Pathway-Specific Targeting of \( \text{GABA}_A \) Receptor Subtypes to Somatic and Dendritic Synapses in the Central Amygdala

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Delaney, Andrew J. and Pankaj Sah. Pathway-specific targeting of \( \text{GABA}_A \) receptor subtypes to somatic and dendritic synapses in the central amygdala. J Neurophysiol 86: 717–723, 2001. Neurons in the central amygdala express two distinct types of ionotropic GABA receptor. One is the classical \( \text{GABA}_A \) receptor that is blocked by low concentrations of bicuculline and positively modulated by benzodiazepines. The other is a novel type of ionotropic GABA receptor that is less sensitive to bicuculline but blocked by the \( \text{GABA}_C \) receptor antagonist (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA) and by benzodiazepines. In this study, we examine the distribution of these two receptor types. Recordings of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) showed a wide variation in amplitude. Most events had amplitudes of <50 pA, but a small minority had amplitudes >100 pA. Large-amplitude events also had rise times faster than small-amplitude events. Large-amplitude events were fully blocked by 10 \( \mu \)M bicuculline but unaffected by TPMPA. Small amplitude events were partially blocked by both bicuculline and TPMPA. Focal application of hypertonic sucrose to the soma evoked large-amplitude mIPSCs, whereas focal dendritic application of sucrose evoked small-amplitude mIPSCs. Thus inhibitory synapses on the dendrites of neurons in the central amygdala express both types of GABA receptor, but somatic synapses expressed purely \( \text{GABA}_A \) receptors. Minimal stimulation revealed that inhibitory inputs arising from the laterally located intercalated cells innervate dendritic synapses, whereas inhibitory inputs of medial origin innervated somatic inhibitory synapses. These results show that different types of ionotropic GABA receptors are targeted to spatially and functionally distinct synapses. Thus benzodiazepines will have different modulatory effects on different inhibitory pathways in the central amygdala.

INTRODUCTION

\( \gamma \)-Aminobutyric acid (GABA) is the principal inhibitory transmitter in the CNS and activates ionotropic \( \text{GABA}_A \) and \( \text{GABA}_C \) receptors, and metabotropic \( \text{GABA}_B \) receptors. Ionotropic GABA receptors are heterooligomeric proteins of which 20 subunits have so far been identified: \( \alpha_1–6, \beta_1–4, \gamma_1–3, \delta, \epsilon, \pi, \theta, \) and \( \rho_1–3 \). Of these, the \( \rho \) subunits assemble to form \( \text{GABA}_C \) receptors, while \( \pi \) subunits are only found outside the CNS. The other subunits coassemble as heteromultimers to form a number of different types of GABA receptor (Barnard et al. 1998). The subunit composition of GABA receptors determines their biophysical and pharmacological properties (MacDonald and Olsen 1994). Thus considerable GABA receptor heterogeneity is expected to be present within the CNS. Immunohistochemical studies have shown that different subunits can be targeted to different regions of the neuronal membrane (Nusser et al. 1996; 1998). It has therefore been suggested that functionally different receptors may be targeted to different synapses. However, there is little direct evidence to support this proposal.

The amygdala is intimately involved in emotional behavior (Kluver and Bucy 1939; LeDoux 2000), and its role in the generation of anxiety and conditioned fear is well known (Davis 1992; LeDoux 1995). Studies of Pavlovian fear conditioning have suggested that the basolateral amygdala is the site for convergence of neural pathways conveying information about conditioned and unconditioned stimuli. This information is processed locally and then transmitted to the central amygdala. Neurons in the central nucleus project to the hypothalamus and brain stem regions, which are important in the behavioral, hormonal, and autonomic aspects of the fear response (LeDoux 2000). Neurons in the central amygdala express two types of ionotropic GABA receptor (Delaney and Sah 1999). One is a typical \( \text{GABA}_A \)-receptor that is blocked by low doses of bicuculline and positively modulated by benzodiazepines, anesthetics, and barbiturates. The other is a novel type of receptor, similar to \( \text{GABA}_C \) receptors first described in the retina (Enz et al. 1995; Qian and Dowling 1994). These receptors are less sensitive to bicuculline and are little affected by anesthetics and barbiturates. However, unlike \( \text{GABA}_C \) receptors, they are inhibited by 1,4 benzodiazepines such as diazepam (Delaney and Sah 1999). In this study, we examine the distribution of these two types of ionotropic GABA receptors. We find that \( \text{GABA}_C \)-like receptors are present on dendritic synapses where they colocalize with \( \text{GABA}_A \) receptors. These synapses are innervated by axons of the intercalated cell masses (ICMs). In contrast, somatic synapses express only \( \text{GABA}_A \) receptors and are innervated by axons of different origin.

METHODS

Wistar rats (17- to 20-days old) were anesthetized with intraperitoneal pentobarbitone (50 mg/kg) and decapitated, and the brain was removed and immersed in ice-cold Ringer. Coronal brain slices (400 \( \mu \)m) were prepared using standard methods. All procedures were in accordance with the Institutional Animal Care and Ethics Committee guidelines. Slices were superfused at 200 ml/h with oxygenated ex-
ternal solution containing (in mM) 118 NaCl, 2.5 KCl, 25 NaHCO₃, 10 glucose, 1.2 NaH₂PO₄, 1.3 MgCl₂, and 2.25 CaCl₂ in a bath volume of 1 ml. Kynurenic acid (2 mM) was included in the external solution to block excitatory glutamatergic transmission. Tetrodotoxin (0.5 μM) was added to block synaptic transmission when recording mIPSCs. Recordings were made from neurons in the lateral division of central amygdala (CeL) using the whole cell patch-clamp method with either the blind approach or infrared differential interference contrast techniques. Borosilicate glass electrodes (3–5 MΩ) were filled with high chloride internal solution containing (in mM) 130 CsCl₂, 1 MgCl₂ · 6H₂O, 10 EGTA, 10 HEPES, 2 Mg₂ATP, and 0.2 Na₂GTP (pH 7.3 with CsOH, 290 mOsM). In experiments where the sucrose solution was applied to the soma and dendrites, Lucifer yellow was also added to the internal solution. Neurons were held in voltage-clamp mode at −60 mV.

Drugs used were bicuculline methiodide, (1,2,5,6-tetrahydropyridine-4-yl) methylphosphonic acid (TPMPA; RBI Research Chemicals), kynurenic acid (Sigma), tetrodotoxin (Alamone), and diazepam (gift from Professor P. Gage). Inhibitory postsynaptic currents (IPSCs) were evoked electrically using stainless steel bipolar stimulating electrodes (Frederick Haer) placed laterally along the edge of CeL or medially in central nucleus medial sector (CeM). Stimuli were 50 μs in duration. Sucrose stimulation of miniature IPSCs (mIPSCs) was performed by pressure ejection of external solution containing 0.5 M sucrose through a 3 MΩ patch pipette to the surface of the slice or by low pressure injection through a 3–5 MΩ patch pipette under visual guidance. Lucifer yellow was included in the sucrose solution for visualization of the spread of the ejected sucrose solution. Signals were recorded using an Axopatch 200B amplifier (Axon Instruments), filtered at 5 kHz, and digitized at 10 kHz (Instrutech, ITC 16). Data were acquired with Axograph (Axon Instruments) on a Macintosh G3 computer. Series resistance (10–30 MΩ) was monitored on-line throughout the experiment, and experiments were rejected if resistance changed by 10%. No series resistance compensation was used. IPSC peak amplitude, 10–90% rise time, half-peak width, and decay time constants were analyzed using Axograph 4.0 and compiled and statistically analyzed using Microsoft Excel. All values are expressed as means ± SE, and all statistical comparisons were done using Student’s t-test. Spontaneous mIPSCs were detected using the variable-amplitude template event-detection program included in Axograph. Measurements of amplitude and kinetics and tracer of mIPSCs shown are averages of between 4 and 10 events for large mIPSCs and between 30 and 50 events for the small-amplitude mIPSCs under control conditions. All experiments were done at room temperature (21–24°C).

R E S U L T S

Whole cell recordings were made from neurons in the lateral division of the central amygdala (Jolkkonen and Pitkanen 1998). We have shown that locally evoked inhibitory synaptic inputs to these neurons activate both GABAₐ and GABAₐ-like ionotropic receptors (Delaney and Sah 1999). To test if both types of GABAₐ receptor were co-localized at all inhibitory synapses, we examined spontaneously occurring mIPSCs that reflect the response to single quanta of transmitter. At a holding potential of −60 mV, mIPSCs in CeL neurons showed a wide variation in amplitude (Fig. 1). Most responses were of small amplitude (<50 pA; mean amplitude, 19 ± 2 pA; n = 8). However, a small number of large-amplitude events (>100 pA; mean amplitude, 123 ± 9 pA; n = 7) were also detected. These large-amplitude events had significantly faster (P < 0.01) 10–90% rise times (0.91 ± 0.09 ms) compared with the rise times of the smaller events (1.51 ± 0.12 ms; n = 4; Fig. 1, C and D).

To increase the frequency of large-amplitude events, we applied a hypertonic Ringer solution containing 0.5 M sucrose, which causes asynchronous release of mini (Fatt and Katz 1952). Application of hypertonic solution to the slice caused a large increase in mIPSC frequency (Fig. 2A) and revealed a much larger proportion of fast large-amplitude mIPSCs. The frequency of large-amplitude events increased from 0.02 ± 0.01 to 0.31 ± 0.01 Hz (n = 6; P < 0.01). Large-amplitude mIPSCs evoked in sucrose had amplitudes and rise times identical to those of large-amplitude events under control conditions (Fig. 2B). The higher frequency of large-amplitude events following application of sucrose increased the amplitude of the average mIPSC from 19.4 ± 1.5 to 35.5 ± 3.4 pA (n = 9; Fig. 3, A and B). Application of the GABAₐ receptor antagonist bicuculline methiodide (BIC, 10 μM) markedly reduced the amplitude (71 ± 6% reduction; n = 6) and frequency of small mIPSCs but completely abolished the large events (Fig. 3A). Furthermore, in the absence of BIC, application of sucrose increased the frequency of mIPSCs with no change in their average amplitude (Fig. 3A). In contrast, application of the GABAₐ-receptor antagonist TPMPA (60 μM) (Ragozzino et al. 1996) reduced the amplitude of the small events (P < 0.01) while having no significant effect (P > 0.05) on the large-amplitude IPSCs (Fig. 3, C and D; n = 3). Application of hypertonic Ringer in the presence of TPMPA increased the frequency of the large-amplitude events (Fig. 3B). In the presence of TPMPA, mIPSCs were completely blocked by 10 μM BIC and application of sucrose did not evoke any IPSCs (data not shown). These results indicate that two types of GABAergic mIPSCs are present in CeL neurons. One consists of large-amplitude fast-rising events that are fully blocked by low doses of BIC but are unaffected by TPMPA, suggesting that they are due to activation of pure GABAₐ receptors. The second population consists of small-amplitude slow-rising events that are partially blocked by BIC and
TPMPA, indicating that both GABA_A receptors and GABA_C-like receptors (Delaney and Sah 1999) are present at these synapses.

What explains the rapid rising phase and large amplitude of the pure GABA_A mIPSCs? One possibility is that these synapses are located electrotonically closer to the soma resulting in less filtering of their kinetic properties (Bekkers and Stevens 1996; Maccaferri et al. 2000). To test this idea, we focally applied hypertonic sucrose solution to the soma and dendrites of CeL neurons. Focal somatic application of sucrose evoked mostly large-amplitude mIPSCs (Fig. 4, A–C). These events had a peak amplitude of 144 ± 13 pA and 10–90% rise time of 0.92 ± 0.04 ms (n = 4). Activation of these large-amplitude events led to a large increase in the average mIPSC (Fig. 4D). In contrast, dendritic application of sucrose evoked mIPSCs that had amplitudes and rise times indistinguishable from those of the small events present before application of sucrose (Fig. 4, E–G). Thus the average mIPSC was not affected by the dendritic application of sucrose (Fig. 4H). In addition, large-amplitude mIPSCs evoked by somatic application of sucrose were fully blocked by 10 μM BIC (n = 3) but unaffected by TPMPA (Fig. 5; 13% reduction in amplitude, n = 2). In contrast, mIPSCs evoked by dendritic sucrose application were reduced in amplitude by TPMPA (Fig. 5; 25% reduction in amplitude, n = 2). Together these results show that fast-rising large-amplitude mIPSCs arise from synapses on or near the soma of CeL neurons that express pure GABA_A receptors. In contrast, the smaller-amplitude slower-rising mIPSCs arise from synapses of more distal dendritic origin that express both GABA_A and GABA_C-like receptors. We next tested if these

![FIG. 2. Application of hypertonic Ringer increases the frequency of large amplitude mIPSCs. A: application of hypertonic (0.5 M) sucrose increased the frequency of large-amplitude events. B: events recorded before (left) and during sucrose application (right) are shown as histograms. Note the increase in number of large-amplitude events in sucrose. C: large-amplitude events evoked by sucrose application have properties identical to those recorded under control conditions (n = 6).](http://jn.physiology.org/)

![FIG. 3. Large fast mIPSCs are due to activation of purely GABA_A receptors, but small mIPSCs are due to activation of 2 types of GABA receptor. A: effect of 10 μM bicuculline application on spontaneous mIPSCs evoked by sucrose. Left: traces are in control condition and average mIPSCs before and after sucrose application (Aii) are shown as histograms. Note the increase in number of large-amplitude events in sucrose. Right: traces show the effects of sucrose application in the presence of 60 μM TPMPA. B, i and ii: average mIPSCs before and after sucrose have been superimposed. C: small-amplitude events are reduced in amplitude by TPMPA (P < 0.01). Average data (n = 3) are shown in the histograms on the right. The IPSC recorded in control Ringer is marked (*). D: large-amplitude mIPSCs evoked by sucrose before and after application of TPMPA have been superimposed. Note that these events are not significantly affected by TPMPA (P > 0.05). Average data are shown in the histograms on the right.)
synapses are innervated by axons of the same or different origin.

We have shown previously that IPSCs evoked by stimulation on the border of the CeL and the lateral amygdala activates IPSCs that contain both GABA_A and GABA_C-like receptors (Delaney and Sah 1999). These inhibitory inputs are thought to arise from GABAergic neurons within the intercalated cell masses of the amygdala (Paré and Smith 1993; Royer et al. 1999). To test if both types of GABA receptors were present at all synapses made by these inputs, we evoked single-fiber IPSCs by using minimal stimulation (Raastad et al. 1992). Glutamatergic inputs were blocked by inclusion of 2 mM kynurenic acid to the perfusing Ringer. Stimulation electrodes were placed on the lateral border of the CE to activate inputs arising from the ICMs (Royer et al. 1999) and medial to the CE to activate inputs that form part of the extended central amygdala (Sun and Cassell 1993) (Fig. 6A). IPSCs evoked from the ICMs had a peak amplitude of 19.3 ± 3.8 pA and a 10–90% rise time of 3.2 ± 0.4 ms (n = 10; Fig. 6B). In contrast, when the stimulating electrode was placed medially, minimal stimulation evoked a more rapidly rising (10–90% rise time 1.7 ± 0.1 ms) large-amplitude (130.8 ± 13.4 pA) IPSCs (Fig. 6, C and D). Laterally evoked IPSCs were partially inhibited by BIC (10 μM, 71 ± 4% block; n = 8) and TPMPA (100 μM, 47 ± 10% block; n = 4), indicating that both types of GABA receptors are present at these synapses (Fig. 7, A and B). Medially evoked IPSCs in the same cells were fully blocked by 10 μM BIC (98 ± 3% block; n = 7; Fig. 7A) and not affected by TPMPA (n = 10; Fig. 7B). This result shows that IPSCs evoked by medial stimulation activate a population of pure GABA_A receptors. In contrast, IPSCs evoked from the ICMs activate both GABA_A and GABA_C-like receptors. In further confirmation of this, application of
diazepam potentiated the amplitude of minimally evoked medial IPSCs from 99\(^6\) to 108\(^5\) pA, whereas it reduced the amplitude of laterally evoked IPSCs from 23\(^6\) to 19\(^5\) pA (Delaney and Sah 1999) (Fig. 8).

**DISCUSSION**

We have shown that two types of GABAergic mIPSCs are present on neurons in the CeL. One type of mIPSC is of large amplitude and has a fast rise time. These mIPSCs are fully blocked by low concentrations of bicuculline (BIC) but are unaffected by the GABA\(_C\) antagonist TPMPA, indicating that they are purely due to activation of GABA\(_A\) receptors. The frequency of these mIPSCs was greatly potentiated by somatic application of hypertonic sucrose solution, indicating that they result from activation of somatic synapses. The other type of mIPSC is of smaller amplitude, has a slower rise time, and is partially blocked by both BIC and TPMPA, indicating that these synapses contain both GABA\(_A\) and GABA\(_C\)-like receptors. The frequency of these mIPSCs is potentiated by dendritic application of hypertonic sucrose solution, indicating that they result from activation of dendritic synapses. Minimal electrical stimulation of inputs that have a medial-origin-evoked IPSC whose amplitude and rise time was similar to those of large mIPSCs evoked by somatic sucrose application. Medially evoked IPSCs were fully blocked by bicuculline but unaffected by TPMPA. These results indicate that inputs that have a medial origin make synapses on the soma of CeL cells, and these synapses contain only GABA\(_A\) receptors. In contrast, stimulation of lateral inputs arising from the intercalated cell masses evoked IPSCs with kinetics and pharmacology similar to those of dendritically located mIPSCs. These IPSCs were partially blocked by BIC and TPMPA, indicating that both GABA\(_A\) and GABA\(_C\)-like receptors are present at these synapses. Together, these data show that neurons in the lateral division of the central amygdala have two types of fast inhibitory synapses. GABAergic inputs arising from the intercalated cell masses make dendritic synapses at which both GABA\(_A\) and GABA\(_C\)-like receptors are present. In contrast, inhibitory inputs arising medial to the CeL (Sun and Cassell 1993) make somatic synapses at which only GABA\(_A\) receptors are present. Thus while GABA\(_A\) receptors are present at both somatic and dendritic synapses,
Average data are shown in the histogram. Bicuculline, while medially evoked IPSCs were fully blocked by bicuculline, only GABA A receptors. Thus one possibility is that the two types of receptor subunit composition is likely to be different (Delaney and Sah 1999). Thus one possibility is that the two types of receptor have different anchoring proteins. In this model, anchoring proteins that bind GABA A-like receptors would have to be present only at dendritic synapses while those for GABA C receptors would be present at somatic as well as dendritic locations.

The amygdala forms a key element of the circuit involved in emotional processing and Pavlovian fear conditioning (L-Doux 2000). Anatomically, the amygdala is divided into a number of subnuclei (McDonald 1999). Cortical (McDonald 1999) and thalamic (Turner and Herkenham 1991) inputs enter the amygdala at the level of the basolateral and central amygdala (Millhouse 1986; Nitecka and Ben-Ari 1987). These cells form an inhibitory interface between the basolateral and central amygdala (Royer et al. 1999). Connections between medial and lateral groups of ICMs, which receive inputs from different regions of the basolateral complex, have been proposed to modulate the traffic of information reaching different regions of the basolateral amygdala (Royer et al. 2000). Our results indicate that the axons of ICM cells make synapses on the dendrites of CeL neurons.

We cannot be certain of the origin of the medial input to CeL neurons. It is unlikely to be a projection originating from CeM as anatomical studies have found this to be rather meager (Jolkkonen and Pitkanen 1998). The axons of GABAergic neurons in the CeL have been found to make synapses on local cells (McDonald 1982), raising the possibility that the medial input may be due to retrograde activation of neurons projecting out of the CeL. However, against this proposal, in our extensive recordings from neurons in the CeL, we have never recorded from neurons that were antidromically activated by medical stimulation (unpublished observations). The central amygdala also receives a number of extraamygdaloid inputs (Pitkänen 2000). However, as the exact nature of these inputs has not been determined, it is not possible to speculate on the identity of medially originating GABAergic inputs.

The presence of GABA C-like receptors at these synapses leads to a reduction in the amplitude of the IPSC by benzodiazepines (Fig. 7), which enhance the IPSC at most GABAergic synapses (Fig. 8). Diazepam potentiates medially evoked IPSCs but reduces laterally evoked IPSCs. A: IPSCs were evoked by minimal stimulation in the region of the ICMs, and diazepam was bath applied at a concentration of 10 μM. The IPSC shows reduction in peak amplitude and a slowing of the remaining IPSC as expected for a synapse expressing both GABA C-like and GABA A receptors. B: IPSC evoked by minimal medial stimulation evokes a large-amplitude event that is potentiated by diazepam as expected for a pure GABA A synapse.

![Image](http://jn.physiology.org/doi-fig/10.220.33.4/.../FIG. 7. Laterally evoked IPSCs activate synapses that contain both GABA A and GABA C-like receptors, but medially evoked IPSCs activate synapses with only GABA A receptors. A: laterally evoked IPSCs were partially blocked by bicuculline, while medially evoked IPSCs were fully blocked by bicuculline. Average data are shown in the histogram. B: laterally evoked IPSCs were partially blocked by TPMPA, while medially evoked IPSCs were unaffected by TPMPA. Average data are shown in the histogram (n = 3 for TPMPA and n = 7 for bicuculline). All recording of 2 inputs are from the same cells and are averages of 6 sweeps.

![Image](http://jn.physiology.org/doi-fig/10.220.33.4/.../FIG. 8. Diazepam potentiates medially evoked IPSCs but reduces laterally evoked IPSCs. A: IPSCs were evoked by minimal stimulation in the region of the ICMs, and diazepam was bath applied at a concentration of 10 μM. The IPSC shows reduction in peak amplitude and a slowing of the remaining IPSC as expected for a synapse expressing both GABA C-like and GABA A receptors. B: IPSC evoked by minimal medial stimulation evokes a large-amplitude event that is potentiated by diazepam as expected for a pure GABA A synapse.)
synapses. This result suggests that processing of information between the input and output stages of the amygdala will be differently modulated by agents that modulate GABA receptors. The axons of neurons in the CeL have been found to have spines in the axon initial segment that are innervated by axons of passage (McDonald 1982). While the nature of this synapse has not been determined, it is tempting to speculate that the medial inhibitory inputs we have recorded may in part be due to the activation of these axonic synapses. The large-amplitude of these inputs would make them potent modulators of the output of CeL neurons. Agents such as benzodiazepines, which amplify these medial inputs would therefore have much larger effects on the output of these cells as compared to its effects on the integration of dendritic inputs.

Disorders of the storage or expression of fear responses are thought to underlie such mental disorders as panic attacks, anxiety, and post traumatic stress disorder. Benzodiazepines, which are commonly used for the treatment of anxiety, are thought to act by enhancing the action of the inhibitory transmitter GABA (Costa and Guidotti 1996; Tallman and Gallagher 1985). The central amygdala has a particularly high density of benzodiazepine binding sites (Sibille et al. 2000). Our results show that benzodiazepines have distinct actions at different GABAergic circuits within the amygdala and highlight the complex nature of the inhibitory control over amygdaloid function. The implications of having two types of inhibitory synapse on GABAergic lateral central amygdaloid neurons must be taken into account when considering the mechanisms of action of benzodiazepines in the treatment of anxiety disorders.

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REFERENCES


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