Synaptic α5 Subunit–Containing \textit{GABA}_A Receptors Mediate IPSPs Elicited by Dendrite-Preferring Cells in Rat Neocortex

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Previous studies indicated that one class of dendrite-prefering hippocampal interneurons inhibits pyramidal cells via α5 γ-aminobutyric acid (\textit{GABA}_A) receptors whereas parvalbumin- and CCK-containing basket cells act via α1 and α2/3 \textit{GABA}_A receptors, respectively. This study asked whether there is selective insertion of different α subunit–containing \textit{GABA}_A receptors at neocortical inhibitory synapses innervated by specific classes of interneurons. The benzodiazepine site pharmacology of inhibitory postsynaptic potentials (IPSPs) elicited in neocortical pyramidal cells by 3 classes of interneurons was explored with dual whole-cell recordings in neocortical slices from juvenile rats (P18–23). Fast IPSPs activated by multipolar interneurons with narrow spikes and nonadapting firing patterns were powerfully enhanced by the α1-prefering agonist zolpidem, suggesting mediation via larger proportion of α1 \textit{GABA}_A receptors than those activated by multipolar, adapting interneurons, which were less strongly enhanced by zolpidem, but equally insensitive to the α5-selective inverse agonist IAalph5 (MSD, Essex, UK) suggesting mediation predominantly via α2/3 \textit{GABA}_A receptors. In contrast, the IPSPs elicited by bitufted, dendrite-prefering interneurons were reduced by IAalpha5 and by zinc and insensitive to zolpidem despite enhancement by the broad-spectrum agonist, diazepam. Thus insertion of \textit{GABA}_A receptors at synapses on neocortical pyramids is input-specific, with proximal inhibition employing α1 and α2/3 \textit{GABA}_A receptors and dendrite-prefering bitufted interneurons activating α5 \textit{GABA}_A receptors.

\textbf{Keywords:} benzodiazepine site, \textit{GABA}_A, interneuron, IPSPs, neocortex, synaptic

\textbf{Introduction}

γ-aminobutyric acid (\textit{GABA}_A) receptors are heteropentameric, ligand-gated ion channels. Nineteen \textit{GABA}_A receptor subunits have been identified to date (α1–6, β1–3, γ1–3, δ, ε, π, θ, for review, see Simon et al. 2004; Wafford 2005). The majority of \textit{GABA}_A receptors in the brain contain α, β, and γ2 subunits in a 2:2:1 stoichiometry (Sieghart and Sperrk 2002). β subunits ensure correct insertion of assembled receptors into the plasma membrane. The γ2 subunit with an adjacent α1, α2, α3, or α5 subunit forms the benzodiazepine site, and the α subunit determines the pharmacological profile of this site. Receptors with the highest affinity for the benzodiazepine type 1 agonist, zolpidem, contain an α1 subunit. Receptors containing an α2 or α3 subunit have a lower affinity for zolpidem (Munakata et al. 1998), whereas α5 subunit-containing receptors display exceedingly low affinity for zolpidem (Puia et al. 1991; Burgard et al. 1996).

In contrast, nonselective benzodiazepine agonists, like diazepam, enhance the \textit{GABA}_A receptor-mediated chloride flux through all α1/α2/α3/α5βγ2 receptors. This potentiation of \textit{GABA}_A receptor activity produces the sedative, anxiolytic, muscle relaxant, anticonvulsant and cognitive-imparing effects of benzodiazepines. Recent studies, using genetically modified animals have shown that \textit{GABA}_A receptors containing α1 subunits mediate the sedative, anticonvulsant and amnestic effects of benzodiazepines, whereas the α2 and α3 subunits mediate their anxiolytic effects (Rudolph et al. 1999; McKernan et al. 2000; Atack et al. 2005).

The α5-containing receptors have been less extensively studied but may play an important role in learning and memory (Maubach 2003). Suppression of α5 \textit{GABA}_A receptors results in an enhanced rate of associative memory acquisition and a slower rate of extinction in hippocampal-dependent learning tasks (Collinson et al. 2002; Chambers et al. 2004). From fear conditioning studies, α5 \textit{GABA}_A receptors have been suggested to be control elements in associative memory (Crestani et al. 2002; Yee et al. 2004). Thus, the selective activation or suppression of \textit{GABA}_A receptors containing different α subunits produces different behavioral profiles (Mohler et al. 2002; Whiting 2003).

This might suggest that the circuitry that activates each \textit{GABA}_A receptor subtype differs from that activating other subtypes. In support of this suggestion, firstly that immunocytochemical and pharmacological studies on CA1 pyramidal cells have shown that fast-spiking parvalbumin-containing basket cells innervate α1 \textit{GABA}_A receptors, whereas adapting, CCK-containing basket cells and axo-axonic cells innervate α2/3 \textit{GABA}_A receptors (Nusser et al. 1996; Fritschy et al. 1998; Loup et al. 1998; Pawelzik et al. 1999; Thomson et al. 2000; Nyiri et al. 2001). Secondly, pyramidal neurons express mRNA for a large number of different \textit{GABA}_A receptor subunits, including the α5 subunit (Wisden et al. 1992; Ruano et al. 1997), and in contrast to α1, 2/3 subunits, the α5-subunit immunoreactivity is more concentrated in the dendrites of hippocampal CA1 pyramidal cells (Fritschy and Mohler 1995; Sperk et al. 1997; Wainwright et al. 2000; Christie et al. 2002). Until recently, these α5 \textit{GABA}_A receptors were thought to be almost exclusively extrasynaptically located and to play a role in the tonic inhibition of CA1 pyramidal cells (Caraiscos et al. 2004). However, a recent study combining immunofluorescence (of cultured hippocampal cells) and electron microscope (EM) postembedding immunogold (in ex vivo hippocampus) has identified synaptic α5 subunits on CA1 pyramidal cell dendrites in addition to the extrasynaptic expression of these receptors (Serwanski et al. 2006).

This study asks whether different \textit{GABA}_A receptor subtypes are selectively inserted at specific synaptic locations on postsynaptic neocortical pyramidal cells, that is, whether insertion of \textit{GABA}_A receptor subtypes correlates with the class
of presynaptic interneuron innervating that synapse and whether any particular class of inhibitory connection is mediated by GABA_A receptors. To this end, paired whole-cell recordings of inhibitory postsynaptic potentials (IPSPs) were challenged with a range of benzodiazepine site ligands and recorded cells were filled with biocytin to allow the interneurons involved to be identified.

Methods

Male Wistar rats (postnatal day 18-22) were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg Euthatal, Merial, Harlow, Essex, UK) and perfused transcardially with 50-100 ml ice-cold modified artificial cerebrospinal fluid (ACSF). This modified ACSF contained (in mM) 248 sucrose, 25.5 NaHCO_3, 3.3 KCl, 1.2 KH_2PO_4, 0.1 MgSO_4, 2.5 CaCl_2 and 15 mg glucose, equilibrated with 95% O_2/5% CO_2. The animals were then decapitated and the brain removed. Coronal sections of cerebral cortex, 300-330 μm thick, were cut using a vibratome (Leica, Wetzlar, Germany). The slices were incubated for 1 h in standard ACSF containing (in mM) 121 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 CaCl_2, 1 MgCl_2, 26 NaHCO_3, 20 glucose, and 5 pyruvate and equilibrated with 95% O_2/5% CO_2. For recordings, slices were transferred to a submerged-style chamber and perfused at 1-2 mL/min with the standard ACSF. These procedures comply with UK Home Office regulations for the use of animals.

Dual whole-cell recordings were made in current clamp in rat somatosensory cortex. Each recorded cell pair included a visually and electrophysiologically identified interneuron and a pyramidal cell. Interneurons were selected according to the shape of their somata; round or oval, or inverted pyramid shaped, visualized using video microscopy under near-infrared differential interference contrast illumination and further characterized by their firing properties. Experiments were conducted at 20-22°C. Patch pipettes (resistance 8-10 MΩ) were pulled from borosilicate glass tubing and filled with an internal solution containing (in mM) 144 K-glutamate, 3 MgCl_2, 0.2 ethylene glycol-bis(aminohexyl ether)-tetraacetic acid, 10 N-glutamylglycine-N 2-ethanesulfonic acid, 2 Na_2ATP, 0.2 Na_2-GTP, and 0.02% w/v of biocytin (pH 7.2-7.4, 300 mOsm).

Single presynaptic action potentials (APs) were elicited in the interneuron by injecting short (5-8 ms) pulses of depolarizing current repeated at 0.33 Hz (SEC OSL/Hi, npi electronics, Tamm, Germany) and postsynaptic responses in pyramidal cells recorded. Recordings were amplified, low-pass filtered at 2 kHz, digitized at 5 kHz using a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK) and recorded on disc for analysis.

Drugs

Zolpidem (Sigma, Aldrich, UK, 0.4 μM) dissolved first in ethanol to a final bath ethanol dilution of 1:20 000; flumazenil (Roche, Welwyn Garden City, UK, 0.4 μM); IA alpha 5 (MSD, UK, 1-1.5 μM); diazepam (RBI, Poole, UK; 1-2 μM), dissolved in ethanol to a final bath ethanol concentration of 1:5000; and zinc chloride (100 μM, Sigma) were bath applied. The IA alpha 5 concentration used (1-1.5 μM) was within the range at which it is reported to act as an inverse agonist with efficacy selective for 5-containing GABA_A receptors (Dawson et al. 2006). The concentration of zolpidem used produces near maximal effects on 21-containing receptors but submaximal effects on 2/3-containing receptors (K_50 of 0.2 μM for 21-containing receptors; 1.5 μM for 2/3-containing receptors, Munakata et al. 1998).

Data Analysis

Data were acquired and analyzed using Signal software (Cambridge Electronic Design, Cambridge, UK) and in-house software. Individual sweeps were occupied and either accepted, edited, or rejected according to the trigger points that would trigger measurements and averaging of the IPSPs during subsequent data analysis. IPSPs were selected within a narrow window of membrane potential of ±20.5 mV, and averaging was triggered from the rising phase of the presynaptic spike. The IPSP 10-90% rise times and width at half amplitude were measured from averaged IPSPs. Single-sweep IPSP amplitudes were measured from the baseline to the peak of the IPSP. Average IPSP amplitudes are given as the mean ± standard deviation of measurements of 50-350 single sweeps. Apparent failures of synaptic transmission were identified by eye and confirmed when averaging of these apparent failures resulted in no measurable postsynaptic response. The electrophysiological characteristics of the recorded cells were measured from their voltage responses to 500 ms current pulses between -0.2 and +0.1 nA in amplitude.

To determine whether the electrophysiological characteristics of the 3 groups of presynaptic interneurons studied differed, measurements of AP characteristics, input resistance, and membrane time constants were compared (1-way analysis of variance followed by pairwise Student’s unpaired t-test to determine which class differed). To determine whether a drug produced a significant change in IPSP amplitude at each individual inhibitory connection tested, single-sweep measurements obtained under control conditions and during drug application were compared (Student’s unpaired t-test). To determine whether the drug had a significant effect on a given population of IPSPs, the mean IPSP amplitudes obtained under control and under drug conditions were compared (Student’s paired t-test).

Slices containing biocytin-filled cells were fixed overnight in 4% paraformaldehyde plus 0.2% saturated picric acid solution in 0.1 M phosphate buffer (PB), pH 7.2 at 4°C. For standard avidin-horseradish peroxidase-Diaminobenzidine (DAB) processing, slices were fixed overnight in 1.25% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M PB. Extensive rinses were carried out between each step using phosphate-buffered saline (0.1 M). Slices were then embedded in gelatin, fixed, and resctioned at 60 μm (vibratome 1000, Intracel Ltd, Herts, UK). The sections were freeze-thawed over liquid nitrogen after cryoprotecting for 2×10 min in 10% sucrose, 2×20 min in 20% sucrose with 6% glycerol and 2×30 min in 30% sucrose with 12% glycerol. The sections were incubated overnight in Vector ABC peroxidase (1:200, Vector Laboratories, Peterborough, UK) at 4°C. The peroxidase group was revealed using 3’3’ diaminobenzidine as the chromogen (Vector DAB kit, Vector Laboratories). The visualized cells were intensified with 0.1% osmium tetroxide, and the sections were cleared by dehydration in an ascending series of alcohols to 100%, then embedded in Durpan resin (Agar Scientific, Cambridge, UK). The cells were reconstructed at 1000x (100x objective, 10x eyepiece) using a Zeiss Axioskop (Carl Zeiss, Göttingen, Germany) with drawing tube attachment.

Results

In 71 dual recording experiments, 16 bitufted interneurons and 32 multipolar interneurons activated IPSPs in postsynaptic pyramidal cells. The probability of finding an inhibitory synaptic connection from a bitufted interneuron to a simultaneously recorded postsynaptic pyramid was 1/4. The probability of finding a connection from a multipolar interneuron to a postsynaptic pyramid within close proximity (5-15 μm) was very high, 1/1, in the pairs reported here. When the 2 neurons were further apart, the probability of finding a synaptic connection was lower. Pharmacological studies were performed on 13 IPSPs activated by bitufted cells and 16 IPSPs activated by multipolar cells, and the data that will be discussed from this point onwards will be those pairs with pharmacological protocols.

Morphology of Bitufted Dendrite-Preferring Interneurons

Bitufted interneurons in layers 2-5 were sparsely located and their somata were usually oval and gave rise to 3-5 vertically orientated primary beaded dendrites from opposite poles (Figs 1Aa and AACaDa). The primary dendrites then branched further, and the larger portion of the dendritic axis was directed down toward deeper layers. Although majority of these dendrites were smooth, 2 bitufted cells included in this
study were sparsely spiny. The fine axons containing small/medium-sized boutons originated from the soma or a primary dendrite and ramified quite extensively around their somata with a vertical distribution preference. All 13 recovered bitufted cells reported in detail here had ascending axons, projecting vertically toward the pia. Four of these cells had axons that extended horizontally in layer 1 and thus resembled Martinotti cells (Martinotti 1889; Kawaguchi and Kubota 1997). Close membrane appositions between the presynaptic axon and postsynaptic neuron typically involved the shafts of second- and third-order pyramidal dendrites.

**Morphology of Multipolar Proximally Targeting Interneurones**

The somata of multipolar cells with an adapting firing pattern either resembled inverted pyramids \((n = 4, \text{Figs 1Ba and 3Bc})\) or were round/oval \((n = 3)\), whereas the somata of non-adapting multipolar cells were usually round \((n = 9, \text{Figs 1Ca and 3Ac})\). Multipolar interneurons had aspiny, typically beaded dendrites that extended radially from their 3–7 primary dendrite. The axons of these cells were more commonly horizontally oriented and ramified densely in the layer of origin \((n = 16)\).

**Electrophysiological Properties of Presynaptic Interneurones**

The bitufted, dendrite-prefering interneurons also displayed an adapting firing pattern (Fig. 1Ab). Their APs were broader with shallower spike after-hyperpolarizations (AHPs) than those of multipolar, nonadapting interneurons. They also displayed higher input resistances and longer membrane time constants than multipolar cells (Table 1).

The intrinsic membrane properties of multipolar, adapting cells were intermediate between those of bitufted interneurons and multipolar, nonadapting cells (Table 1, Fig. 1Bb). Nonadapting cells displayed the fastest APs, which were terminated with deep, fast-spike AHPs, and trains of spikes showed little accommodation or spike frequency adaptation (Fig. 1Cb). The multipolar, nonadapting cells displayed the lowest mean input resistances and the fastest membrane time constants (Table 1). None of these 3 classes displayed a “sag” in voltage responses to hyperpolarizing current.

**Synaptic Properties of Bitufted and Multipolar Interneurones**

Bitufted, dendrite-prefering interneurons with ascending axons, innervated small- to medium-sized pyramidal cells.
Reciprocal connections were also seen between bitufted interneurones and pyramidal cells \( (n = 4) \). The excitatory postsynaptic potentials (EPSPs) elicited in bitufted interneurons in this study, like those reported previously (Thomson et al. 1995; Porter et al. 1998; Reyes et al. 1998; Silberberg et al. 2005; Ali et al. 2007), displayed paired pulse and brief train facilitation (Fig. 1A).

Four of the 16 multipolar to pyramidal cell pairs were reciprocally connected. As reported previously (Reyes et al. 1998; Angulo et al. 1999; Thomson et al. 2002; Silberberg et al. 2005; Ali et al. 2007), a common feature of both subclasses of multipolar cells was that the EPSPs they received displayed paired pulse and brief train depression (Fig. 1B).

The time course of IPSPs elicited by the 3 types of interneurons studied correlated with the presynaptic spike width (Fig. 2D) (correlation coefficient, \( r = 0.76, n = 29 \)). The fastest IPSPs were elicited by multipolar, nonadapting cells (rise time 5.2 ± 0.73, width at half amplitude 53.5 ± 2.4 ms, amplitude 0.60 ± 0.38 mV). Multipolar cells with an adapting firing pattern elicited IPSPs with an intermediate time course (10–90% rise time 9 ± 1.4, width at half amplitude 82 ± 31 ms, amplitude 1.2 ± 0.92 mV), whereas the bitufted cells elicited the slowest IPSP (rise time 14.3 ± 3.0, width at half amplitude 96.3 ± 24 ms, amplitude 0.81 ± 0.4 mV, Table 2).

### Table 1: Electrophysiological properties of presynaptic multipolar and bitufted interneurons

<table>
<thead>
<tr>
<th>Presynaptic'Neill</th>
<th>AP properties</th>
<th>Input resistance (MΩ)</th>
<th>Time constant (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike HW (ms)</td>
<td>AHP amplitude (mV)</td>
<td>AHP HW (ms)</td>
<td></td>
</tr>
<tr>
<td>Multipolar, nonadapting cells, mean ± SD</td>
<td>1.0 ± 0.25 a (n = 6)</td>
<td>13.0 ± 1.8 a (n = 6)</td>
<td>4.6 ± 1.4 a (n = 6)</td>
</tr>
<tr>
<td>Multipolar, adapting cells, mean ± SD</td>
<td>1.4 ± 0.13 a,b (n = 10)</td>
<td>9.4 ± 2.60 a,b (n = 10)</td>
<td>5.6 ± 1.1 a,b (n = 10)</td>
</tr>
<tr>
<td>Bitufted cells, mean ± SD</td>
<td>1.7 ± 0.44 a,b (n = 13)</td>
<td>6.0 ± 2.3 a,b (n = 13)</td>
<td>6.0 ± 1.2 a,b (n = 13)</td>
</tr>
</tbody>
</table>

Note: HW = half width; SD = standard deviation.

*Significantly different from the other 2 subclasses of interneurons, 1-way analysis of variance, \( P < 0.05 \).
*bSignificantly different from multipolar, nonadapting cells, unpaired \( t \)-test, \( P < 0.05 \).

Figure 2. Differences in sensitivity to 0.4 \( \mu \)M zolpidem of IPSPs activated by different types of interneurons in postynaptic pyramidal cells. (A) A multipolar, nonadapting interneuron, elicited fast IPSPs that were powerfully enhanced by zolpidem (gray). (B) A multipolar neuron with an adapting firing pattern elicited IPSPs that were less powerfully enhanced by zolpidem. (C) A bitufted interneuron with an adapting firing pattern elicited slowly rising zolpidem-insensitive IPSPs. (D) Correlation between presynaptic spike width and 10–90% rise time of the IPSPs, the narrower the spike width the faster the IPSP rise time. (E) The fastest IPSP rise times were correlated with the strongest zolpidem effect (black triangles and gray squares represent multipolar, adapting, and nonadapting cell IPSPs, respectively, gray circles represent bitufted cell IPSPs).
Table 2
Pharmacological properties of IPSPs generated by presynaptic multipolar and bitufted cells

<table>
<thead>
<tr>
<th>Pair</th>
<th>ISPS properties</th>
<th>Control</th>
<th>Zolpidem</th>
<th>Alphax5</th>
<th>Flum</th>
<th>Zinc</th>
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<tbody>
<tr>
<td></td>
<td>Amp</td>
<td>RT</td>
<td>HW</td>
<td>Amp</td>
<td>RT</td>
<td>HW</td>
</tr>
<tr>
<td>Multipolar, nonadapting cells</td>
<td>160805b</td>
<td>0.34 ± 0.08</td>
<td>5.5</td>
<td>51</td>
<td>0.54 ± 0.1*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>170805a</td>
<td>0.23 ± 0.06</td>
<td>6</td>
<td>56</td>
<td>0.39 ± 0.1*</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>130906a</td>
<td>0.7 ± 0.15</td>
<td>4</td>
<td>53</td>
<td>1.1 ± 0.16*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>251006c</td>
<td>0.84 ± 0.05</td>
<td>5</td>
<td>57</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>120906a</td>
<td>1.2 ± 0.32</td>
<td>6</td>
<td>52</td>
<td>2.0 ± 0.43*</td>
<td>6.2</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.2 ± 0.38 mV</td>
<td>5.2 ± 0.76 ms</td>
<td>53.5 ± 2.4 ms</td>
<td>+82 ± 30%</td>
<td>+13 ± 9%</td>
<td>+18 ± 9%</td>
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<tr>
<td>Multipolar, adapting cells</td>
<td>230806a</td>
<td>0.73 ± 0.2</td>
<td>8</td>
<td>60</td>
<td>1.0 ± 0.3*</td>
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<tr>
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<td>130906b</td>
<td>2.3 ± 0.3</td>
<td>10</td>
<td>83</td>
<td>3.5 ± 0.6*</td>
<td>11</td>
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<td>1.3 ± 0.2</td>
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<td>110</td>
<td>1.6 ± 0.25*</td>
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<td>7</td>
<td>55</td>
<td>0.3 ± 0.1*</td>
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<td>181005</td>
<td>2.8 ± 0.44</td>
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<td>155</td>
<td>3.8 ± 0.6*</td>
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<tr>
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<td>210306b</td>
<td>0.76 ± 0.25</td>
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<td>60</td>
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<td>60</td>
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<td></td>
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<td>0.44 ± 0.1</td>
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<td></td>
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<td>8</td>
<td>63</td>
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<tr>
<td>Mean ± SD</td>
<td>1.2 ± 0.92 mV</td>
<td>9 ± 1.4 ms</td>
<td>82 ± 31 ms</td>
<td>+36 ± 14%</td>
<td>+6 ± 6%</td>
<td>+20 ± 14%</td>
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<td>Bitufted, dendrite-preferring cells</td>
<td>191005c</td>
<td>0.5 ± 0.15</td>
<td>17</td>
<td>100</td>
<td>0.52 ± 0.16*</td>
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<tr>
<td></td>
<td>251005a</td>
<td>1.2 ± 0.27</td>
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<td>130</td>
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<td>115</td>
<td>1.1 ± 0.4*</td>
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<td>061205a</td>
<td>1.2 ± 0.38</td>
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<td>68</td>
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<td></td>
<td>120105d</td>
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<td>125</td>
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<td></td>
<td>200105a</td>
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<td>0.71 ± 0.28*</td>
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<td>161106b</td>
<td>0.91 ± 0.3</td>
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<td>96</td>
<td>0.92 ± 0.3*</td>
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<td>060307a</td>
<td>1.1 ± 0.18</td>
<td>14</td>
<td>96</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mean ± SD (%)</td>
<td>0.81 ± 0.47 mV</td>
<td>14.7 ± 3.0 ms</td>
<td>96.3 ± 24 ms</td>
<td>+7.8 ± 5.7%</td>
<td>0%</td>
<td>+1 ± 1%</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (SD) of percentage change are given.

Amp = amplitude (mV), RT = 10–90% rise time (ms), HW = width at half amplitude (ms), + = increase, - = decrease.

*Significantly different from control, unpaired t-test, *P* ≤ 0.05, shown in bold.

#Significantly different from control, paired t-test, *P* ≤ 0.05, shown in bold.

Not significantly different from the control, unpaired t-test, *P* > 0.05.

Not significantly different from the control, paired t-test, *P* > 0.05.
Differential Sensitivity to Zolpidem of IPSPs Activated by Different Classes of Interneurons

Figure 2 illustrates the differential sensitivity to zolpidem (0.4 μM) of IPSPs elicited by the 3 classes of interneurons studied. Increases in amplitude stabilized within 8–12 min and did not increase further within 20–30 min. The enhancement produced by zolpidem was inversely related to the rise time of the IPSP (r = 0.8, n = 16), with multiform, nonadapting cells eliciting the IPSPs that were most strongly enhanced. Multipolar, adapting cells elicited IPSPs that were moderately enhanced by zolpidem. In contrast, although the IPSPs elicited by bitufted cells were enhanced by the broad-spectrum benzodiazepine agonist, diazepam, they were almost insensitive to zolpidem, and average peak IPSP amplitudes after 20–40 min bath application of zolpidem were not significantly different from control (Table 2). The 90% rise times and widths at half amplitude of IPSPs were measured from averaged records in each condition. Zolpidem either had no measurable effect or slightly increased 10–90% rise times and widths at half amplitude of IPSPs, as elicited by multipolar interneurones of both types. Zolpidem had no significant effect on the time course of IPSPs elicited by bitufted cells. However, the broad-spectrum benzodiazepine site agonist, diazepam, which enhanced the amplitudes of these IPSPs (to 153 ± 23%, n = 3, P < 0.05, Student’s unpaired t-test), also produced small, but not significant, increases in their 10–90% rise times (9.6 ± 3.8%, n = 3, P > 0.05, Student’s unpaired t-test) and widths at half amplitude (12.0 ± 10.8%, n = 3, P > 0.05), whereas IAalpha5, which decreased the amplitudes of these IPSPs (see below) also resulted in small reductions in their time course.

5-Selective Inverse Agonist, IAalpha5

Consistent with the finding that the IPSPs elicited by bitufted dendrite-preferring cells were insensitive to the 5/2/3 subunit-selective agonist, zolpidem (Figs 2, 4, and 5), these IPSPs were sensitive to IAalpha5 (1 μM, Fig. 4Ab, Bd, C). IAalpha5 reduced the amplitudes of these IPSPs and their time course. There was a correlation between the rise time of the IPSP and the sensitivity to IAalpha5 (Fig. 4C). The slower the IPSP rise time, the more sensitive the IPSPs were to IAalpha5 (r = 0.91, n = 13). The IPSPs activated by multipolar cells were insensitive to the 5-subunit-selective inverse agonist IAalpha5 (1 μM, P > 0.05, Students unpaired t-test, Fig. 3Aa, Ba, Table 2). These IPSPs were, however, reduced by flumazenil (0.4 μM 19 ± 2.2%), reported in some studies to be a broad-spectrum benzodiazepine site inverse agonist (De Vry and Slanger 1985; King et al. 1985; File et al. 1986), indicating that effects of inverse agonists on multipolar cell-elicited IPSPs were detectable in these experiments (Fig. 3Ab, Bd, Table 2).

Zinc (100 μM, Fig. 4F), which reduces responses to GABA that are mediated by GABA receptors lacking the (2 subunit and those mediated by (2-containing receptors that also contain the 5 subunit (Draguhn et al. 1990; Sieghart 1995; Burgard et al. 1996; Knoflach et al. 1996; Krishek et al. 1998), also reduced IPSPs elicited by bitufted cells but not the IPSPs elicited by a multipolar interneuron (Table 2, Fig. 4E).

The Effects of Benzodiazepine Site Ligands Including IAalpha5 Appear to Be Mediated Postsynaptically

To determine whether the changes in IPSP amplitude produced by bath application of zolpidem, IAalpha5, or zinc were of postsynaptic or presynaptic origin, 2 tests were applied. First, the proportions of failures of transmission before and after drug addition were calculated. If the effects of IAalpha5 were mediated presynaptically, an increase in failure rate would be expected to parallel the decrease in IPSP amplitudes observed. However, addition of IAalpha5 was followed by a small decrease (1–2%, not significant, Students paired t-test, P > 0.05, n = 7) in the proportion of failures in the majority of bitufted cell-elicited IPSPs (Fig. 5A), indicating that the decrease in amplitude was not mediated presynaptically. Zolpidem did not change the proportion of failures (Fig. 5A).

For the second test, a simple binomial model of release was assumed. In this model, a change in the mean amplitude (M = npq) due to a change in the quantal amplitude (q) would be accompanied by no change in the inverse square of the coefficient of variation (CV^2 = np/[1–p]), resulting in a slope of zero in the plot of normalized CV^2 against normalized mean amplitude. A change in CV^2 that is proportional to the change in the mean (a slope of 1) indicates a change in n, and a greater proportional change in CV^2 than in mean (a slope >1) indicates a change in p.

In this study, all data points fell on slopes close to zero (mean slopes of -0.24 ± 0.34 and -0.17 ± 0.28 for the effects of IAalpha5 and zinc on bitufted cell IPSPs, respectively, and 0.11 ± 0.26 for the effects of zolpidem on multipolar cell IPSPs, Fig. 5B). This indicates a predominantly postsynaptic site for the changes in IPSP amplitude with bath application of zolpidem, IAalpha5, or zinc.

Discussion

This study compares 3 classes of inhibitory connections in the neocortex and the actions of GABA receptor modulators, zolpidem, IAalpha5, flumazenil, diazepam, and zinc on the unitary IPSPs that they elicit in postsynaptic pyramidal cells. The data presented suggest that different 5 subunits are selectively inserted at specific synaptic locations on postsynaptic pyramidal cells and that the insertions of these GABA receptor types are input dependent and correlates with the class of presynaptic interneuron.

Presynaptic Interneuron-Dependent Insertion of Postsynaptic GABA Receptor Subtype in Pyramids

The presynaptic interneurons studied here were classified into 3 groups, bitufted dendrite-preferring cells, which included Martinotti cells, and 2 groups of multipolar cells, those with adapting and those with nonadapting firing patterns. The bitufted cells in this study displayed spike frequency adaptation and accommodation. Close membrane appositions, or putative synapses, between the presynaptic bitufted cell axons and postsynaptic pyramidal cells involved second- and third-order dendrites of pyramidal cells. These interneurons are typically classed as dendrite preferring, although they may also, on occasion, innervate more proximal dendrites and somata of pyramidal cells (Wang et al. 2004). The multipolar cells studied here resembled basket cells (Kawaguchi and Kubota 1997), which target somata and proximal dendrites of postsynaptic pyramidal cells.

Interestingly, the inhibitory synapses innervated by these 3 classes of interneurons were differentially sensitive to GABA receptor modulators. Bitufted dendrite-preferring cell IPSPs were insensitive to the benzodiazepine type 1 agonist zolpidem.
but were reduced by an α5-selective inverse agonist, Iαalpha5, suggesting that IPSPs elicited by bitufted dendrite-prefering cells are mediated by α5-subunit-containing GABA$_\alpha$ receptors. Consistent with these findings, zinc also reduced bitufted cell IPSPs. The inhibitory effects of zinc require exclusion of the γ2 subunit in α1-, α2-, or α3-containing GABA$_\alpha$ receptors (Draguhn et al. 1990; Seighart 1995; Krishek et al. 1998) or inclusion of either α4 or α5 subunits (Burgard et al. 1996; Knopfach et al. 1996). Because these IPSPs were enhanced by diazepam, confirming the inclusion of a γ2 subunit these data suggest that at least 1 class of dendritic IPSPs involves α5-subunit-containing receptors. This is consistent with pharmacological studies in the hippocampus (Pawelzik et al. 1999; Thomson et al. 2000) and with recent immunofluorescence and EM immunogold studies demonstrating synaptically located α5 subunits (Serwanski et al. 2006) in addition to those located extrasynthetically (Brunig et al. 2002; Christie et al. 2002; Crestani et al. 2002; Serwanski et al. 2006).

Previous studies in hippocampal pyramidal cells have demonstrated that the small tonic inhibitory currents that are mediated by extrasynaptic GABA$_\alpha$ receptors (Banks and Pearce 2000; Petrini et al. 2004) involve an α5 GABA$_\alpha$ receptor-mediated component (Caraiscos et al. 2004). These studies were however performed in the presence of a GABA uptake blocker or exogenous GABA. In the absence of uptake inhibitors, the tonic current in hippocampal pyramids was found largely to be due to high-affinity delta-subunit-containing receptors, α5 receptors being recruited only when ambient GABA levels were increased (Semyanov et al. 2003; Scimemi et al. 2003). It is perhaps not surprising therefore, that we observed no change in either membrane potential or input resistance associated with the application of benzodiazepine site ligands in this study because these drugs do not modify the activity of delta-subunit-containing receptors. This does not, however, discount the possibility that increased neuronal activity may result in the release of sufficient GABA to activate extrasynaptic α5-containing receptors.

The potentiation by zolpidem of IPSPs elicited by multipolar, nonadapting fast-spiking interneurons was stronger than of IPSPs elicited by multipolar, adapting cells. This, in combination with the lack of sensitivity of IPSPs elicited by both of these classes of putative basket cells to the α5-selective inverse agonist, Iαalpha5 or to zinc, suggests that proximally located inhibitory synapses delivered by multipolar, nonadapting cells are predominantly mediated by α1-subunit-containing GABA$_\alpha$ receptors, whereas multipolar, adapting cell IPSPs are mediated predominantly by α2/3-subunit-containing GABA$_\alpha$ receptors. These IPSPs are unlikely to be mediated by α4-containing receptors at which flumazenil acts as a partial benzodiazepine site agonist (Whittemore et al. 1996) because in this and in previous studies (Thomson et al. 2000) flumazenil reduced the amplitudes of IPSPs by 10–20%.

There are 2 possible explanations for this effect; either that flumazenil is a partial inverse agonist as suggested by previous in vivo and acute slice studies (De Vry and Slagen 1985; King et al. 1985; File et al. 1986), an activity that may not be demonstrable at GABA$_\alpha$ receptors expressed in nonneuronal systems. Alternatively, there is endogenous activation of the benzodiazepine site in our control conditions in vitro and in some in vivo models. The data presented here cannot, however, distinguish between these 2 possibilities.

This is also consistent with previous immunohistochemical studies in hippocampus showing that the majority of synapses supplied by basket cells contain the α1 subunit (Nusser et al. 1996; Fritschy et al. 1998; Loup et al. 1998). Also consistent with the present study was the demonstration that a subset of hippocampal parvalbumin-negative (putative CCK-containing) basket cell terminals innervated synapses with 5 times more immunoreactivity for α2 subunits than synapses innervated by parvalbumin-positive terminals (Nyiri et al. 2001). Our preliminary immunocytochemical data indicate that multipolar, nonadapting cells similar to those reported here contain parvalbumin, whereas the adapting multipolar cells contain CCK (Ali and Thomson, unpublished data, see also, Kawaguchi and Kubota 1997).

Interestingly, other studies using freeze fracture and double immunolabeling have shown that the majority of synapses on pyramidal cell somata colocalize α1 and α2 subunits (Kasagai et al. 2006). Synaptic colocalization of α subunits could either result from the insertion of more than 1 type of receptor, each containing 2 identical α subunits, or from insertion of a single receptor type that contains 2 different α subunits. The presence of 2 different subunits does not preclude a selective benzodiazepine site pharmacology because the benzodiazepine site involves only the α subunit that is adjacent to the γ2 subunit. Benzodiazepine type 1 pharmacology predominates whenever α1 is colocalized with α2 or α3 (Araujo et al. 1996). This suggests that the selective pharmacology observed here could as easily result from coexpression of α1 and α2 subunits in receptors activated by nonadapting (probably parvalbumin-containing) basket cells as from selective insertion of receptors containing 2 α1 subunits. The more α2/3 type pharmacology seen at synapses supplied by multipolar, adapting (presumed CCK-containing) basket cells would by this argument require the exclusion of α1 subunits from the majority of receptors at these synapses. The absence of measurable effect of an α5-selective compound on proximal IPSPs indicates that if α5 subunits are inserted at proximal synapses, they are in the minority. In contrast, the absence of zolpidem sensitivity at dendritic synapses, that are nevertheless strongly augmented by diazepam and depressed by Iαalpha5 and by zinc, suggests either that at more distal synapses, receptors containing α5 subunits are inserted, or that where α5 coexists with another α subunit, α5 pharmacology predominates as indicated by

Figure 3. Pharmacology of multipolar cell IPSPs. (Aa) Plot of the peak IPSP amplitudes elicited by a multipolar, nonadapting cell in a pyramidal cell during the time course of an experiment in control conditions and during bath application of zolpidem, Iαalpha5 (1 μM, α5-selective inverse agonist) and flumazenil (0.4 μM, a broad-spectrum benzodiazepine site inverse agonist). (Aa) The average of the IPSPs in each condition. (Aa) In another multipolar, nonadapting interneuron to pyramidal cell connection (reconstruction of this pair is illustrated in Aa) Iαalpha5 was applied before zolpidem. Iαalpha5 did not affect the IPSP amplitudes or the subsequent effects of zolpidem on this IPSP. (Bb) IPSP amplitudes elicited by a multipolar, adapting cell plotted against time. IPSP in control conditions and during bath application of Iαalpha5 and subsequent addition of zolpidem are shown (reconstruction shown Fig. 1Bb). These connections were also insensitive to Iαalpha5 and sensitive to zolpidem. Average IPSPs in each condition are shown in (Bb). (Bb) illustrates another connection of this type in control conditions and during the bath application of zolpidem and subsequent addition of flumazenil (reconstruction of this pair is shown in Bb). Zolpidem enhanced the IPSPs, which were then reduced by flumazenil. Average control IPSPs are superimposed (dotted lines).
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binding studies on double immunpurified α1/α5 GABA_A receptors (Araujo et al. 1999).

**IPSP Time Course**

That dendritic filtering may play a role in determining IPSP duration (as recorded at the postsynaptic soma) is indicated by the long rise times of IPSPs elicited by dendrite-preferring bitufted interneurons. Simple cable theory predicts that in passive dendrites, excitatory/inhibitory postsynaptic potential rise times will be more strongly influenced by dendritic filtering than their typically slower decay phases (Rall 1959). Indeed, the rise times of bitufted cell-elicited IPSPs were significantly longer than those of adapting multipolar cell-elicited IPSPs (P < 0.001, Student’s unpaired t-test), while their widths at half amplitude were similar (P > 0.05). Until recordings are obtained from the immediate postsynaptic site, the duration of events at these dendritic synapses and the extent to which they are slowed during their passage to the soma can only be surmised. Similarly, although the postsynaptic target was in all cases a pyramidal cell and the properties of these postsynaptic cells (recorded at the soma) were similar, the extent to which voltage-dependent currents and ionic gradients in different subcellular compartments may differentially influence IPSP amplitude and time course, remains unclear. At this stage, therefore, it is possible to propose only that the differences in time course between IPSPs elicited by bitufted cells and those elicited by adapting multipolar cells are likely to be due in part to dendritic filtering.

In contrast, both the rise times and the widths at half amplitude of IPSPs elicited by multipolar adapting interneurons were significantly longer than those of IPSPs elicited by fast-spiking multipolar cells (P < 0.001). Because both cell classes typically innervate relatively proximal locations, dendritic filtering cannot be invoked to account for this difference. Developmental (Dunning et al. 1999; Bosman et al. 2002) and knockout studies (e.g., Goldstein et al. 2002) indicate that IPSCs mediated by α1-subunit-containing GABA_A receptors are briefest than those involving other receptor subtypes while inclusion of the α1, rather than the α2 (Lavoie et al. 1997) or α3 subunit (Gingrich et al. 1995) increases the deactivation rate of recombinant GABA_A receptors. The current data indicating a strong negative correlation between zolpidem sensitivity and IPSP time course are consistent with these findings.

**Conclusion**

These data demonstrate that different α subunit-containing GABA_A receptor subtypes are selectively inserted at specific postsynaptic locations on pyramidal cells in the neocortex. These suggest moreover that insertion maybe presynaptic input dependent. Synapses innervated by dendrite-preferring, bitufted cells utilize α5 GABA_A receptors whereas more proximal, IAalpha5-insensitive synapses innervated by nonadapting putative parvalbumin-positive and adapting CCK-positive basket cells respectively include a larger proportion of α1 or appear to be mediated predominantly α2/α3 GABA_A receptors.
This study suggests a structural basis for the different behavioral profiles associated with manipulation of different α subunits (Möller et al. 2002; Whiting 2003). For example, α1 GABA\(_{A}\) receptors have been shown to mediate the sedative effects of benzodiazepines (Rudolph et al. 1999; McKernan et al. 2000). Enhancing the activity of fast-spiking, parvalbumin-positive basket cells would therefore be predicted to generate sedation.

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**References**


Review.

anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha subtype. Nat Neurosci. 3:587-592.


