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Localization and developmental expression of $GABA_B$ receptors in the rat olfactory bulb

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Abstract

In this study, we investigated the distribution and developmental expression of the $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, in the main and accessory olfactory bulbs of the rat. Antibodies raised against these subunits strongly labelled the glomerular layer, suggesting that olfactory and vomeronasal nerve fibers express functional $GABA_B$ receptors. Using postembedding immunogold cytochemistry, we found that $GABA_B$ receptors can be present at both extrasynaptic and presynaptic sites of olfactory nerve terminals, and in the latter case they are preferentially associated with the peripheral part of the synaptic specialization. Olfactory nerve fibers expressed $GABA_{B1}$ and $GABA_{B2}$ at early developmental stages, suggesting that $GABA_B$ receptors may play a role in olfactory development. Output and local neurons of the main and accessory olfactory bulbs were also labelled for $GABA_{B1}$ and $GABA_{B2}$, although the subcellular distribution patterns of the two subunits were not completely overlapping. These results indicate that presynaptically located $GABA_B$ receptors modulate neurotransmitter release from olfactory and vomeronasal nerve fibers and that, in addition to this presynaptic role, $GABA_B$ receptors may regulate neuronal excitability in infraglomerular circuits.

Introduction

GABA is the major inhibitory neurotransmitter in the olfactory bulb, and activates both ionotropic type A receptors and metabotropic type B receptors. While $GABA_A$ receptors mediate neuronal inhibition at various levels in the bulb, $GABA_B$ receptors have been considered to be primarily involved in glomerular microcircuits. Autoradiographic studies have shown that $GABA_B$ receptors are highly concentrated in the glomerular layer (Bowery *et al.*, 1987; Chu *et al.*, 1990), and activation with baclofen reduces the olfactory bulb field potential evoked by electrical stimulation of the olfactory nerve (Potapov, 1985; Nickell *et al.*, 1994; Keller *et al.*, 1998; Wachowiak & Cohen, 1999; Aroniadou-Anderjaska *et al.*, 2000). Immunolabelling for $GABA_B$ receptors has been found in axon terminals of the olfactory nerve (Bonino *et al.*, 1999), suggesting that activation of presynaptic $GABA_B$ receptors inhibits excitatory neurotransmission from olfactory nerve axons to second-order neurons. It is presently unknown, however, what is the precise localization of $GABA_B$ receptors with regard to the glutamate release sites in olfactory nerve terminals.

Recent studies indicate that, in addition to their presynaptic role, $GABA_B$ receptors may exert other functions in the olfactory bulb. Patch-clamp recordings from the olfactory bulbs of frogs and rats have shown that baclofen suppresses the spontaneous activity of mitral and tufted cells (Duchamp-Viret *et al.*, 2000; Palouzier-Paulignan *et al.*, 2002). In addition, $GABA_B$ receptors have been found to modulate dendrodendritic synaptic transmission between granule cells and mitral/tufted cells in the external plexiform layer (Isaacson & Vitten, 2003). Based on these observations, one would assume that $GABA_B$ receptors are expressed both by the principal neurons and by local interneurons, but this remains to be shown by anatomical techniques.

Two different genes encoding the $GABA_B$ receptor subunits $GABA_{B1}$ (which occurs in at least two alternatively spliced forms designated $GABA_{B1(a)}$ and $GABA_{B1(b)}$) and $GABA_{B2}$ have so far been identified

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(Kaupmann *et al.*, 1997; Isomoto *et al.*, 1998; Pfaff *et al.*, 1999; Schwarz *et al.*, 2000; Bowery *et al.*, 2002). Recombinant expression studies have demonstrated that the assembly of $GABA_{B1}$ and $GABA_{B2}$ into heterodimers is essential for the formation of functional $GABA_B$ receptors with properties similar to those of native receptors (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998; Kuner *et al.*, 1999). Previous immunocytochemical analyses have shown that $GABA_{B1}$ is expressed by different neuronal populations of the olfactory bulb (Bonino *et al.*, 1999; Margeta-Mitrovic *et al.*, 1999). However, it is unclear whether the same neurons also express the $GABA_{B2}$ subunit.

In several brain regions, $GABA_B$ receptors have been found to be expressed early during development (Turgeon & Albin, 1993, 1994; Fritschy *et al.*, 1999; López-Bendito *et al.*, 2002) and have been implicated in distinct developmental processes, such as neuronal migration (Behar *et al.*, 1998, 2000, 2001; López-Bendito *et al.*, 2003) and axon pathfinding (Xiang *et al.*, 2002). This raises the possibility that $GABA_B$ receptors may play a role in the formation and/or the refining of olfactory sensory projections. However, the expression pattern of $GABA_B$ receptors during olfactory development has not been investigated yet.

In the present study, we used subunit-specific antibodies to investigate the distribution and developmental expression of $GABA_{B1}$ and $GABA_{B2}$ in the main (MOB) and accessory olfactory bulb (AOB) of the rat. We show that $GABA_{B1}$ and $GABA_{B2}$ are colocalized in the glomerular layer of both the MOB and AOB, suggesting that they are expressed presynaptically in olfactory nerve and vomeronasal nerve fibers. Using postembedding immunogold cytochemistry, we provide evidence that $GABA_B$ receptors can be located close to the glutamate release sites in olfactory nerve terminals. In addition, we show that $GABA_{B1}$ and $GABA_{B2}$ are widely distributed in the olfactory bulb, supporting additional functions of $GABA_B$ receptors in bulbar neurons. Finally, we describe the pre- and postnatal development of $GABA_B$ receptors and show that $GABA_B$ receptor subunits are present in the olfactory nerve at the initial stages of the formation of olfactory projections.

Materials and methods

ANTIBODIES

Two antibodies were used to detect the $GABA_{B1}$ subunit. One antibody (AB1531, Chemicon, Temecula, California) was raised in guinea pigs against a synthetic peptide which is common to both the $GABA_{B1(a)}$ and $GABA_{B1(b)}$ splice variants. In the present study it was used diluted 1:5000. Another antibody (B17; kindly provided by Prof. Ryuichi Shigemoto, National Institute for Physiological Sciences, Okazaki, Japan) was raised in rabbits against a fusion protein containing a C-terminal sequence of $GABA_{B1(a/b)}$ (Kulik *et al.*, 2002). This antibody was used diluted 1:500 for light microscopy and 1:50 for postembedding electron microscopy. The two $GABA_{B1}$ antibodies produced identical staining patterns (not shown). For $GABA_{B2}$, we used an antiserum (AB5394, Chemicon; diluted 1:1000) raised in guinea pigs against a synthetic peptide corresponding to a C-terminal sequence.

IMMUNOFLUORESCENCE

All the experiments described here were performed on Wistar rats raised in the authors' Animal House Facilities. The care and handling of the animals prior to and during the experimental procedures followed European Union regulations, and were approved by the Animal Care and Use Committees of the authors' institutions.

Adult rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine-xylazine 1:1 (0.1 ml/kg body weight) and decapitated. The olfactory bulbs were dissected and cut manually in coronal slabs ($\sim$1mm) that were fixed in 4% paraformaldehyde for 30 minutes. After fixation, the slabs were rinsed in 0.1M phosphate buffer (PB, pH 7.4), cryoprotected in sucrose (10%, 20% and 30%) and sectioned with a cryostat. The sections were then incubated overnight in primary antibodies diluted in phosphate-buffered saline (PBS, pH 7.4) containing 5% normal serum and 0.05% Triton X-100. For double-immunofluorescence, the sections were incubated with a mixture of the rabbit $GABA_{B1}$ antiserum and the guinea pig $GABA_{B2}$ antiserum. The sections were then rinsed with PBS and incubated 1 hour with secondary antibodies conjugated to either Alexa®-488 (Molecular Probes, Eugene, Oregon) or the cyanine-derived fluorochrome Cy3 (Jackson Immunoresearch, West Grove, Pennsylvania). Finally, the sections were rinsed and coverslipped with Mowiol. They were observed with a fluorescence microscope (Eclipse 800; Nikon, Japan) equipped with a CCD camera (Axiocam HRc; Zeiss, Germany) or with a confocal laser scanning microscope (Fluoview FV3; Olympus, Japan), using sequential dual channel recording to eliminate possible cross-talk between the recorded channels.

PREEMBEDDING ELECTRON MICROSCOPY

The rats were deeply anesthetized and perfused with 4% paraformaldehyde in 0.1 M PB. After perfusion, the olfactory bulbs were washed in PB, embedded in agar and cut into 70-μm coronal sections with a vibratome (VT1000S; Leica, Germany). The sections were then immunolabelled with the avidin-biotin-peroxidase method as described in detail previously (Sassoè-Pognetto *et al.*, 1994). After incubation in the primary (guinea pig anti-$GABA_{B1}$) and secondary antibodies, they were treated with 3,3'-diaminobenzidine (DAB), and the reaction product was silver intensified and gold toned. The sections were then postfixed with 1% osmium tetroxide in cacodylate buffer for 1 hour, dehydrated in acetone and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM-1010 electron microscope (Jeol, Japan) equipped with a side-mounted CCD camera (Mega View III; Soft Imaging System, Germany).

POSTEMBEDDING ELECTRON MICROSCOPY

The rats were anesthetized and perfused with 4% paraformaldehyde using the "Ph-shift protocol" (Nagelhus *et al.*, 1998). Small specimens, isolated from the olfactory bulbs, were cryoprotected in glycerol (10%, 20% and 30% in PB) and rapidly frozen in liquid propane in a cryofixation unit (KF80; Reichert, Austria). This was followed by freeze substitution with methanol and embedding in Lowicryl HM20. Ultrathin sections were collected on adhesive-coated (Electron Microscopy Sciences, Ft. Washington, Pennsylvania; catalog No. 70624) nickel grids and immunolabelled as described previously (Sassoè-Pognetto & Ottersen, 2000). Briefly, the sections were etched with a saturated solution of NaOH in absolute ethanol for 2–3 sec, rinsed with double-distilled water, and incubated sequentially in the following solutions (at room temperature): (1) 0.1% sodium borohydride and 50 mM glycine in Tris buffer (5 mM) containing 0.1% NaCl and 0.1% Triton X-100 (TBST; 10 min); (2) 2% human serum albumin (HSA) in TBST (10 min); (3) rabbit anti-$GABA_{B1}$ diluted in TBST containing 2% HSA (overnight); (4) TBST (several rinses) and 2% HSA in TBST (10 min); (5) goat anti-rabbit Fab fragments coupled to 10 nm colloidal gold particles (British BioCell International, Cardiff, UK), diluted 1:20 in TBST with 2% HSA and 0.05% polyethyleneglycol (2 h). The grids were then rinsed several times in double-distilled water and counterstained with uranyl acetate and lead citrate.

DEVELOPMENTAL STUDY

The animals used for the developmental analysis ($n = 3$ for each stage) were from different litters and were grouped as follows: E16, E18, postnatal day 0 (P0, day of birth), P2, P7, P10, P12, P15 and P21. Fetuses were collected by caesarean section after anesthesia of the dam with an i.p. injection of ketamine-xylazine 1:1 (0.1 ml/kg body weight). The animals were deeply anesthetized by hypothermia (E16-P7) or by i.p. injection of ketamine-xylazine 1:1 (0.1 ml/kg body weight), and perfused with 4% paraformaldehyde and 15% (v/v) saturated picric acid made up in 0.1 M PB. After perfusion, the olfactory bulbs were dissected, washed thoroughly in PB for several hours, embedded in 4% agarose and sectioned at 60 μm with a vibratome (Leica VT1000S). The sections were then immunolabelled using the avidin-biotin-peroxidase method and observed and photographed with a Leica DMRS photomicroscope equipped with a digital imaging camera (Leica DC 200; for details, see López-Bendito *et al.*, 2002). Double-immunofluorescence was performed by incubating the sections in a mixture of the rabbit $GABA_{B1}$ antiserum and guinea pig anti-$GABA_{B2}$. The binding sites of the primary antibodies were revealed with specific secondary antibodies coupled to either Alexa®-488 or Cy3. The sections were analysed with a confocal laser scanning microscope (Leica TLSCM).

CONTROLS

To test method specificity in the procedures for light and electron microscopy, the primary antibodies were omitted or replaced with 5% (v/v) non-immune serum. Under these conditions, no selective labelling was observed. When double-immunofluorescence labelling was carried out, some sections were incubated with only one primary antibody and the full complement of secondary antibodies to test for any cross-reactivity. No cross-immunoreactivity that would influence the results was detected.

Results

DISTRIBUTION OF $GABA_B$ RECEPTORS IN THE MAIN AND ACCESSORY OLFACTORY BULBS

The antibodies raised against the two $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, produced labelling patterns that were similar in the MOB and in the AOB. Immunoreactivity for $GABA_{B1}$ was particularly pronounced in the glomerular layer and had a typical patchy appearance (Fig. 1A and C). We have shown previously in the MOB (Bonino *et al.*, 1999) that this labelling is associated with synaptic boutons of the olfactory nerve (see below). In sections incubated with the $GABA_{B2}$ antibody, there was also an intense staining of the glomerular layer (Fig. 1B and D), and confocal analysis of double-labelled sections showed that the glomerular labelling pattern was similar for $GABA_{B1}$ and $GABA_{B2}$ (Fig. 2A and B). These data indicate that both $GABA_B$ receptor subunits are colocalized in olfactory and vomeronasal nerve terminal boutons. No immunoreactivity was observed in the olfactory nerve layer or in the vomeronasal nerve layer, indicating that $GABA_B$ receptors are concentrated in nerve terminals.

In addition to the glomerular layer, the antibodies against $GABA_{B1}$ also labelled virtually every neuron in the MOB and AOB. This staining was particularly evident in the AOB, where granule cells were intensely immunopositive (Fig. 1C). At higher magnification, immunoreactivity for $GABA_{B1}$ was localized in the cytoplasm and extended into the proximal segment of the major dendrites (Fig. 2). In addition to this, there was also a faint labelling of the neuropil, which presented small clusters superimposed over a more diffuse labelling (Fig. 2E). In sections labelled with the $GABA_{B2}$ antibody, there was no obvious staining of cell bodies, but there was a rather diffuse immunoreactivity that was more pronounced in the granule and mitral cell layers (Fig. 2F) and was more evident in the AOB compared to the MOB (Fig. 1B and D). In double-labelled sections analysed by confocal laser scanning microscopy, it was evident that most of the $GABA_{B1}$-positive neurons also contained $GABA_{B2}$, albeit at a much lower level. Rather than filling the cytoplasm of neurons, $GABA_{B2}$-immunoreactivity appeared to be located at the cell surface and formed small clusters that frequently colocalized with $GABA_{B1}$ (Fig. 2). Double-labelled clusters were also present in the neuropil. A faint immunoreactivity for $GABA_{B2}$ was also observed in the nuclei of neurons (Fig. 2G).

ULTRASTRUCTURAL LOCALIZATION OF $GABA_B$ R1 IN OLFACTORY NERVE AXONS

Preembedding electron microscopy (Fig. 3A) confirmed our previous findings indicating that immunoreactivity

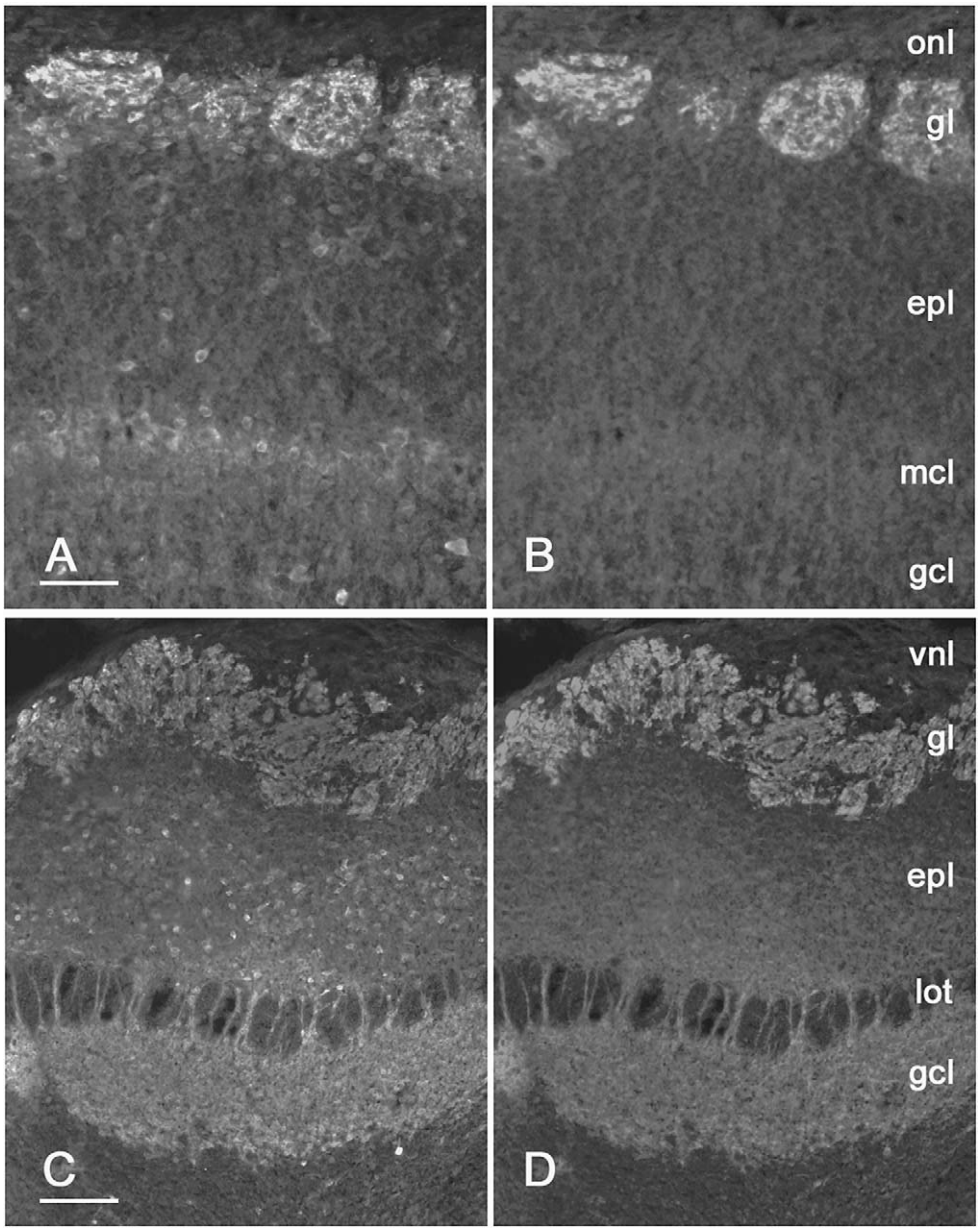

Fig. 1. Double-immunofluorescence micrographs showing the distribution of $GABA_{B1}$ (A and C; Alexa®-488 fluorescence) and $GABA_{B2}$ (B and D; Cy3 fluorescence) on cryostat sections of the main (A and B) and accessory (C and D) olfactory bulbs. The glomerular layer (gl) is strongly labelled, with both $GABA_{B1}$ and $GABA_{B2}$ showing a similar distribution pattern, but there is practically no immunoreactivity in the olfactory nerve layer (onl) and in the vomeronasal nerve layer (vnl). Immunoreactivity for $GABA_{B1}$ is also visible in neuronal perikarya, whereas $GABA_{B2}$ immunostaining is weaker and is more evident in the accessory olfactory bulb than in the main olfactory bulb. Epl, external plexiform layer; gcl, granule cell layer; lot, lateral olfactory tract; mcl, mitral cell layer. Scale bars, 90 μm (A and B) and 180 μm (C and D).

for $GABA_{B1}$ is localized in olfactory nerve terminals and is associated with the cytoplasmic face of the plasma membrane (Bonino *et al.*, 1999). However, the preembedding method suffers from the limitation that the peroxidase reaction product is diffusible, and thus prevents a precise localization of the epitopes. Therefore, we used a postembedding immunogold method to assess whether $GABA_B$ receptors can be associated with the presynaptic membrane of olfactory nerve terminals. In general, metabotropic receptors have proved to be refractory to visualization with postembedding techniques (Luján *et al.*, 1996; Ottersen & Landsend, 1997), and the antibody against $GABA_{B2}$ yielded only weak immunogold signals in the present study. Therefore, our analysis was limited to the distribution of $GABA_{B1}$. In agreement with the preembedding results, we found that gold particles decorated the plasma membrane of olfactory nerve terminals.

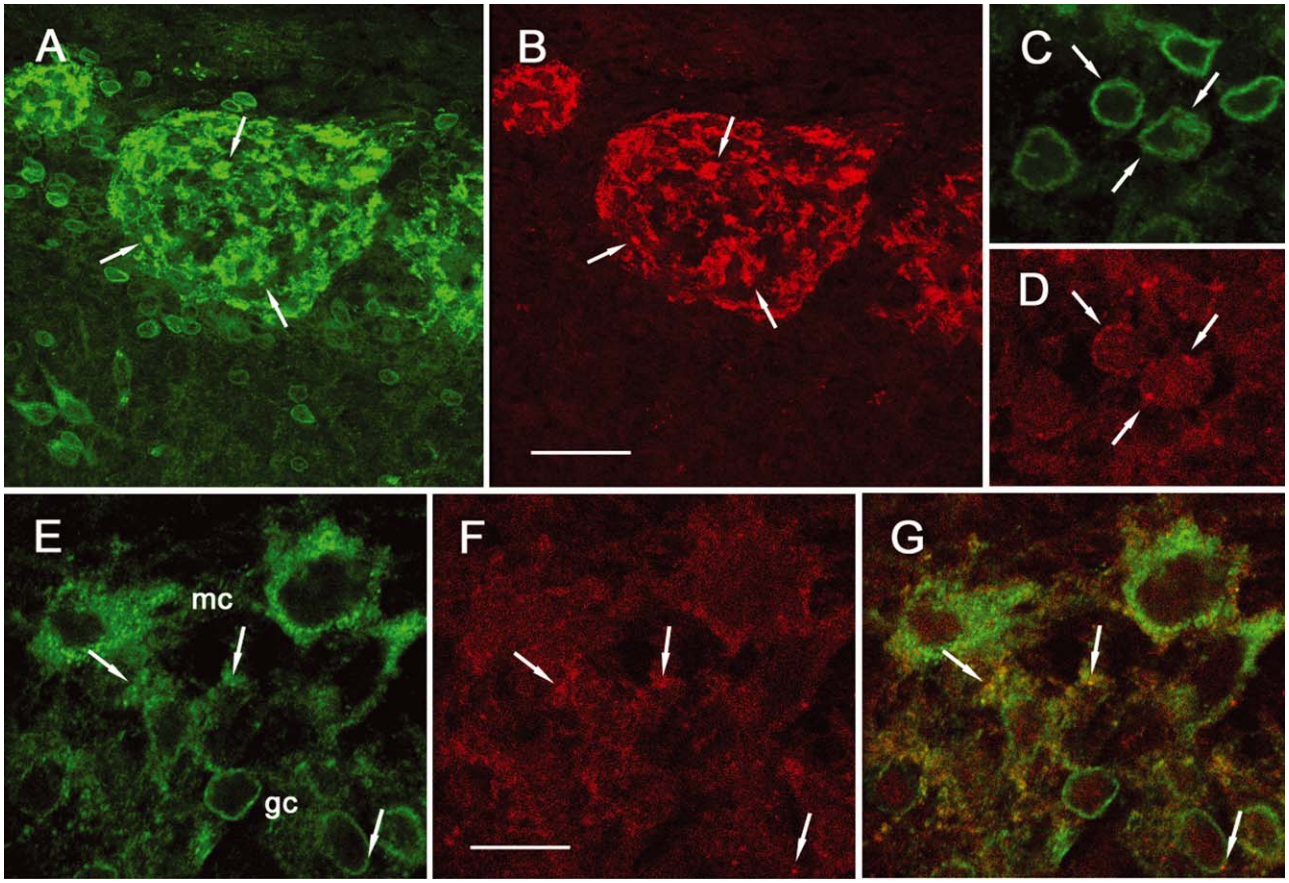

Fig. 2. Confocal images showing the subcellular distribution of $GABA_{B1}$ (A, C, E; Alexa®-488 fluorescence) and $GABA_{B2}$ (B, D, F; Cy3 fluorescence) in the main olfactory bulb. A and B show the extensive colocalization (arrows) of the two $GABA_B$ receptor subunits within the glomerular neuropil. Immunoreactivity for $GABA_{B1}$ is also evident in juxtaglomerular neurons. At higher magnification, coexpression of $GABA_{B1}$ (C) and $GABA_{B2}$ (D) becomes evident in periglomerular cells. Note that immunoreactivity for $GABA_{B2}$ is present in the form of small clusters (arrows) at the cell surface. E–G, $GABA_{B1}$ and $GABA_{B2}$ are also partially colocalized in mitral cells (mc), granule cells (gc) and the surrounding neuropil (arrows). Note the faint $GABA_{B2}$ labelling in the cell nuclei. Scale bars, 60 μm (A and B) and 20 μm (C–G).

Although many immunoparticles were located on the extrasynaptic plasma membrane (52%, $n = 65/125$), we frequently observed immunolabelling of presynaptic active zones (Fig. 3B–D). In a sample of 52 axon terminals which established asymmetric synapses, we found that 36 synapses (69.2%) contained at least one gold particle in presynaptic or perisynaptic position, defined as the region located within 150 nm from the edge of the synaptic specialization. Synaptic and perisynaptic particles accounted for about half of the total number of membrane-associated particles (60 out of 125 in our sample). As shown in Figure 3, gold particles were almost invariably located close to the edge of the synaptic specialization, indicating that $GABA_B$ receptors tend to be excluded from the central core of the presynaptic active zone. These data indicate that $GABA_B$ receptors are located at both synaptic and extrasynaptic sites in olfactory nerve terminals and those presynaptic receptors are preferentially accumulated at the periphery of the synaptic specialization.

DEVELOPMENTAL DISTRIBUTION OF $GABA_B$ RECEPTORS

At the earliest stages studied (E16–E18), the antibodies against $GABA_{B1}$ and $GABA_{B2}$ labelled the surface of the developing olfactory bulbs (Fig. 4). Notably, the labelling was concentrated in a superficial layer which plays an important role in glomerular formation (Bailey *et al.*, 1999; Treloar *et al.*, 1999). This staining was initially restricted to the rostral part of the olfactory bulbs and extended caudally as development proceeded (not shown). This suggests that olfactory nerve fibers may express $GABA_B$ receptors shortly after they have reached the olfactory bulb primordium and before they establish synaptic connections (Gesteland *et al.*, 1982; Doucette, 1990; Valverde *et al.*, 1992; Gong & Shipley, 1995; Treloar *et al.*, 1996). The immunoreactivity persisted and increased during postnatal development, as shown for $GABA_{B2}$ in Figure 5. During the first postnatal week, it was not easy to detect individual glomeruli, but starting from P12 adult-like glomerular formations became evident (Fig. 5E).

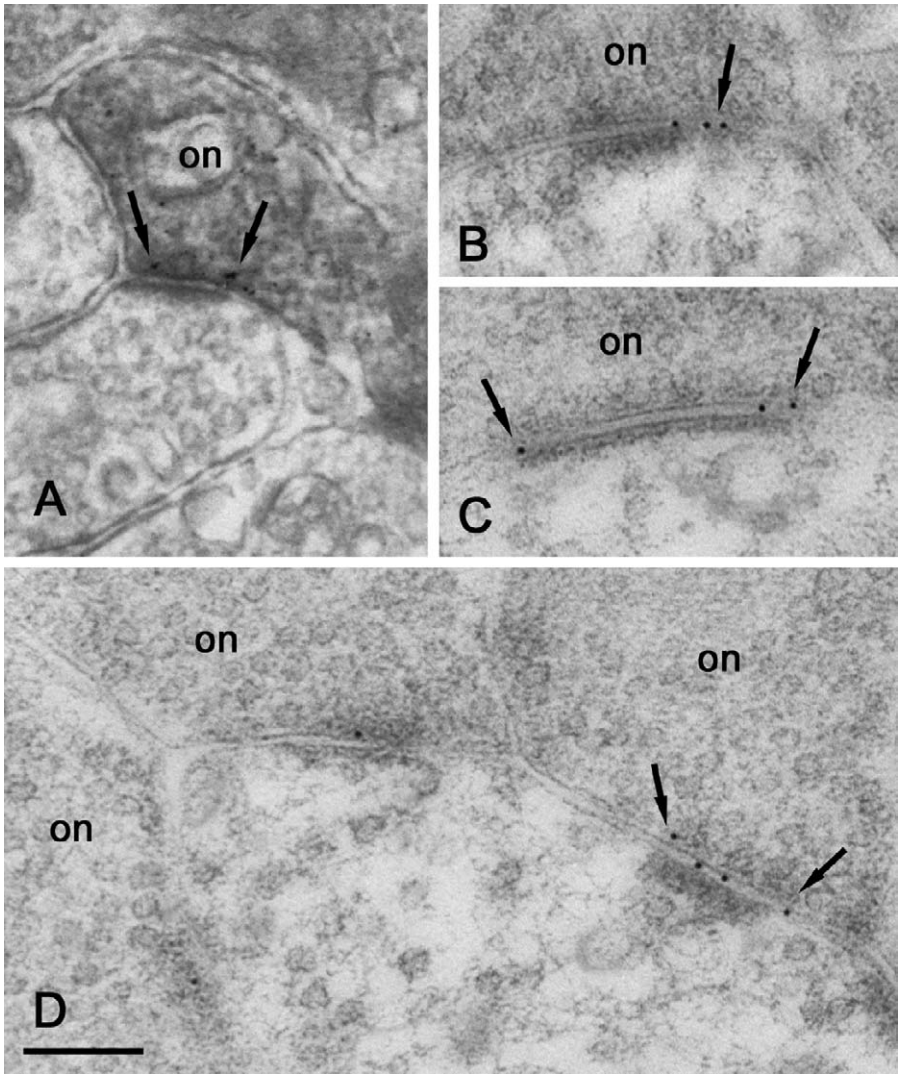

Fig. 3. Electron-microscopic localization of $GABA_B$ receptors in olfactory nerve terminals (on), as detected by preembedding (A) and postembedding (B–D) methods. All micrographs show $GABA_{B1}$-immunoreactivity at asymmetric synapses. Note that immunoparticles (gold-toned silver particles in A and colloidal gold particles in B–D) are localized mainly at the edge of the presynaptic specialization (arrows). Scale bar, 0,3 μm.

In the other layers of the olfactory bulb, immunoreactivity for $GABA_{B2}$ was always weak. In contrast, the antibody against $GABA_{B1}$ labelled cell bodies, in addition to the olfactory nerve fibers (Fig. 6), indicating that olfactory bulb neurons express high levels of $GABA_{B1}$ throughout development.

Discussion

$GABA_B$ RECEPTORS IN OLFACTORY AND VOMERONASAL NERVES

The present results demonstrate that the $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, are colocalized in terminal boutons of the olfactory nerve. This conclusion is based on the electron microscopic finding that $GABA_{B1}$ is present in olfactory nerve terminals (Bonino *et al.*, 1999 and present results), coupled with evidence from confocal laser scanning microscopy that $GABA_{B1}$ and $GABA_{B2}$ immunoreactivities are precisely colocalized in olfactory glomeruli. Although the antibodies used in the present study did not distinguish between the two $GABA_{B1}$ splice variants, it is likely that $GABA_B$ receptors of the olfactory nerve are composed of the $GABA_{B1(a)}$ and $GABA_{B2}$ subunits, because an antibody specific for the $GABA_{B1(b)}$ isoform did not label olfactory glomeruli (Shigemoto *et al.*, 1998). The localization of $GABA_B$ receptor proteins in the accessory olfactory bulb was not investigated at the ultrastructural level, but the labelling pattern (Fig. 1C and D) provides a strong argument in favour of the presence of $GABA_{B1}$ and $GABA_{B2}$ in vomeronasal axons. Because native $GABA_B$ receptors are believed to be heterodimers of $GABA_{B1}$ and $GABA_{B2}$ (Jones *et al.*, 1998;

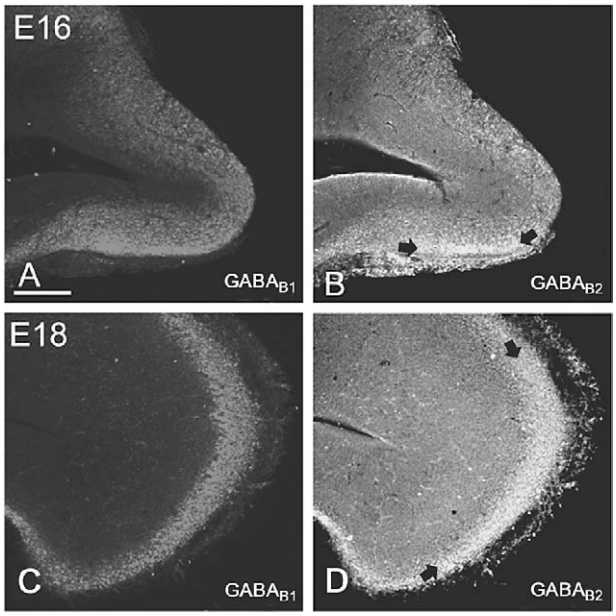

Fig. 4. Confocal micrographs showing $GABA_{B1}$ (Cy3 fluorescence) and $GABA_{B2}$ (Alexa®-488 fluorescence) immunoreactivities in the olfactory bulbs of E16 (A and B) and E18 (C and D) rat embryos. Note that the labelling is concentrated in a superficial layer of the developing olfactory bulbs (thick arrows). Scale bar, 500 μm.

Kaupmann *et al.*, 1998; White *et al.*, 1998; Kuner *et al.*, 1999), our findings provide strong evidence that olfactory and vomeronasal nerve fibers express functional $GABA_B$ receptors.

This conclusion is in agreement with functional analyses indicating that activation of $GABA_B$ receptors by baclofen reduces or suppresses synaptic transmission from the olfactory nerve to second-order neurons (Potapov, 1985; Nickell *et al.*, 1994; Keller *et al.*, 1998). It has also been shown that baclofen reverses paired-pulse depression of olfactory nerve evoked responses into paired-pulsed facilitation, which suggests that $GABA_B$ receptors act presynaptically to reduce the probability of glutamate release from olfactory nerve terminals (Aroniadou-Anderjaska *et al.*, 2000). Such a mechanism may contribute to maintain synaptic efficacy during high frequency stimulation (Brenowitz *et al.*, 1998), like for instance in the occurrence of repetitive sniffing activity. Similarly, there is evidence that $GABA_B$ receptors may regulate information flow from the vomeronasal nerve to postsynaptic neurons in the accessory olfactory system (Sugai *et al.*, 1999).

The ultrastructural localization of $GABA_{B1}$ immunoreactivity in olfactory nerve terminals has important implications for the mechanisms by which $GABA_B$ receptors suppress neurotransmission from the olfactory nerve. A well established mechanism by which $GABA_B$ receptors modulate neurotransmitter release is the reduction of Ca^{2+} influx through voltage-activated calcium channels (Huston *et al.*, 1995; Dittman & Regehr, 1996; Wu & Saggau, 1997; Takahashi *et al.*, 1998). Another mechanism, which involves cyclic AMP and Ca^{2+}/calmodulin, has been described recently (Sakaba & Neher, 2003). Similarly, studies in the turtle olfactory bulb have shown that $GABA_B$ receptors may reduce Ca^{2+} influx in olfactory nerve boutons (Wachowiak & Cohen, 1999). Our immunogold findings, showing the presence of $GABA_{B1}$ at short distance from the presynaptic active zone, indicate that activation of $GABA_B$ receptors may be effectively coupled to inhibition of presynaptic calcium channels. While extrasynaptic receptors may be less effective in acutely suppressing neurotransmitter release from the olfactory nerve, tonic activation of these receptors may contribute to set a constant level of inhibition in glomerular activity patterns.

The absence of postsynaptic sites on olfactory nerve terminals raises the question of what is the source of the $GABA_B$ receptor endogenous agonist (see also Aroniadou-Anderjaska *et al.*, 2000). The only source of GABA in the glomerular neuropil is represented by periglomerular cell dendrites. Periglomerular cells release GABA at dendrodendritic synapses established with mitral and tufted cells (Hinds, 1970; Pinching & Powell, 1971; Ribak *et al.*, 1977; Mugnaini *et al.*, 1984; Liu *et al.*, 1989; Sassoè-Pognetto *et al.*, 1993). However, the majority of dendrodendritic synapses occur in a dendritic compartment of the glomerular neuropil from which olfactory nerve axons are excluded (Kasowski *et al.*, 1999). Alternatively, GABA may be released through nonsynaptic mechanisms (Gaspary *et al.*, 1998) from segments of periglomerular cell dendrites located closer to olfactory nerve terminals. Interestingly, dendritic GABA release and $GABA_B$ receptor activation has been reported recently in the neocortex (Zilberter *et al.*, 1999). Whatever the mechanism of GABA release, a diffuse action of GABA is consistent with the observation that olfactory nerve terminals are only loosely packed with glial cell processes (Chao *et al.*, 1997; Kasowski *et al.*, 1999). In addition, $GABA_B$ receptors have a high affinity for GABA and may be activated even by low concentrations of this neurotransmitter (Sodickson & Bean, 1996). Taurine, which is contained in olfactory nerve axons (Didier *et al.*, 1994; Kratskin *et al.*, 2000), is another possible endogenous agonist of $GABA_B$ receptors, and recent electrophysiological data indicate that taurine activates presynaptic $GABA_B$ receptors and reduces glutamate release from olfactory nerve terminals (Belluzzi and Kratskin, personal communication).

$GABA_B$ RECEPTORS IN OLFACTORY BULB NEURONS

It has been shown recently that olfactory bulb neurons may express functional $GABA_B$ receptors. Palouzier-Paulignan *et al.* (2002) have proposed that postsynaptic

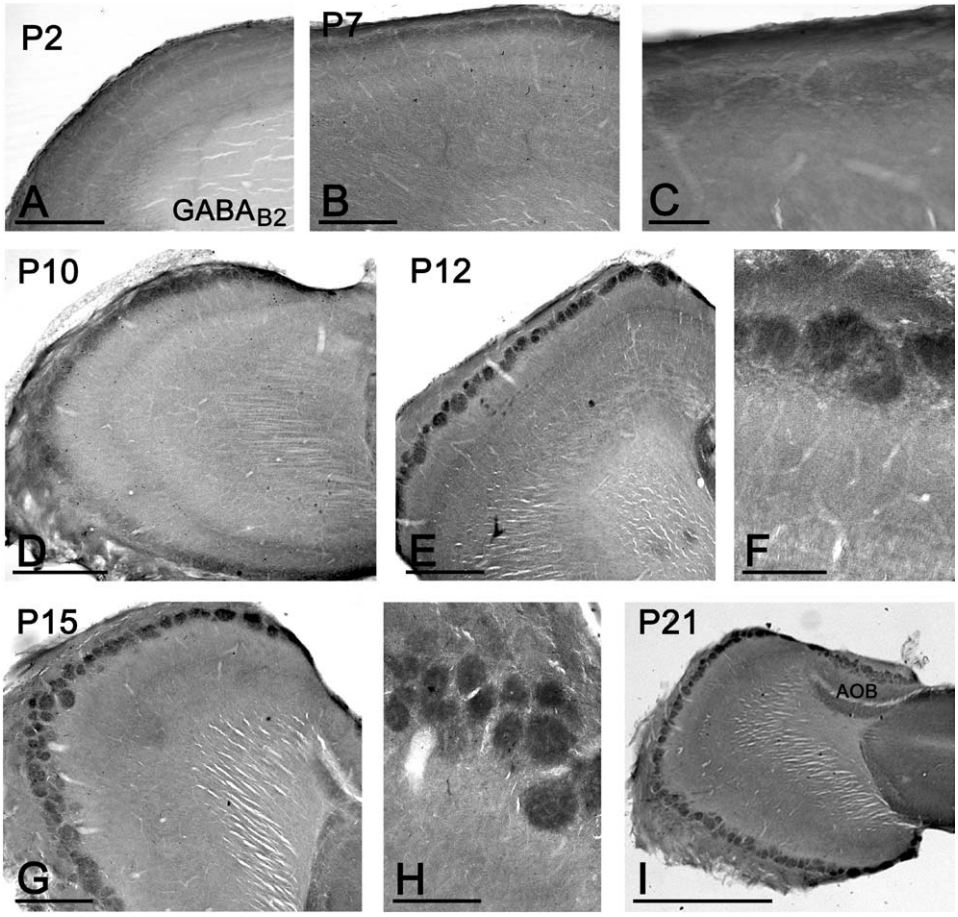

Fig. 5. Immunoreactivity for $GABA_{B2}$ in the olfactory bulb during postnatal development using a pre-embedding immunoperoxidase method. Sagittal sections of the olfactory bulb at P2, P7, P10, P12, P15 and P21 are shown. Note the progressive increase in the intensity of labelling in the glomerular layer and the appeareance of adult-like glomeruli at P12. AOB, accessory olfactory bulb. Scale bars: 500 μm (A, B, D, E and I); 300 μm (G); 200 μm (F, H); 100 μm (C).

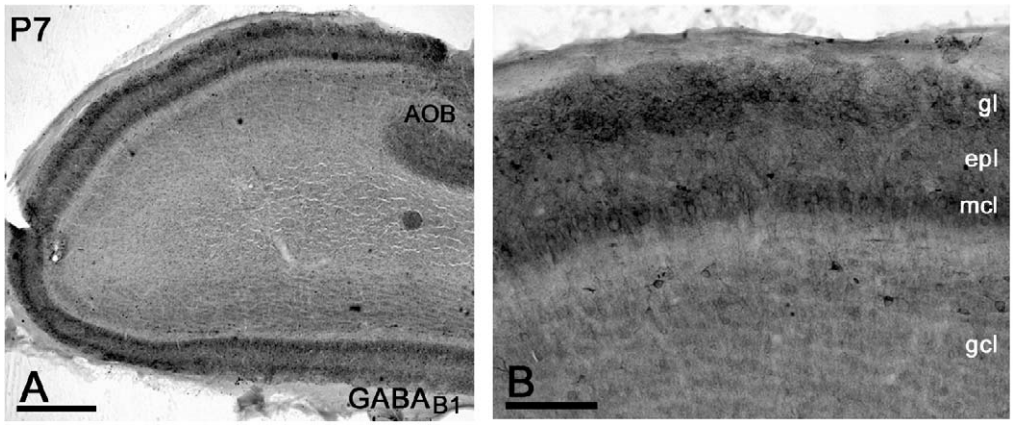

Fig. 6. Immunoreactivity for $GABA_{B1}$ in the olfactory bulb of P7 rat pups. Note the intense immunoreactivity of neuronal cell bodies, in addition to that of the glomerular layer (gl). $GABA_{B1}$ immunolabelling is particularly pronounced in the mitral cell layer (mcl). AOB, accessory olfactory bulb; epl, external plexiform layer; gcl, granule cell layer. Scale bars: 300 μm (A), 200 μm (B).

receptors located on mitral/tufted cells may be primarily involved in the control of spontaneous activity and contribute to make odor responses more salient by reducing background noise. According to Isaacson and Vitten (2003), granule cells may also express $GABA_B$ receptors. These authors have suggested that $GABA_B$ receptors located on granule cell spines may modulate GABA release at dendrodendritic synapses with mitral/tufted cells in the external plexiform layer. Our present findings do not rule out an involvement of $GABA_B$ receptors in infraglomerular circuits, but also raise several questions about the molecular composition of native $GABA_B$ receptors.

We have shown that distinct neuronal populations of the olfactory bulb, including periglomerular cells, mitral and tufted cells and granule cells, express the $GABA_{B1}$ subunit (see also Bonino *et al.*, 1999; Margeta-Mitrovic *et al.*, 1999). In addition, we found a faint immunolabelling for $GABA_{B2}$ in the same neurons, as well as a partial colocalization of $GABA_{B1}$ and $GABA_{B2}$ in the neuropil. However, the weak $GABA_{B2}$ immunostaining of bulbar neurons, compared to the strong labelling of the glomeruli, suggests that $GABA_B$ receptor expression in infraglomerular circuits is rather low. This is consistent with radioligand binding studies showing that the glomeruli have the highest concentration of $GABA_B$ binding sites (Bowery *et al.*, 1987; Chu *et al.*, 1990). Furthermore, of all layers of the olfactory bulb, the external plexiform layer showed the weakest immunoreactivity, suggesting that the contribution of $GABA_B$ receptors to dendrodendritic microcircuits may be little. Similar considerations also apply to the accessory olfactory bulb, which however showed a stronger cellular immunolabelling than the MOB.

A notable finding of this study was that the $GABA_{B1}$ and $GABA_{B2}$ proteins were only partially colocalized in neuronal perikarya. $GABA_{B1}$ immunoreactivity was mostly concentrated in the cytoplasm, whereas a faint labelling for $GABA_{B2}$ was present at the cell surface and in the nucleus. Nuclear staining for $GABA_B$ receptors has been reported previously (Charles *et al.*, 2001; Gonchar *et al.*, 2001). Interestingly, yeast two-hybrid studies have demonstrated that both $GABA_{B1}$ and $GABA_{B2}$ can associate with the transcription factors CREB2 and ATFx (Nehring *et al.*, 2000; White *et al.*, 2000). This raises the possibility that $GABA_B$ receptors may translocate to the nucleus and participate in the regulation of activity-dependent gene expression (Davies *et al.*, 1991; Mott & Lewis, 1991).

Differences in the subcellular distribution of $GABA_{B1}$ and $GABA_{B2}$ immunoreactivities similar to those described in the present study have been reported also in other locations (Ige *et al.*, 2000; Charles *et al.*, 2001; Ng & Yung, 2001; López-Bendito *et al.*, 2002). Furthermore, the expression of $GABA_{B2}$ is much lower than that of $GABA_{B1}$ in some brain regions (Durkin *et al.*, 1999; Clark *et al.*, 2000; Poorkhalkali *et al.*, 2000; Kulik *et al.*, 2002; López-Bendito *et al.*, 2002), as well as in several peripheral organs (Castelli *et al.*, 1999). Such observations are perplexing, if one considers that association of the C-terminal α-helices of the two proteins is essential for surface trafficking of $GABA_B$ receptors (Filippov *et al.*, 2000; Calver *et al.*, 2001; Pagano *et al.*, 2001). While studies of *knockout* mice have revealed that the $GABA_{B1}$ subunit is absolutely essential for the formation of functional $GABA_B$ receptors (Prosser *et al.*, 2001; Schuler *et al.*, 2001), it cannot be excluded that there are other putative binding partners of $GABA_{B1}$ which may substitute for $GABA_{B2}$ as a chaperone and/or in the assembly of a functional receptor complex (Billinton *et al.*, 2001; Enna, 2001). Indeed, recent studies in *Drosophila* and in mammals have identified novel G-protein coupled receptors with a high sequence homology to $GABA_{B1}$ and $GABA_{B2}$, which potentially represent new $GABA_B$ receptor subunits (Mezler *et al.*, 2001; Calver *et al.*, 2003). The availability of mouse models with targeted mutations of the $GABA_{B2}$ gene may help to shed light on this issue.

DEVELOPMENT OF $GABA_B$ RECEPTORS

The distribution pattern of $GABA_{B1}$ and $GABA_{B2}$ during pre- and postnatal development was not significantly different from that observed in adult animals. Immunoreactivity for $GABA_B$ receptors was concentrated in the superficial layers of the olfactory bulb at E16 and increased with further development. Therefore, olfactory nerve axons may express functional $GABA_B$ receptors when they reach the olfactory bulb and establish the first synaptic connections (Hinds & Hinds, 1976a, b; Valverde *et al.*, 1992). These data suggest that $GABA_B$ receptors may play a role in the development of the olfactory system, although demonstration of this will require further analyses.

Previous studies have unraveled a highly ordered projection pattern of olfactory nerve axons to the olfactory bulb: olfactory sensory neurons that express a given odorant receptor send their axons to only a few defined glomeruli in each bulb (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). In addition, because sensory neurons are replaced throughout life (Graziadei & Monti-Graziadei, 1979), precise glomerular convergence must be maintained by the newly generated axons. It is tempting to speculate that $GABA_B$ receptors located in nerve terminals may play a role in these processes.

Indeed, studies in other brain regions have indicated that $GABA_B$ receptors are involved in the regulation of various developmental events, such as neuronal migration (Behar *et al.*, 1998, 2000; López-Bendito *et al.*, 2003), chemoattraction (Behar *et al.*, 2001) and axonal pathfinding (Xiang *et al.*, 2002). Furthermore, *in vitro* analyses have revealed that $GABA_B$ receptors regulate neurotransmitter-induced calcium elevations in

developing hypothalamic neurons (Obrietan & van den Pol, 1998, 1999). Mutant mice lacking $GABA_{B1}$ have no residual $GABA_B$ receptor activity in the brain (Prosser *et al*., 2001; Schuler *et al*., 2001), and should be an ideal system in which to investigate whether the presence of functional $GABA_B$ receptors is essential for the correct assembly of neuronal circuits. The analysis of the glomerular pattern of termination of olfactory nerve fibers in these mutant mice may be instrumental in determining whether $GABA_B$ receptor activity affects the formation and refinement of synaptic connections.

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