increase in EC_{50}, Amin & Weiss 1993). To examine the effect of this mutation on the spontaneous activity and the two GABA components of the $\alpha_6\beta_2\delta$ receptor, we coinjected cRNA of α_6, β^{Y157F} and δ subunits into oocytes at low, intermediate, and high levels of expression and determined the respective GABA concentration–response relationship (Fig. 8*A*). These oocytes exhibited spontaneous current activity of

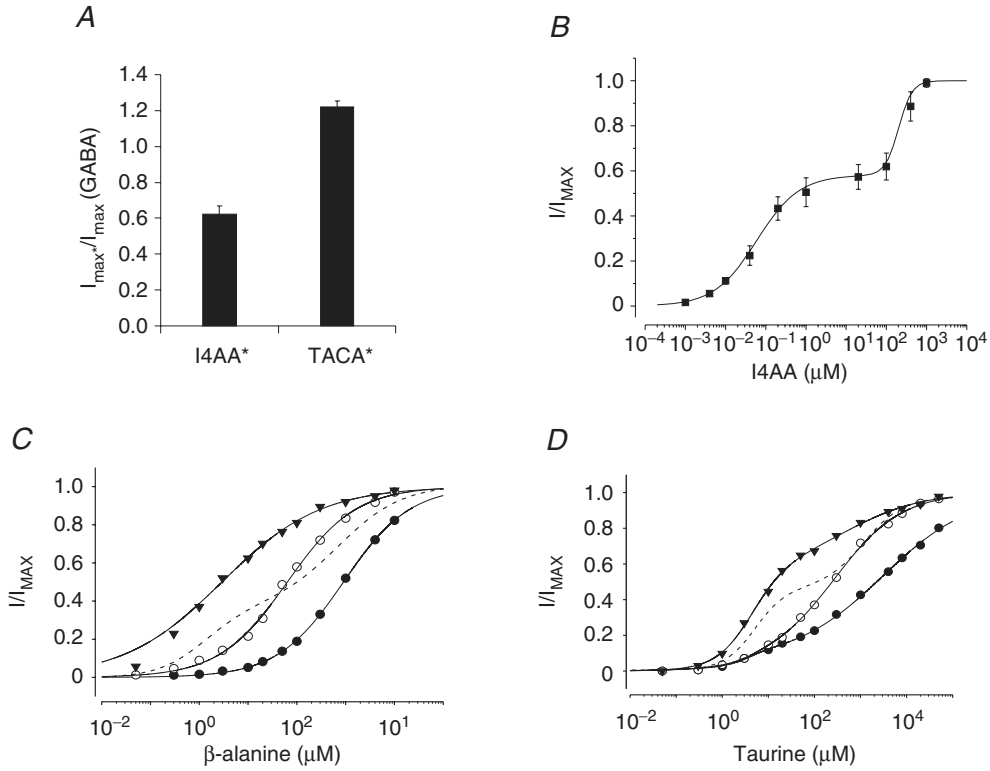

Figure 5. Comparison of the I4AA and TACA maximal-induced currents as well as β-alanine and taurine concentration-response relationship for $\alpha_6\beta_2\delta$ receptors
A, comparison of the I4AA and TACA maximal-induced currents relative to GABA for $\alpha_6\beta_2\delta$ receptors. *B*, concentration–response relationship for I4AA and the fit of a sum of two Hill equations to these data points. The $\alpha_6\beta_2\delta$ receptors response to I4AA concentrations exhibited two components with marked difference in apparent affinity. *C*, the β-alanine concentration–response relationships for oocytes with high (▼), intermediate (○) and low (●) $\alpha_6\beta_2\delta$ expression. The dashed line shows the plot of the fit (average from group data) of sum of two Hill equations to the β-alanine concentration–response data point for wild-type $\alpha_6\beta_2\delta$ receptor at intermediate expression condition. *D*, taurine concentration–response relationships for $\alpha_6\beta_2\delta$ receptors at low (●), intermediate (○), or high (▼) expression condition. The dashed line shows the plot of the fit (average from group data) using a sum of two Hill equations to the taurine data points for the $\alpha_6\beta_2\delta$ receptor at intermediate expression condition.

30 nA, 70 nA and 180 nA at low, intermediate and high expression levels, respectively. Thus, $\alpha_6\beta^{Y157F}\delta$ receptors exhibited a spontaneous current similar in magnitude to that of the wild-type receptors. Fitting these data points to the sum of two Hill equations yielded EC_{50} values of 0.37 and 105 μM GABA (see Table 1). Comparison of the EC_{50} values for the wild-type and mutant receptors showed that the β^{Y157F} mutation had produced an approximately 50-fold reduction in the apparent affinity for GABA for both components.

We then examined $\alpha_6\beta^{Y157F}\delta$ receptors at the injected cRNA ratio of $1\alpha_6 : 0.1\beta^{Y157F}1.8\delta$. Under these expression conditions, $\alpha_6\beta^{Y157F}\delta$ receptors persisted in the low-affinity state (no spontaneous channel activity), with an EC_{50} of 53 μM representing a 50-fold lower sensitivity to GABA in comparison to the $\alpha_6\beta_2\delta$ at $0.1\beta_2$ (see Table 1).

In summary, mutation of the crucial Tyr within the β_2 subunit produced similar impairment of both the low- and high-affinity components, but the appearance of spontaneous activity was unaffected by the β^{Y157F} mutation. These findings suggest that the domains important in establishment of the spontaneous current may be distinct from those crucial for GABA-dependent activation.

δ^{R287M} mutation attenuates both spontaneous activity and the GABA maximal current

Studies of families with a history of generalized epilepsy associated with febrile seizures have identified a single mutation at Lys289 to Met, a residue which is located within the extracellular loop between the second and third transmembrane spanning domains (TM2 and TM3) of the γ_{2S} subunit. This mutation significantly decreases the magnitude of the maximal GABA-induced current in $\alpha_1\beta_2\gamma^{K289M}$ receptors (Baulac *et al.* 2001). To examine the role of δ subunits in the expressional characteristics of $\alpha_6\beta_2\delta$ receptors, we mutated the equivalent residue of the δ subunit, Arg287, to Met. GABA concentration–response relationship experiments for $\alpha_6\beta_2\delta^{R287M}$ receptors yielded an EC_{50} and n_H values of 3.71 μM and 0.69, respectively (Table 1), values which are comparable to the EC_{50} and n_H parameters found for the low-affinity components

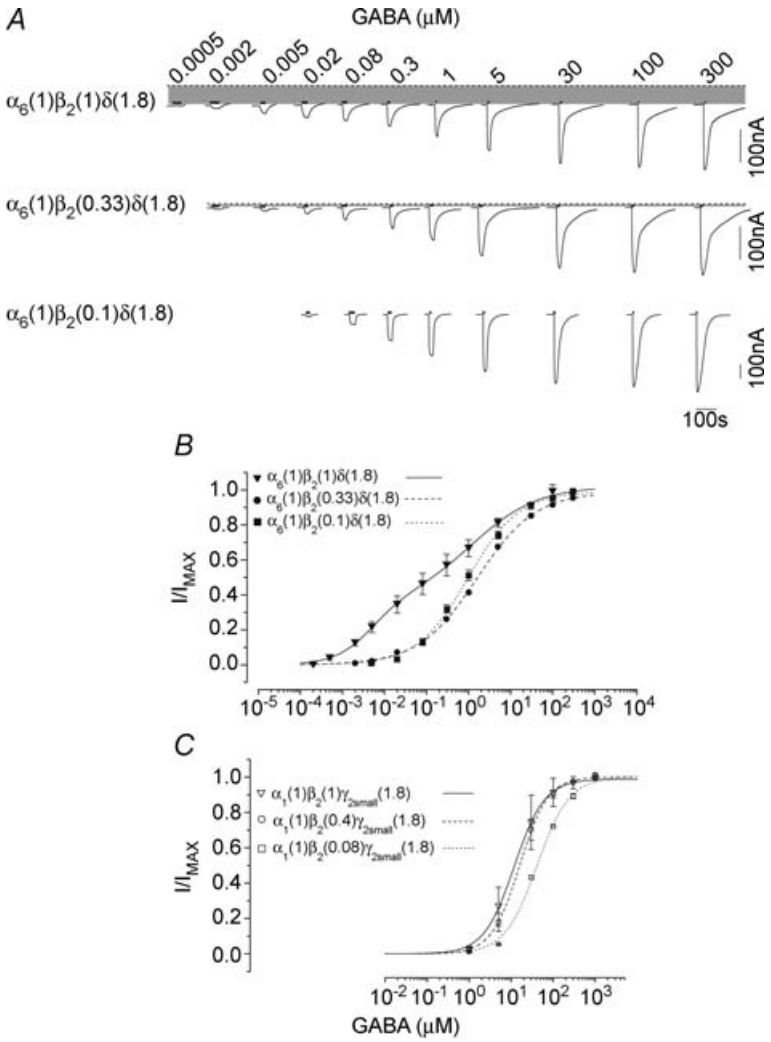

Figure 6. The effect of change in β_2 subunit ratio on the expression of $\alpha_6\beta_2\delta$ and $\alpha_1\beta_2\gamma_2$ receptors
A, the current traces of receptor–channels from the expression of $1\alpha_6 : 0.1\beta_2 : 1.8\delta$, $1\alpha_6 : 0.3\beta_2 : 1.8\delta$, or control $1\alpha_6 : 1\beta_2 : 1.8\delta$ ratios. The dotted line indicates the zero-current level; the shaded area represents the spontaneous activity. The thick lines above the current traces represent the duration of GABA application. *B*, the GABA concentration–response relationship for the three tested ratios of α_6, β_2, and δ subunits. The data derived from $1\alpha_6 : 0.1\beta_2 : 1.8\delta$ and $1\alpha_6 : 0.3\beta_2 : 1.8\delta$ were fitted with a single Hill equation, while the data for $1\alpha_6 : 1\beta_2 : 1.8\delta$ were fitted with a sum of two Hill equations. *C*, the concentration–response relationship for $1\alpha_1 : 0.08\beta_2 : 1.8\gamma_{2S}$, $1\alpha_1 : 0.4\beta_2 : 1.8\gamma_{2S}$ and control$1\alpha_1 : 1\beta_2 : 1.8\gamma_{2S}$ subunit combination.

of the wild-type receptor. Analogous to the effect of the γ^{K289M} mutation within $\alpha_1\beta_2\gamma^{K289M}$ receptors, the δ^{R287M} mutation appeared to induce a diminished maximal GABA-evoked current (Table 1). Accordingly we compared both the spontaneous current and the maxima of GABA-evoked (300 μM) currents of wild-type and $\alpha_6\beta_2\delta^{R287M}$ receptors under equivalent expression conditions (Fig. 8*B*). Under these conditions, the wild-type receptors showed significant spontaneous activity (150 ± 41 nA, $n = 7$; ANOVA one-way analysis F ratio $= 14.31$, $P < 0.001$) as compared to the absence of spontaneous activity (2 ± 4 nA, $n = 8$, corrected for control leak current of 14 ± 3 nA, $n = 7$; see Methods) in $\alpha_6\beta_2\delta^{R287M}$ receptors. Further, we observed that the maximal-induced GABA current in the mutant receptors was also significantly diminished. The GABA maximal current exhibited by $\alpha_6\beta_2\delta^{R287M}$ receptors was 107 ± 23 nA ($n = 7$) as compared to 530 ± 23 nA ($n = 8$) for the wild-type receptors. These experiments demonstrated that the δ subunit contributes to the appearance of the high-affinity state in $\alpha_6\beta_2\delta$ receptors.

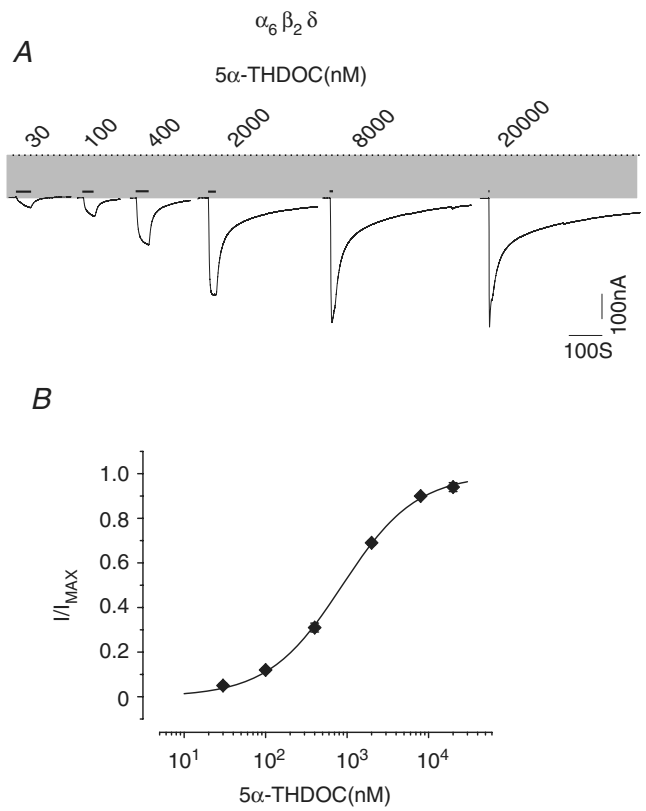

Figure 7. The 5α-THDOC directly activates $\alpha_6\beta_2\delta$ receptors
A, 5α-THDOC-induced current traces. The dotted line indicates the zero-current level; the shaded area represents the spontaneous activity. The thick lines above the current traces represent the duration of 5α-THDOC applications. *B*, the 5α-THDOC concentration–response relationship and fit of data point to a Hill equation.

Discussion

The $\alpha_6\beta_2\delta$ receptors show two distinct states of agonist affinity

We have demonstrated that coexpression of α_6, β_2 and δ subunits produces receptor–channels which display

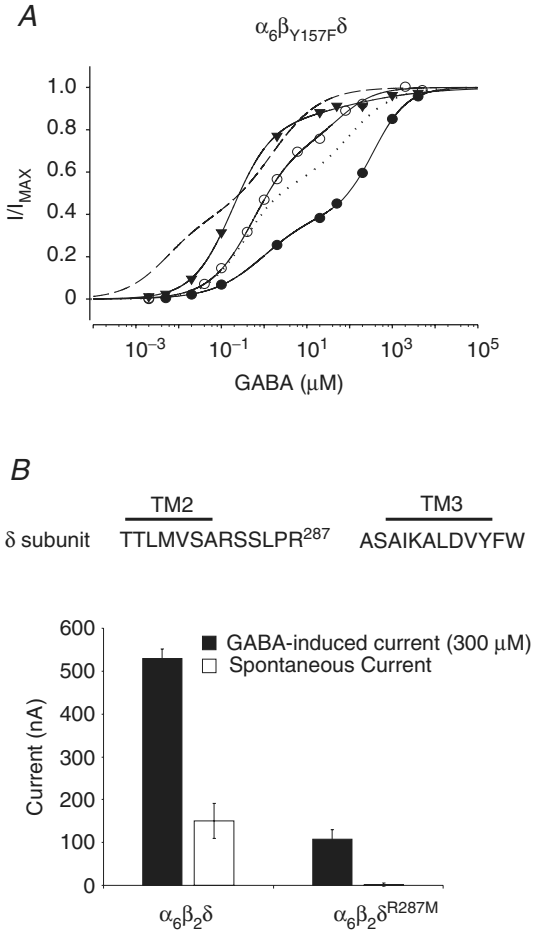

Figure 8. The effect of β_2 or δ subunit mutation on the expression of $\alpha_6\beta_2\delta$ receptors
A, the GABA concentration–response relationship for $\alpha_6\beta^{Y157F}\delta$ receptors for three oocytes with 30 nA (●), 70 nA (○) and 180 nA (▼) of spontaneous current. The mutation within the GABA-dependent activation domain ($\alpha_6\beta_Y{}^{157F}\delta$) did not abolish the spontaneous activity. The continuous lines represent the fit of the GABA data points with a sum of two Hill equations for $\alpha_6\beta^{Y157F}\delta$ receptors at different expression levels. The dashed line shows the overall plot of the fit of the GABA concentration–response relationship data with a sum of two Hill equations for the wild-type $\alpha_6\beta_2\delta$ receptor (intermediate expression). The dotted line shows a plot of the fit (group data) of the GABA concentration–response relationship data with a sum of two Hill equations for the $\alpha_6\beta^{Y157F}\delta$ receptor. *B*, the δ^{R287M} mutation attenuated the spontaneous activity and the GABA maximal current. Top shows the amino acid sequence between the TM2 and TM3 domains of the δ subunit, indicating the position of Arg287. At equivalent expression conditions, the wild-type receptor showed high levels in comparison to no spontaneous activity for $\alpha_6\beta_2\delta^{R287M}$ receptors (corrected for control leak, see methods). The maximal-induced GABA current for mutated receptor was significantly reduced as compared to that for wild-type receptor.

two distinct and separable states of agonist affinity with nanomolar and micromolar sensitivity to GABA. In the high-affinity state, $\alpha_6\beta_2\delta$ receptors showed a significant level of spontaneous activity. Increasing the expression level, or the ratio of β_2 subunit, shifted the equilibrium of the receptor subtypes from the low- to the high-affinity state.

Previous studies of granule neurons in both culture and slice recordings from the cerebellum have demonstrated a significant increase in GABA sensitivity during the maturation of granule neurons (Mathews *et al.* 1994; Puia *et al.* 1994; Zempel & Steinbach, 1995; Tia *et al.* 1996*b*, 1996*a*; Ueno *et al.* 1996; Hevers & Luddens, 2002). However, the degree of shift in GABA sensitivity and spontaneous current shown here has not been reported in studies within granule neuron or with the transient expression of $\alpha_6\beta_3\delta$ receptors in tissue-culture systems (Saxena & Macdonald, 1996). First, the detection of such a current may be hindered by the presence of a mixture of $GABA_A$ receptor subtypes within granule cells, most of which exhibit a significantly lower sensitivity of GABA than the $\alpha_6\beta_2\delta$ receptors. Second, the predominant expression of $\alpha_6\beta_{2\text{-}3}\delta$ receptors in the high-affinity state may occur less frequently and only in fully developed granule cells. Such neurons may be more difficult to culture or to study electrophysiologically in slice recordings. Third, in transient transfection studies the level of expression is difficult to control and electrophysiological experiments are often carried out only 24 hours after transfection, thereby limiting the expression level. Finally, in slice recordings, the persistence of low concentrations of GABA may mask the presence of spontaneous channel activity of $\alpha_6\beta\delta$ receptors.

The presense of two state of agonist affinities or a spontaneous current shown for $\alpha_6\beta_2\delta$ receptors, has precedent within the ligand-gated ion channel family (Khrestchatisky *et al.* 1989; Sigel *et al.* 1989; Krishek *et al.* 1996; Zwart & Vijverberg, 1998; Neelands *et al.* 1999; Buisson & Bertrand, 2001; Mortensen *et al.* 2003; Taleb & Betz, 1994). For example, among $GABA_A$ receptors, $\alpha_1\beta_2\varepsilon$ exhibit a high sensitivity to GABA as well as spontaneous activity (Zwart & Vijverberg, 1998; Neelands *et al.* 1999; Mortensen *et al.* 2003).

Mutation of β_2 *versus* δ subunits

Earlier reports have shown that the β^{Y157F} mutation impairs the GABA-dependent activation of $\alpha_1\beta^{Y157F}\gamma_{2S}$ receptors yet produces little effect on pentobarbital-dependent activation, indicating that the pentobarbital- and GABA-dependent activation domains are distinct (Amin & Weiss, 1993; Amin, 1999). Within the $\alpha_6\beta_2\delta$ receptors, mutation of β^{Y157F} impaired the low- and the high-affinity GABA components to similar extents, without attenuating the spontaneous activity of the $\alpha_6\beta_2\delta$ receptors, suggesting that the domain important for spontaneous activity may also be different from that of the agonist-binding domain.

The mutation of Lys298 to Met within the γ_2 subunit and of the comparable residue in the δ subunit significantly decreased the magnitude of the GABA-induced maximal current within both $\alpha_1\beta_2\gamma_2$ receptor (Baulac *et al.* 2001) and $\alpha_6\beta_2\delta$ receptors. The mutation within the δ subunit also abolished the spontaneous channel activity within $\alpha_6\beta_2\delta$ receptors, indicating the contribution of the δ subunits to the unique properties of $\alpha_6\beta_2\delta$ receptors.

Alternative scenarios for expression of $\alpha_6\beta_2\delta$ receptors within two states of agonist affinities

Earlier reports have shown that $GABA_A$ receptors composed of α_1, β_2 and γ subunits have a fixed stoichiometry in which $2\alpha_1$, $2\beta_2$ and $1\gamma_2$ subunits assemble in a pentameric configuration (Chang *et al.* 1996; Kellenberger *et al.* 1996; Baumann *et al.* 2001; Tretter *et al.* 2001). Comparison of the amino acid sequence among the subunits of $GABA_A$ receptors shows that the δ subunit has a greater homology to the β subunit than the γ subunit. At very high expression levels, the β_2 subunit can form a homo-oligmeric receptor–channels with very low maximal current. The β subunit is also essential for the expression of $GABA_A$ receptors since the cRNA combinations lacking β do not express ligand-gated ion channels within oocytes. This may explain the apparent correlation between the increase in the β_2 levels (by changing the ratio or increasing the amount of cRNA injected) and the level of $\alpha_6\beta_2\delta$ expression. Given the significance of the β_2 subunit in expression of $GABA_A$ receptors, a greater homology of the δ to the β subunit may be an important clue undelying the unique kinetic signature of $\alpha_6\beta_2\delta$ receptors.

Recent studies have shown that the kinetics of $GABA_A$ receptors within hippocampal neurons changes upon clustering of these receptor–channels (Petrini *et al.* 2003; Petrini *et al.* 2004). Additional studies with recombinant $GABA_A$ receptors demonstrate that coexpression of $GABA_A$ receptor-associated proteins markedly alters the kinetics of the $GABA_A$ receptors increasing their conductance (Everitt *et al.* 2004). We propose two alternative scenarios that could account for expression of $\alpha_6\beta_2\delta$ receptors into two distinct states of affinity. First, the $\alpha_6\beta_2\delta$ receptors could assemble with two different stoichiometries (e.g. $2\alpha_6$, $2\beta_2$ and $1\delta_2$ *versus* $1\alpha_6$, $3\beta_2$ and $1\delta_2$). Second, the $\alpha_6\beta_2\delta$ receptors may have a high propensity to cluster where close interaction with the neighbouring receptor-channels may energetically favour the channel configuration in the open state (Fig. 9).

Pharmacological parallel between the spontaneous activity and the tonic inhibition

The spontaneous activity of $\alpha_6\beta_2\delta$ receptors exhibited a pharmacology corresponding to that of tonic inhibition in adult rat granule cells. The following support this notion. First, similar to the block of spontaneous current of $\alpha_6\beta_2\delta$ receptors shown here, bicuculline, gabazine (SR95531) and picrotoxinin all inhibit the tonic chloride current within the granule neurons (Kaneda *et al.* 1995; Brickley *et al.* 1996; Wall & Usowicz, 1997; Rossi & Hamann, 1998; Hamann *et al.* 2002). Second, earlier reports have demonstrated that the ability of bicuculline to inhibit the tonic current diminishes by adulthood. For example, Wall & Usowicz (1997) showed that bicuculline does not completely block the tonic current within a subpopulation of granule neurons at the adult stage. In addition, gabazine does not completely block the tonic current in every neuron tested (Hamann *et al.* 2002). In our studies, bicuculline and gabazine did not completely block the spontaneous current of $\alpha_6\beta_2\delta$ receptor. Third, furosemide, a specific antagonist for $GABA_A$ receptors containing the α_6 subunit (Korpi *et al.* 1995; Korpi & Luddens, 1997), blocks the tonic inhibition (Hamann *et al.* 2002) to a similar extent to the block of spontaneous activity of the $\alpha_6\beta_2\delta$ receptors. Fourth, the kinetics of washouts were significantly slower for bicuculline than for furosemide block of the spontaneous activity of $\alpha_6\beta_2\delta$ receptors (Fig. 2). This is analogous to the markedly slower return of current to the baseline after bicuculline wash than after furosemide wash in studies of tonic inhibition from adult animals (Hamann *et al.* 2002). The very slow wash of the competitive antagonist bicuculline in these studies may indicate the presence of $GABA_A$ receptor within the high-affinity state. Fifth, β-alanine in the micromolar concentration range can activate $\alpha_6\beta_2\delta$ receptors. Earlier reports have demonstrated that β-alanine, in micromolar concentrations, can also induce a bicuculline-sensitive current in adult cerebellar granule cells exhibiting a tonic current (Wall & Usowicz, 1997). Finally, nanomolar concentrations of 5α-THDOC can enhance tonic conductance through α_6-containing receptors (Stell *et al.* 2003). In parallel, 5α-THDOC at nanomolar concentrations can directly activate $\alpha_6\beta_2\delta$ receptors in the high-affinity state.

Potential contribution of the high-affinity state of $\alpha_6\beta_{2\text{-}3}\delta$ receptors to granule neuron

The developmentally controlled expression of $\alpha_6\beta_2\delta$ receptors may be an important component in the formation of motor memory in the CNS. We hypothesize that the intrinsic spontaneous activity from $\alpha_6\beta_2\delta$ receptors contributes to some extent to the formation of motor memory at later stages of rat development. Conceivably, the level of $\alpha_6\beta\delta$ expression alone could have a key function in the formation of single-cell memory, producing a graded but ligand-independent tonic activity in cerebellar granule neurons, where spillover of GABA can further modulate its strength. A few findings support this hypothesis. (1) The expression of α_6, β and δ subunits in the cerebellum increases during postnatal development, reaching a markedly high level by adulthood (Laurie *et al.* 1992; Jones *et al.* 1997). The high expression of α_6, β and δ subunits increases the likelihood of the transition of $\alpha_6\beta_{2\text{-}3}\delta$ receptors to the high-affinity state. (2) There is an extensive pharmacological similarity between spontaneous activity of the $\alpha_6\beta_2\delta$ receptor and the tonic inhibition in rat granule neurons. (3) In an α_6-knockout animal model in which motor functions remain normal, a spontaneously open K^+ channel appears to subsume the role of the α_6-containing $GABA_A$ receptors (Brickley *et al.* 2001). (4) The $\alpha_6\beta_{2\text{-}3}\delta$ receptors are extrasynaptically located (Nusser *et al.* 1998). This is consistent with the view that these receptor–channels may, to some extent, be independent from neurotransmitters for conveying their entire functional spectrum. (5) Electrophysiological studies have demonstrated the existence of $GABA_A$ receptor exhibiting spontaneous current in cultured rat neurons (Birnir *et al.* 2000). (6) Recent binding studies have shown a bimodal distribution of GABA affinity with components in the nanomolar and micromolar range within membrane preparations of

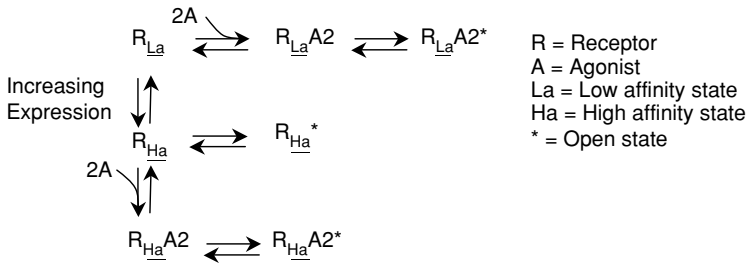

Figure 9. A model showing different states of $\alpha_6\beta_2\delta$ receptors upon clustering

cerebellum (Maksay & Biro, 2005). We found that the derived GABA-binding values were similar to the EC_{50} values for the two GABA components of $\alpha_6\beta_2\delta$ receptors (Table 1). Two distinct affinity states were also shown for taurine with binding values matching closely with the EC_{50} of the two taurine components of $\alpha_6\beta_2\delta$ receptors (Table 1). In comparison, the high-affinity component was altogether absent in membrane preparations from the hippocampus, where α_6 and δ subunits are absent.

We have demonstrated that $\alpha_6\beta_2\delta$ receptors can exist in low- and high-affinity states in which the expression level can affect the equilibrium between the two states. The high-affinity state is always accompanied by a spontaneous current that exhibited an overall pharmacology similar to that of tonic inhibition. Additional studies are needed to determine the relative contributions of the neurotransmitter-dependent and spontaneous currents arising from $\alpha_6\beta_{2\text{-}3}\delta$ receptors in mediating tonic inhibition in the adult cerebellum.

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