

# Molecular mechanisms supporting a paracrine role of GABA in rat adrenal medullary cells

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GABA is known to produce membrane depolarization and secretion in adrenal medullary (AM) cells in various species. However, whether the GABAergic system is intrinsic or extrinsic or both in the adrenal medulla and the role that GABA plays are controversial. Therefore, these issues were addressed by combining a biochemical and functional analysis. Glutamic acid decarboxylase (GAD), a GABA synthesizing enzyme, and vesicular GABA transporter (VGAT) were expressed in rat AM cells at the mRNA and protein levels, and the adrenal medulla had no nerve fibre-like structures immunoreactive to an anti-GAD Ab. The double staining for VGAT and chromogranin A indicates that GABA was stored in chromaffin granules. The  $\alpha 1$ ,  $\alpha 3$ ,  $\beta 2/3$ ,  $\gamma 2$  and  $\delta$  subunits of GABA<sub>A</sub> receptors were identified in AM cells at the mRNA and protein levels. Pharmacological properties of GABA-induced Cl<sup>-</sup> currents, immunoprecipitation experiments and immunocytochemistry indicated the expression of not only  $\gamma 2$ -, but also  $\delta$ -containing GABA<sub>A</sub> receptors, which have higher affinities for GABA and neurosteroids. Expression of GATs, which are involved in the clearance of GABA at GABAergic synapses, were conspicuously suppressed in the adrenal medulla, compared with expression levels of GABA<sub>A</sub> receptors. Increases in Ca<sup>2+</sup> signal in AM cells evoked trans-synaptically by nerve stimulation were suppressed during the response to GABA, and this suppression was attributed to the shunt effect of the GABA-induced increase in conductance. Overall Ca<sup>2+</sup> responses to electrical stimulation and GABA in AM cells were larger or smaller than those to electrical stimulation alone, depending on the frequency of stimulation. The results indicate that GABA functions as a paracrine in rat AM cells and this function may be supported by the suppression of GAT expression and the expression of not only  $\gamma 2$ -, but also  $\delta$ -GABA<sub>A</sub> receptors.

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GABA is the major inhibitory neurotransmitter in the central nervous system, and it has two types of receptors, the A and B types (Inoue *et al.* 1985*b*; Blein *et al.* 2000). The A type GABA (GABA<sub>A</sub>) receptor, which is a pentameric assembly of subunits (Barnard *et al.* 1998), forms a Cl<sup>-</sup> channel by itself. Nineteen GABA<sub>A</sub> receptor subunits ( $\alpha 1$ – $\alpha 6$ ,  $\beta 1$ – $\beta 3$ ,  $\gamma 1$ – $\gamma 3$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho 1$ – $\rho 3$ ) have been cloned from the mammalian genome (Farrant & Nusser, 2005), and most of GABA<sub>A</sub> receptors in the brain are formed by combinations of both the  $\alpha$  and  $\beta$  subunits plus one or more of the  $\gamma$ ,  $\delta$  or  $\epsilon$  subunit (McKernan & Whiting, 1996). Although

these combinations will allow a potentially enormous molecular heterogeneity of GABA<sub>A</sub> receptor subtypes, the number of receptor subtypes expressed in brain neurones is limited (McKernan & Whiting, 1996) and the expression of receptor subtypes is regulated fairly strictly in a cell- and site-specific manner (Wisden *et al.* 1992; Farrant & Nusser, 2005). In the major part of the brain, GABA released from nerve terminals binds GABA<sub>A</sub> receptors in the postsynaptic membrane with the consequent generation of an inhibitory postsynaptic potential. This inhibitory synaptic transmission will be terminated by uptake of GABA into neighbouring glia and nerve terminals through GABA transporters (GAT) (Ribak *et al.* 1996; Farrant & Nusser, 2005). On the other hand, in granule cells of the cerebellum and the dentate gyrus (CG and DGG cells), GABA spilling over from

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the synaptic cleft continuously stimulates extrasynaptic GABA<sub>A</sub> receptors, resulting in tonic inhibition (Semyanov *et al.* 2004; Farrant & Nusser, 2005). It has been shown that synaptic and extrasynaptic GABA<sub>A</sub> receptors in CG and DGG cells have different compositions of subunits (Nusser *et al.* 1998; Farrant & Nusser, 2005). While  $\gamma$ -containing GABA<sub>A</sub> receptors are enriched in the subsynaptic membrane,  $\delta$ -containing GABA<sub>A</sub> receptors are exclusively or predominantly localized extrasynaptically. These  $\gamma$ - and  $\delta$ -GABA<sub>A</sub> receptors differ conspicuously in their biophysical and pharmacological properties (Quirk *et al.* 1995; Fisher & Macdonald, 1997; Wohlfarth *et al.* 2002; Bianchi & Macdonald, 2003; Belelli & Lambert, 2005), which may reflect multiple roles of GABA in the brain.

GABA and its receptors are present not only in the brain, but also in the peripheral tissue (Inoue *et al.* 1986; Chessler *et al.* 2002; Geigerseder *et al.* 2003). In bovine AM cells, GABA has been shown to produce depolarization through stimulating GABA<sub>A</sub> receptors, resulting in facilitation of catecholamine secretion (Peters *et al.* 1989; Xie *et al.* 2003). There is, however, much controversy over how GABA modulates catecholamine secretion or an increase in  $[Ca^{2+}]_i$  evoked by splanchnic nerve stimulation or secretagogues, i.e. inhibition (Kataoka *et al.* 1986), facilitation (Kitayama *et al.* 1990), or no effect (Xie *et al.* 2003). It has not been settled yet whether these differences are attributable to a difference in species or stimulation parameter. Furthermore, precisely where GABA in the adrenal medulla is synthesized remains an open question. Immunohistochemistry with an anti-GABA Ab revealed that GABA-like immunoreactive materials were present in nerve fibre-like structures and AM cells in the mouse adrenal medulla (Iwasa *et al.* 1998). In cultured bovine AM cells, an anti-GAD Ab labelled one third of the adrenaline cells (Kataoka *et al.* 1984; Castro *et al.* 2003). In contrast, in human and rat adrenal glands, GAD-like immunoreactivity was found to be distributed in adrenal cortex (AC) cells, but not in AM cells, and an Ab against VGAT, which is involved in uptake of GABA into secretory granules (Ebihara *et al.* 2003), was also immunologically detected in AC cells, but not AM cells (Metzeler *et al.* 2004). It is not known whether these differences in localization of GABA in the adrenal gland are due to a difference in species or to non-specific immunoreactivity of the Abs used. The first aim of the present experiment was to elucidate the source of GABA in the rat adrenal medullae by combining immunohistochemistry with immunoblotting and RT-PCR. The second aim was to clarify the effects of GABA on an increase in  $[Ca^{2+}]_i$  trans-synaptically evoked using  $Ca^{2+}$  imaging and to elucidate the molecular mechanisms responsible for the GABA function in the adrenal medulla. The results indicate that GABA is stored in chromaffin granules of AM cells and plays a paracrine or autocrine role for catecholamine secretion. The molecular

basis for this GABA function may be the suppression of GAT expression and the expression of  $\delta$ - and  $\gamma$ 2-GABA receptors.

## Methods

Male Wistar rats weighing 200–400 g ( $n = 67$ ) were used. All experiment procedures involving animals were approved by the Institutional Animal Care and Use Committee of University of Occupational and Environmental Health.

## Immunoblot

The animals were killed by cervical dislocation, and the brain, the heart and the adrenal glands were excised and immediately put into ice-cold  $Ca^{2+}$ -deficient balanced salt solution in which 1.8 mM  $CaCl_2$  was simply omitted from standard saline. The standard saline contained 137 mM NaCl, 5.4 mM KCl, 1.8 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 0.53 mM  $NaHPO_4$ , 5 mM D-glucose, 5 mM Hepes and 4 mM NaOH (pH 7.4). The adrenal cortex was removed from the adrenal gland using microscissors and forceps under stereoscopic observations. The preparations were minced and homogenized with a Potter-Elvehjem homogenizer in 10 volumes of a solution containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and a protease inhibitor cocktail (set 1: Calbiochem, San Diego, CA, USA). Homogenates were centrifuged at 500 g for 10 min at 4°C to remove the nuclei, then the post-nucleus supernatants were mixed with equal volumes of a SDS buffer containing 25 mM Tris-HCl (pH 6.8), 4% SDS and 20% glycerol. Protein concentrations in samples were determined using a BSA protein assay kit (Pierce, Rockford, IL, USA). After addition of 5% (v/v) 2-mercaptoethanol and 1% (v/v) bromophenol blue to the sample, proteins were separated by 10% (w/v) SDS-PAGE, and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% (w/v) fat-free powdered milk dissolved in PBS-T solution, which contained 2 mM  $NaH_2PO_4$ , 8 mM  $Na_2HPO_4$  and 145 mM NaCl, and 0.1% Tween 20. The PVDF membrane was incubated with a rabbit, mouse or goat Ab. The immunoreaction was detected by incubating the membrane with a respective secondary Ab linked to horseradish peroxidase (Amersham, Buckinghamshire, UK), and then with ECL-Plus (Amersham). Immunoblotting was repeated at least three times for each Ab. The neutralization of an Ab with its antigen was performed according to the manufacturer's instructions.

The immunoprecipitation assay was performed in a manner similar to that described elsewhere (Lin *et al.* 2005) in cases where Abs made in host animals of different species were used for immunoprecipitation and immunoblotting.

Briefly, post-nuclear supernatants were solubilized in an immunoprecipitation buffer (10  $\mu\text{M}$  deoxycholate, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and the protease inhibitor cocktail) to bring the final protein concentration to 1–2  $\mu\text{g } \mu\text{l}^{-1}$ . The sample was centrifuged at 12000  $g$  for 3 min at 4°C to pellet insoluble materials. The supernatant was incubated with an anti-GABA<sub>A</sub>  $\beta$ 2/3 monoclonal Ab (mAb) (clone: 62-3 G1) (Upstate, Lake Placid, NY, USA), a rabbit anti-GABA<sub>A</sub>  $\delta$  Ab (SC-25705; Santa Cruz, Santa Cruz, CA, USA), or a rabbit IgG coupled with protein G-sepharose at 4°C for 3 h. The mixture was washed three times in buffer I (1% NP-40, 150 mM NaCl, 20 mM Tris, 2 mM EDTA, pH 7.5). Immunoprecipitated receptor subunits were dissociated from beads by incubation in Laemmli sample buffer for 30 min at 37°C and then subjected to immunoblot analysis. In cases where Abs in immunoprecipitates hindered detection of the target protein, a Size X Protein G Immunoprecipitation kit (Pierce) was used to cross-link the Ab to protein G.

### Whole-cell recording

Unless otherwise stated, the whole-cell current was recorded in an isolated rat AM cell using the nystatin perforated patch method, as described elsewhere (Inoue & Imanaga, 1995; Inoue *et al.* 2008). Briefly, adrenal medullae were treated with collagenase for 30 min and AM cells were dissociated mechanically with fine needles. The standard pipette solution contained 120 mM potassium isethionate, 20 mM KCl, 10 mM NaCl, 10 mM Hepes and 2.6 mM KOH (pH 7.2), and in low Cl<sup>-</sup> solution 20 mM KCl was equimolarly replaced with potassium isethionate. On the day of the experiment, nystatin dissolved in dimethyl sulfoxide (5 mg in 100  $\mu\text{l}$ ) was added to the pipette solution at a final concentration of 100  $\mu\text{g } \text{ml}^{-1}$ . In some experiments, gramicidin instead of nystatin was added to pipette solutions at final concentrations of 4–40  $\mu\text{g } \text{ml}^{-1}$ , since nystatin pores pass Cl<sup>-</sup> as well as monovalent cations (Achilles *et al.* 2007). All chemicals were bath applied. The membrane potential was corrected for a liquid junction potential of –3 mV between the standard pipette solution and saline. For the analysis of the dose-dependent production of GABA-induced Cl<sup>-</sup> current ( $I_{\text{Cl}}$ ), Sigma plot (7.0: SPSS, Chicago, IL, USA) was used to fit a peak value ( $I$ ) of  $I_{\text{Cl}}$  to the logistic equation  $I = (I_{\text{max}} \times \chi^a) / (EC_{50}^a + \chi^a)$ , where  $I_{\text{max}}$  represents the maximum value of  $I$ ,  $\chi$  is the concentration of GABA,  $EC_{50}$  is the concentration of GABA responsible for half the maximum current, and  $a$  is a slope factor (corresponding to a Hill coefficient). The peak  $I_{\text{Cl}}$  in response to GABA was expressed relative to the maximum of 30  $\mu\text{M}$  GABA-induced  $I_{\text{Cl}}$  in the same cells. Experiments were carried out at  $26 \pm 2^\circ\text{C}$ .

### Perfusion experiment

The adrenal glands were removed from rats under pentobarbital (60 mg kg<sup>-1</sup> i.p.) anaesthesia, and then perfused retrogradely via the adrenal vein with saline at a rate of 0.45 ml min<sup>-1</sup> (Warashina & Satoh, 2001). The glands were subjected to a 40 min recurrent perfusion with 1 ml saline containing 10  $\mu\text{M}$  fluo-4 AM and 0.2% Pluronic F127, and then part of the adrenal cortex covering the medulla was carefully removed by microscissors. The adrenal gland was placed between one pair of silver circles for electrical stimulation and then the gland was transferred to a chamber with the naked medulla on the glass bottom. The chamber was mounted on the stage of a confocal laser scanning microscope (LSM410; Carl Zeiss, Oberkochen, Germany), and the adrenal gland was continuously perfused at 25–28°C. Illumination with 488 nm was provided by an argon laser and emission was monitored above 510 nm, and fluorescence images were acquired every 5 s. The extent of photobleaching was estimated by a curve fitting of the intensities of responsive areas at the resting state with a polynomial function ( $at^2 + bt + c$ , where  $a$ ,  $b$  and  $c$  are constants, and  $t$  is time). The intensity in each frame was then corrected for photobleaching. The effects of GABA or muscarine on the Ca<sup>2+</sup> signal in response to electrical stimulation were examined in the preparations where trans-synaptically evoked Ca<sup>2+</sup> signals did not diminish by more than 15% after washout of the agent.

### Immunocytochemistry

Immunostaining of dissociated AM cells was performed, as previously described (Inoue *et al.* 2008). For indirect immunofluorescence studies, cells were treated overnight with an anti-GABA<sub>A</sub> receptor  $\alpha$ 1 Ab diluted at 1:100 (AGA-001: Alomone, Jerusalem, Israel), an anti-GABA<sub>A</sub> receptor  $\alpha$ 3 Ab at 1:1000 (provided by W. Sieghart), an anti-GABA<sub>A</sub> receptor  $\delta$  Ab at 1:50 (Santa Cruz), an anti-GABA<sub>A</sub> receptor  $\gamma$ 2 Ab at 1:20 (AGA-005: Alomone), an anti-GABA<sub>A</sub> receptor  $\beta$ 2/3 mAb (BD17) at 1:100 (MAB341: Chemicon), an anti-synaptophysin mAb (SY38) at 1:50 (61012: Progen, Heidelberg, Germany), an anti-VGAT Ab at 1:100 (PA1-4701: Affinity BioReagents, Golden, CO, USA), an anti-chromogranin A Ab at 1:100 (SC-13090; Santa Cruz), or an anti-dopamine- $\beta$ -hydroxylase Ab at 1:500 (AB1538; Chemicon). After the incubation, the cells were washed three times in PBS and then treated with a respective secondary Ab conjugated with Alexa 488 or 546 (Molecular Probe, Eugene, OR, USA). The fluorescence was observed using a laser scanning confocal microscope or inverted fluorescence microscope (Axiovert 135; Carl Zeiss). The objective lens was an oil-immersion lens with a magnification of  $\times 63$  and fluorescence was observed

**Table 1. Primer sequences used for PCR of GABA<sub>A</sub> receptors, GAD, VGAT, GAT and  $\beta$ -actin**

Target	Forward	Reverse	PCR product size (bp)
GABA <sub>A</sub>			
$\epsilon$	5'-CGATGCGAAGAACAACCTTGG-3'	5'-TTAGCACGGCTATTGGTTGG-3'	360
GAD			
67	5'-GTCAAGGAAAAGGGTATACTCCAAGG-3'	5'-GTGTGCTCAGGCTCACCATTG-3'	280
65	5'-GTGATGAGAGAGGGAAAATG-3'	5'-TGCATCAGTCCCTCTCTGACC-3'	337
VGAT	5'-ACGCCATTGAGGGCATGTTCTGTGC-3'	5'-GGTTGCCGCTCACCCTACGTACAAG-3'	303
GAT			
1	5'-CAATGTGTACAGGGACTCCATCA-3'	5'-AATGCCACCCTGGGTGATGTTAG-3'	391
3	5'-GCCTGGACAGTCAGTTTGTGTG-3'	5'-AAGATGCCCGCACAGATCCCA-3'	364
$\beta$ -actin			
Long	5'-AGGCACCAGGGTGTGATGGTGG-3'	5'-CTCAAACATGATCTGGGTCATC-3'	261
Short	5'-CCTGGGTATGGAATCCTGTGGCAT-3'	5'-GGAGCAATGATCTTGATCTTC-3'	198

with appropriate filter sets. To examine the specificity for the immunoreaction, the preparation was treated with a non-immune serum instead of a primary Ab, and almost no immunoreactivity was observed under the same conditions as used for a primary Ab.

### Immunohistochemistry

The rats were anaesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup> I.P.) and perfused through the ascending aorta with 30 ml of saline, then 250 ml of Zamboni's fixative. The adrenal glands were removed from the rats and post-fixed in the fixative overnight at 4°C. After fixation and rinsing in PBS, they were dehydrated through a graded ethanol series and embedded in paraffin (Histosec: Merck, Germany). Thin sections 5  $\mu$ m in thickness were obtained with a microtome, mounted on glass slides (MAS-coated Superfrost: Matsunami, Japan), dried overnight, and deparaffinized. Next, the sections were rinsed for 10 min in PBS. Endogenous peroxidase activity was inhibited by pre-treatment with 0.1% hydrogen peroxide in methanol for 20 min. After treatment with 0.2% casein for 60 min to reduce the non-specific binding, the sections were incubated overnight with an anti-GAD65/67 Ab diluted at 1:1000 (AB1511; Chemicon). After rinsing in PBS, the immunoreaction was examined with the indirect immunoperoxidase method (Histofine Simple Stain Max-PO: Nichirei, Japan). The peroxidase complex was visualized by treatment with a freshly prepared solution of diaminobenzidine tetrahydrochloride (DAB) (DAB substrate kit: Nichirei), and the diaminobenzidine reaction was enhanced by addition of nickel ammonium sulphate. Immunohistochemistry was repeated on sections obtained from three animals. The specificity of immunohistochemical staining was confirmed by replacing an Ab with PBS.

### RT-PCR

Poly(A)<sup>+</sup>RNA was isolated from rat brain, adrenal medulla and adrenal cortex using the Micro-fast track

kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Oligo dT primer was utilized for the reverse transcriptase (RT) reaction to obtain cDNAs. PCR reactions were carried out with 1.25  $\mu$ l of DNA template, 4 pmol of primer, 2 mM of dNTPs, 0.5 units of rTaq (Takara, Otsu, Japan), and PCR buffers supplied with the kit in a final volume of 20  $\mu$ l. Table 1 lists the primers for the PCR, except for the GABA<sub>A</sub> receptor subunits  $\alpha$ 1–6,  $\beta$ 1–3,  $\gamma$ 1–3 and  $\delta$ , for which the primers designed by Akinci & Schofield (1999) were used. The PCR protocol used started with an initial 3 min denaturation step at 94°C, followed by 30–40 cycles of the profile consisting of 30 s of denaturation at 94°C, 30 s of annealing at 54 to 60°C and 30 s of extension at 72°C. To obtain the maximum fidelity, a hot-start procedure was used. In each PCR reaction, either a 198 or 261 bp PCR product of  $\beta$ -actin mRNA was co-amplified and used as an internal standard. To ensure that  $\beta$ -actin mRNA would not reach the plateau phase earlier than the target gene, addition of  $\beta$ -actin primers was delayed. During the first six PCR cycles, only the target gene primers were present. After completion of the sixth elongation phase, the PCR reaction was halted and the reaction mixture was cooled to 4°C. Then,  $\beta$ -actin primers were added and the reaction was resumed, starting with a 2 min denaturation step at 94°C, followed by cycles of the same PCR profile. Under these conditions both the target gene and the reference were amplified in the exponential range; therefore, the relative amounts of target mRNAs were expressed as fractions of amounts of  $\beta$ -actin PCR products (Lin *et al.* 2005). The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Data were expressed as the mean  $\pm$  S.E.M., and statistical significance was determined using Student's paired or unpaired *t* test.

### Source of agents

Muscarine chloride, Pluronic F 127, baclofen, bicuculline methiodide and GABA were obtained from Sigma-Aldrich

(St Louis, MO, USA); an anti-GABA<sub>A</sub> receptor  $\alpha 3$  Ab (AGA-003) was from Alomone; an anti-GABA<sub>A</sub> receptor  $\delta$  Ab (AB5643), anti-GABA transporter-1 (GAT1) Ab (AB1570W), and anti-GAT3 Ab (AB1574), and anti-actin mAb (MAB1501R) were from Chemicon; fluo-4 AM was from Dojindo (Kumamoto, Japan); collagenase was from Yakult (Tokyo, Japan); BODIPY-FL-thapsigargin was from Molecular Probe.

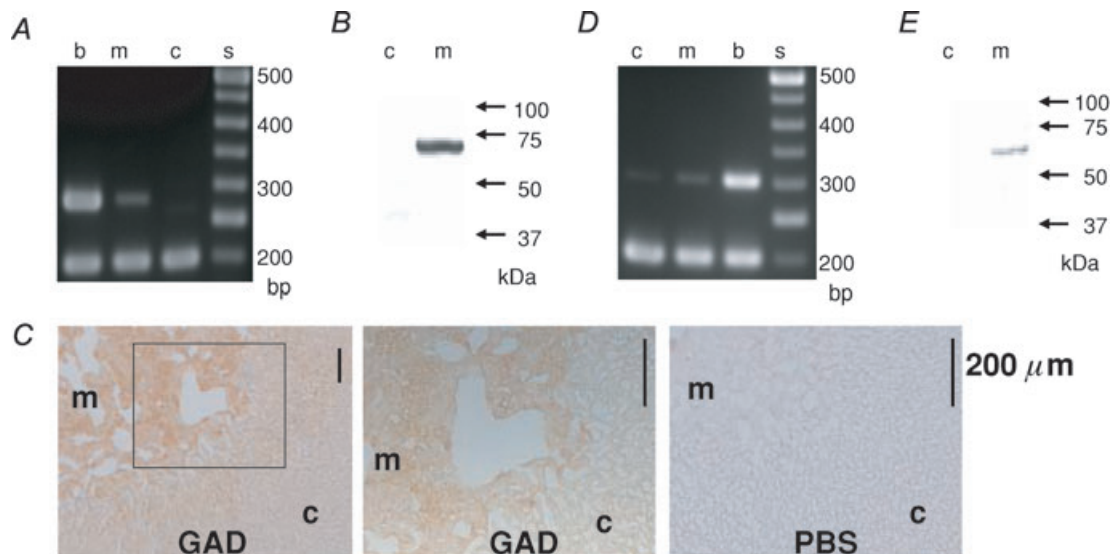
## Results

### Presence of GAD and VGAT in AM cells

The production of GABA in mammals is mediated by GAD65 and/or GAD67 (Soghomonian & Martin, 1998; Ji *et al.* 1999). Therefore, the presence of GAD65 and GAD67 in the rat adrenal medulla was assayed using RT-PCR (Fig. 1A). PCR products of 280 bp for GAD67 were detected in the cDNA samples of rat brain and adrenal medulla, but not in adrenal cortex, whereas PCR products of 337 bp for GAD65 were observed in the brain but not in either the adrenal medulla or the adrenal cortex (not shown). Consistent with the PCR results, an anti-GAD65/67 Ab specifically recognized a band of 65–67 kDa in the homogenates of rat adrenal medulla, but

not adrenal cortex (Fig. 1B). This result led us to examine the localization of the immunoreactivities for GAD in the rat adrenal gland. As shown in Fig. 1C, immunoreactivities for GAD were found to be present in the adrenal medulla. Detailed inspection did not reveal any staining of nerve fibre-like structures or nerve terminals surrounding AM cells (middle in Fig. 1C), which has been observed in the immunodetection of pituitary adenylate cyclase-activating polypeptide (PACAP) in the guinea-pig adrenal medulla (Inoue *et al.* 2000). The detection of GAD mRNA and protein indicates that GABA is produced in rat AM cells.

If that is the case, then VGAT, which is involved in the uptake of GABA into synaptic vesicles in the brain, may be expressed selectively in AM cells. Thus, the expression of VGAT mRNA and protein was determined. PCR products of 303 bp were detected clearly in the cDNA samples of rat brain and adrenal medulla and slightly in that of adrenal cortex (Fig. 1D). On the other hand, an anti-VGAT Ab detected a protein of about 60 kDa in the homogenates of the adrenal medulla, but not the adrenal cortex (Fig. 1E). Figure 2 reveals that the VGAT-like immunoreactivity (IR) coincided with chromogranin A-like IR and the chromogranin-like IR agreed with dopamine- $\beta$ -hydroxylase-like IR, which did not coincide with the synaptophysin-like IR, indicating



**Figure 1. The presence of GAD67 and VGAT in rat AM cells**

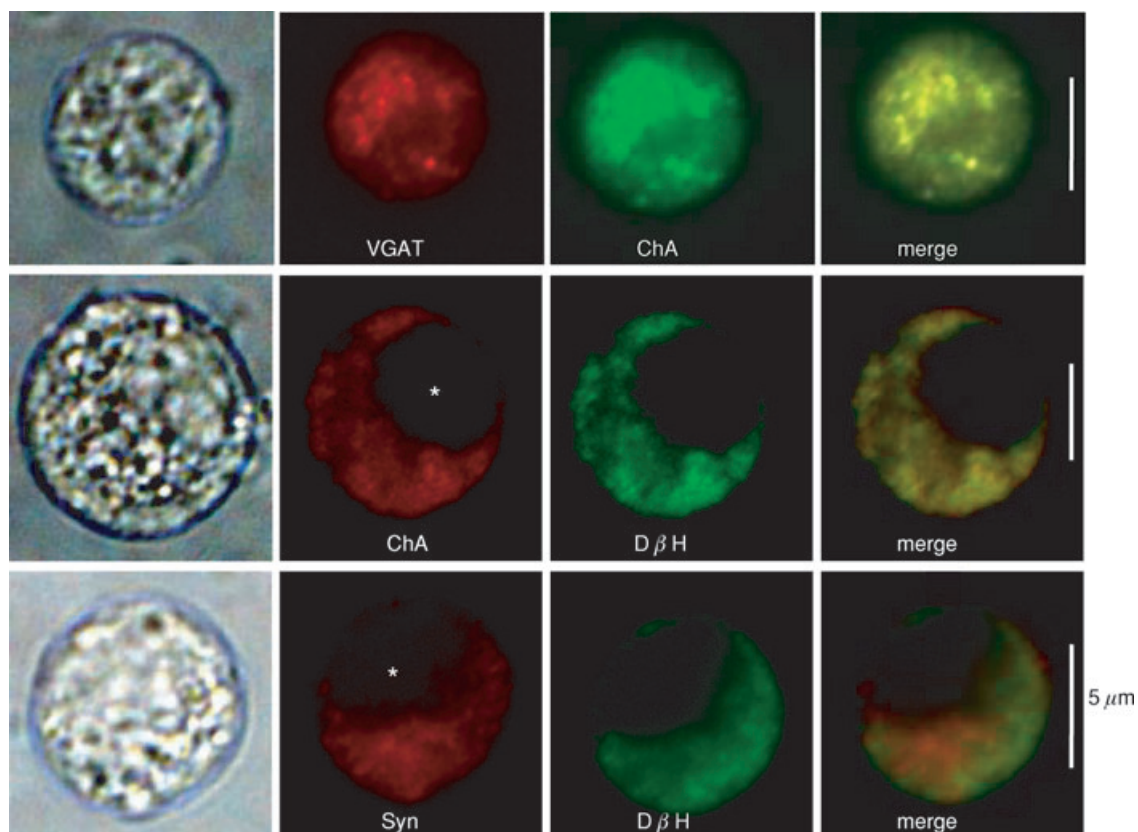
A, electrophoresis of PCR products for GAD67 and  $\beta$ -actin. The PCR product of 280 bp for GAD67 was clearly observed in cDNA samples of the rat brain (b) and adrenal medulla (m), but not in those of the adrenal cortex (c), whereas the 198 bp PCR products for  $\beta$ -actin were observed at similar levels in all three tissues. s stands for standard ladder of DNAs in this and following figures. B, immunoblot for GAD. A band of 65–67 kDa was detected in a homogenate of rat adrenal medulla, but not adrenal cortex. C, immunohistochemistry for GAD. Sections of rat adrenal glands were incubated overnight in PBS with (GAD) and without (PBS) a rabbit anti-GAD Ab. The immunoreaction was detected with the indirect immunoperoxidase method. The middle panel represents an enlargement of the area indicated by the square in the left panel. D, electrophoresis of PCR products for VGAT and  $\beta$ -actin. PCR products of 303 bp were clearly observed in cDNAs of the rat adrenal medulla (m) and brain (b) and faintly in those of the adrenal cortex (c). The lower bands represent PCR product of 198 bp for  $\beta$ -actin as an internal control. E, immunoblot for VGAT. A band of about 60 kDa was detected in a homogenate of rat adrenal medulla, but not adrenal cortex.

that GABA is stored in chromaffin granules but not in synaptic-like microvesicles (SLMV) (Thomas-Reetz & De Camilli, 1994).

### Subunit composition of GABA<sub>A</sub> receptor

RT-PCR and immunoblotting were used to elucidate the subunit composition of GABA<sub>A</sub> receptors in the rat adrenal gland. PCR products with the size expected for each of the  $\alpha$  subunits were observed in rat brain cDNA samples with the specific set of primers (Fig. 3A), indicating that each primer set was appropriate for respective  $\alpha$  subunits. In the rat adrenal medulla, PCR products for  $\alpha 1$  and  $\alpha 3$  subunits were detected, whereas in the rat cortex none of the  $\alpha$  subunits were observed (Fig. 3A). Figure 3B shows the immunodetection of the  $\alpha 1$  and  $\alpha 3$  subunits in the adrenal medulla at the protein level. An anti- $\alpha 1$  Ab recognized proteins of about 50 and 40 kDa in rat brain homogenates and a protein of about 50 kDa in the rat adrenal medulla, whereas a protein of about 48 kDa

was detected in the rat adrenal cortex homogenates and no protein was labelled in the rat heart. Neutralization of the Ab with the antigen peptide abolished the 50 kDa bands in the brain and adrenal medulla immunoblots, but not the 40 kDa protein in the brain and the 48 kDa protein in the adrenal cortex. These results indicate that the  $\alpha 1$  subunit is expressed in the rat adrenal medulla, but not the cortex. The presence of the  $\alpha 3$  subunit in the rat adrenal medulla was also examined at the protein level. An anti- $\alpha 3$  Ab detected a protein of about 58 kDa in the brain and adrenal medulla homogenates and this detection was abolished by pre-incubation of the Ab with the antigen peptide. Figure 4A shows RT-PCR for the  $\beta 1$ – $3$ ,  $\gamma 1$ – $3$  and  $\delta$  subunits in the rat brain and adrenal medulla. The PCR of the brain cDNA with the specific set of primers produced single bands with the size expected for  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 3$  and  $\delta$  subunits and double bands with the expected sizes for the  $\gamma 2S$  and  $\gamma 2L$  (Whiting *et al.* 1990), whereas PCR of the adrenal medulla cDNA with the same set of primers amplified single bands with the expected sizes for



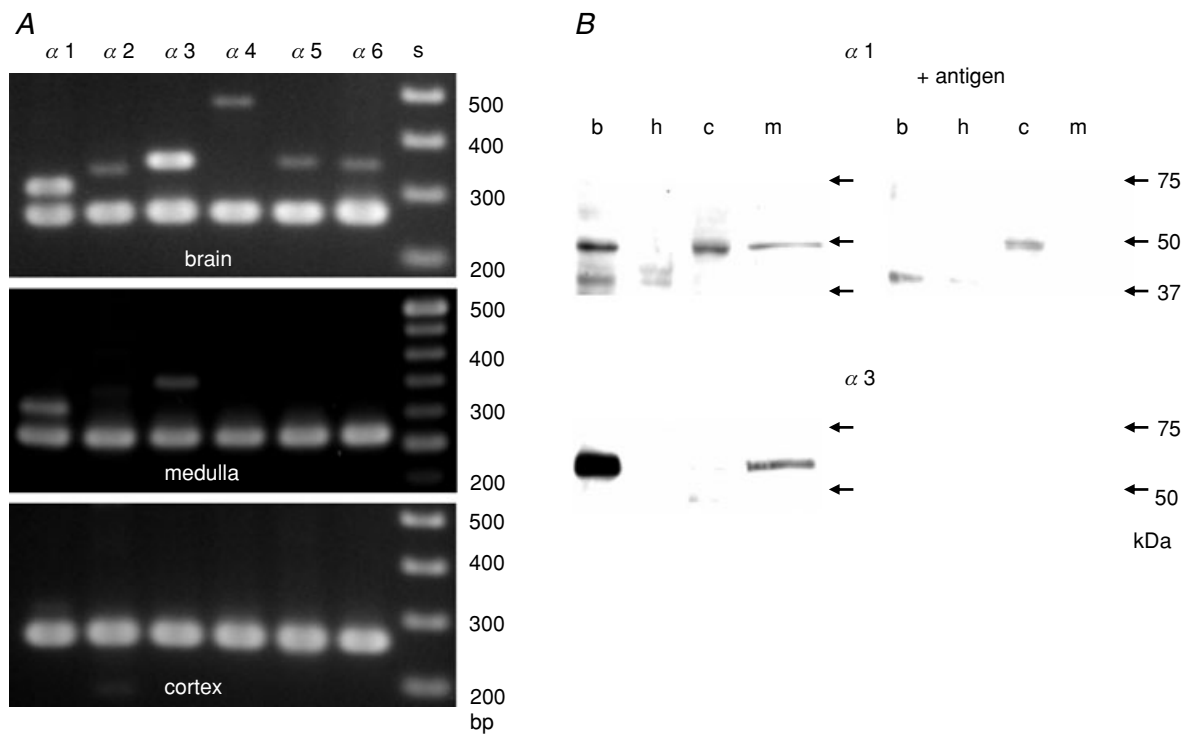
**Figure 2.** The presence of VGAT in chromaffin granules

1st column represents transparent images for each rat AM cell; the 2nd and 3rd columns show rhodamine-like and FITC-like fluorescence images for immunoreaction. 4th column indicates merge of the 2nd and 3rd column images. Colocalization of immunoreactions with different Abs is shown in yellow. Cells were treated with a rabbit anti-VGAT Ab and then with goat anti-chromogranin A (ChA) or with rabbit anti-dopamine- $\beta$ -hydroxylase (D $\beta$ H) and then the anti-ChA Ab or a mouse anti-synaptophysin (Syn) mAb. Asterisks indicate the nucleus. The experiments were repeated at least three times.

the  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2S$  and  $\delta$ . PCR products for the  $\epsilon$  subunit were detected in brain cDNAs, but not in medulla (not shown). These results suggest that the  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2S$  and  $\delta$  subunits are expressed in the adrenal medulla. Therefore, this possibility was explored using an anti- $\gamma 2$  Ab, an anti- $\delta$  Ab, and the anti- $\beta 2/3$  mAb, BD17, which recognized both the  $\beta 2$  and  $\beta 3$  subunits. Figure 4B shows that the 53 kDa  $\beta 2/3$ , 52 kDa  $\gamma 2$  and 50 kDa  $\delta$  subunits were detected in brain and adrenal medulla homogenates.

Immunoblotting and RT-PCR suggest that rat AM cells may express GABA<sub>A</sub> receptor subtypes including  $\alpha 3$  and  $\delta$  subunits other than the  $\alpha 1\gamma 2$  combination, the predominant subtype expressed in brain (Mckernan & Whiting, 1996). Thus, we examined with the immunoprecipitation technique whether the  $\alpha 3$  and  $\delta$  subunits constitute GABA<sub>A</sub> receptors with the  $\beta 2/3$ . As shown in Fig. 5A, the  $\alpha 1$ ,  $\alpha 3$  and  $\delta$  subunits were identified in the immunocomplexes precipitated with the anti- $\beta 2/3$  mAb, 62-3G1, from the lysates of brain and adrenal medulla, whereas an anti- $\delta$  Ab precipitated  $\beta 2/3$  subunits from the preparations. These results are consistent with possible

combinations of  $\alpha 1\beta 2/3\delta$  and  $\alpha 3\beta 2/3\delta$ . Thus, these possibilities were explored in immunocomplexes, which were precipitated with the anti- $\delta$  Ab and subjected to cross-linking. Immunoblotting with a secondary anti-rabbit IgG Ab alone did not produce any band, whereas that with the secondary Ab and either anti- $\alpha 1$  or anti- $\alpha 3$  Ab resulted in clear bands of 50–54 kDa. These results indicate that the  $\alpha 1\beta 2/3\delta$  and  $\alpha 3\beta 2/3\delta$  were expressed in the adrenal medulla. Figure 5C shows that immunostainings for  $\alpha 1$ ,  $\alpha 3$ ,  $\beta 2/3$  and  $\delta$  subunits were mainly localized at the cell periphery whereas part of materials immunoreactive to the anti- $\gamma 2$  Ab were present at the cell periphery, but the majority were distributed in a reticular manner in the cytoplasm, which resembles the distribution of the endoplasmic reticulum (ER) (Lin *et al.* 2005). In fact, in double staining the  $\gamma 2$ -like IR in the cytoplasm agreed well with the binding sites of BODIPY-FL-thapsigargin, which is known to be a marker of the ER (Endo *et al.* 2006) (not shown). Since  $\gamma$  and  $\delta$  subunits constitute GABA<sub>A</sub> receptors in a mutually exclusive manner (Araujo *et al.* 1998),



**Figure 3. The presence of  $\alpha 1$  and  $\alpha 3$  subunits of GABA<sub>A</sub> receptors in rat adrenal medulla**

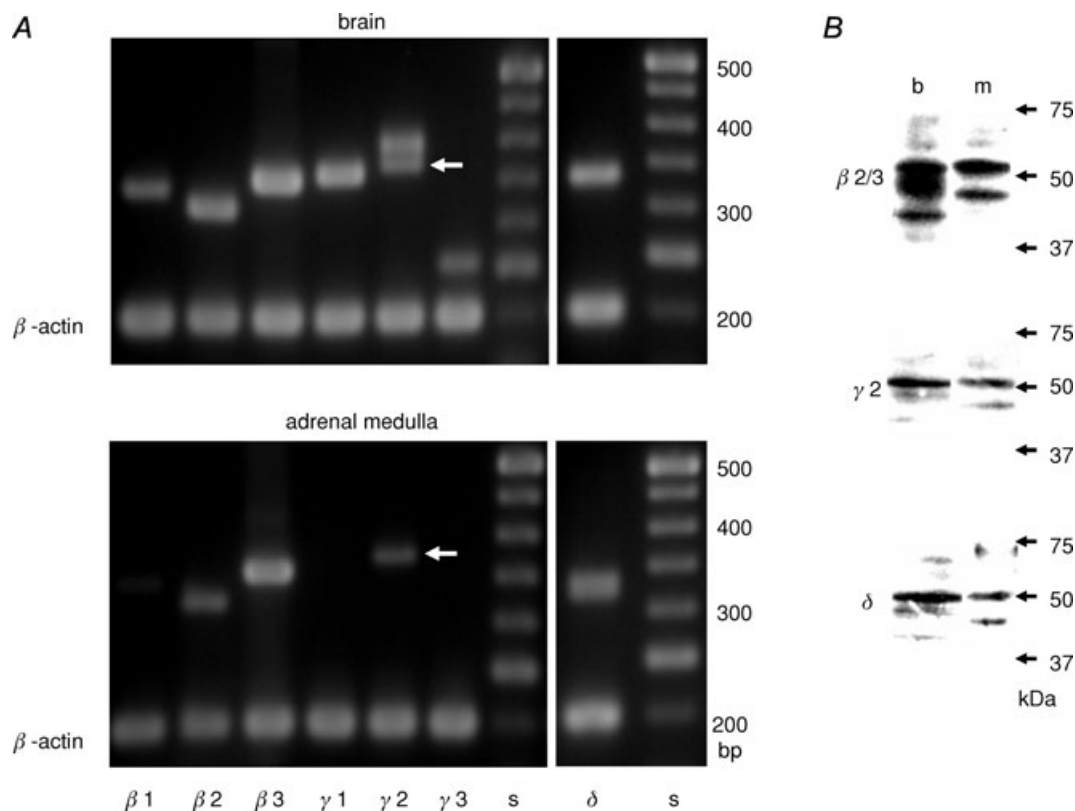
A, electrophoresis of PCR products of rat brain, adrenal medulla and adrenal cortex cDNAs for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and  $\alpha 6$  subunits of GABA<sub>A</sub> receptors and  $\beta$ -actin. PCR products of 304, 333, 351, 478, 338 and 348 bp for  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and  $\alpha 6$  subunits, respectively, were observed in the brain samples, whereas 304 and 351 bp for  $\alpha 1$  and  $\alpha 3$  subunits were observed in the adrenal medulla. None of the PCR products for the six  $\alpha$  subunits were detected in the adrenal cortex. The lower bands represent PCR products of 261 bp for  $\beta$ -actin. B, immunoblots for  $\alpha 1$  and  $\alpha 3$  subunits with and without pre-absorption of Abs by antigens. b, h, c and m stand for homogenates of rat brain, heart, adrenal cortex and adrenal medulla. Alomone-produced Abs were used with or without each antigen peptide. The same amount of proteins (6  $\mu$ g) was loaded for each lane.

immunocytochemical and immunoprecipitation experiments suggest that  $\gamma 2$ - and  $\delta$ -GABA<sub>A</sub> receptors are present in the plasma membrane of rat AM cells.

### Current recordings of GABA<sub>A</sub> receptor

The  $\alpha\beta\gamma$  and  $\alpha\beta\delta$  subtypes of GABA<sub>A</sub> receptors have been shown to differ in affinity for GABA and desensitization kinetics (Fisher & Macdonald, 1997). Therefore, the properties of GABA<sub>A</sub> receptors in AM cells were directly examined with the perforated patch clamp method. The whole-cell current was recorded at  $-70$  mV in an acutely dissociated rat AM cell. Bath application of  $30 \mu\text{M}$  GABA produced an inward current with an increase in current noise in 21 of 56 cells examined (38%), whereas that of  $30 \mu\text{M}$  baclofen, a GABA<sub>B</sub> receptor agonist, did not in any of seven cells (Fig. 6A). This GABA-induced current was reversibly suppressed by  $10 \mu\text{M}$  bicuculline, a GABA<sub>A</sub> receptor antagonist, and the GABA-induced current reversed in the direction at

about  $-40$  mV (not shown). These results clearly indicate that rat AM cells functionally express GABA<sub>A</sub> receptors. To estimate  $[\text{Cl}^-]_i$ , GABA-induced  $\text{Cl}^-$  currents (GABA  $I_{\text{Cl}}$ ) were recorded with gramicidin. The equilibrium potential for  $\text{Cl}^-$  ions ( $E_{\text{Cl}}$ ) determined under the two conditions where  $[\text{Cl}^-]$  in the pipette solution was 10 and 30 mM was  $-35.8 \pm 1.9$  mV ( $n = 5$ ) and  $-40.2 \pm 3.6$  mV ( $n = 6$ ), respectively, and there was no statistical difference between the two values (overall average,  $-38.2$  mV; estimated  $[\text{Cl}^-]_i$ , 33 mM). Figure 6B and C shows two examples of GABA  $I_{\text{Cl}}$  in two different AM cells: in Fig. 6B, GABA  $I_{\text{Cl}}$  did not increase in amplitude with an increase in GABA concentration from  $10 \mu\text{M}$  to  $30 \mu\text{M}$  and the current did not decline during the application, whereas in Fig. 6C GABA  $I_{\text{Cl}}$  increased markedly and showed a remarkable desensitization with increasing GABA concentrations. These results indicate that GABA<sub>A</sub> receptors are heterogeneous among the AM cells, as expected. Responses to several concentrations of GABA were examined in 15 cells, and GABA  $I_{\text{Cl}}$  was expressed as a fraction of the maximum of  $30 \mu\text{M}$  GABA-induced  $I_{\text{Cl}}$



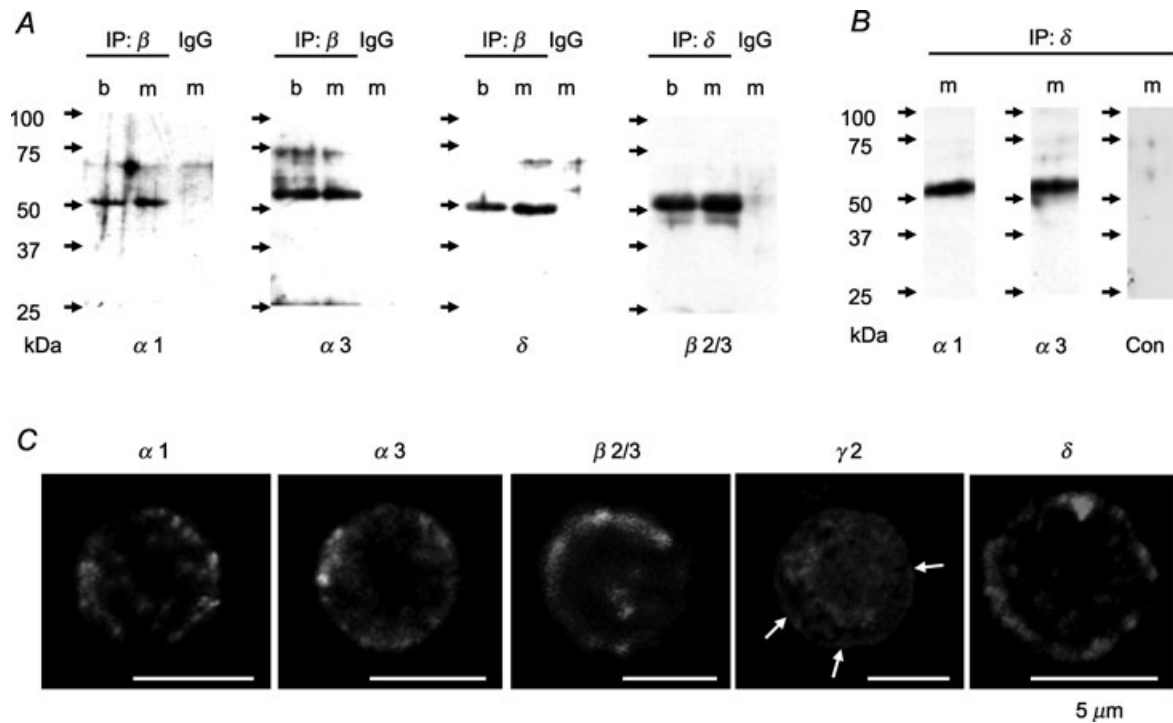
**Figure 4. The presence of  $\beta 2/3$ ,  $\gamma 2$  and  $\delta$  subunits in rat adrenal medulla**

A, electrophoresis of PCR products of rat brain and adrenal medulla cDNAs for  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 2$  (arrows),  $\gamma 2\text{L}$ ,  $\gamma 3$ , and  $\delta$  subunits and  $\beta$ -actin. PCR products of 341, 317, 355, 360, 374, 398, 255 and 333 bp for  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 1$ ,  $\gamma 2\text{S}$ ,  $\gamma 2\text{L}$ ,  $\gamma 3$  and  $\delta$  subunits, respectively, were observed in brain cDNAs, whereas  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2\text{S}$  and  $\delta$  PCR products were observed in adrenal medulla cDNAs. PCR products for  $\beta$ -actin are 198 bp. B, immunoblots for  $\beta 2/3$ ,  $\gamma 2$  and  $\delta$  subunits of homogenates of rat brain (b) and adrenal medulla (m). 53 kDa  $\beta 2/3$ , 52 kDa  $\gamma 2$  and 50 kDa  $\delta$  proteins were detected in brain and adrenal medulla by mouse anti- $\beta 2/3$  mAb (BD17), rabbit anti- $\gamma 2$  Ab and rabbit anti- $\delta$  Ab (Chemicon), respectively.



in the same cells (Fig. 6D). The continuous line in Fig. 6D represents a logistic equation with an  $EC_{50}$  of  $40 \mu M$  and a Hill coefficient of 1.4, which fits the set of data from  $30$  to  $300 \mu M$ , whereas the dashed line represents an equation with an  $EC_{50}$  of  $10 \mu M$  and a Hill coefficient of 2.0, which approximates the set of data from  $6$  to  $30 \mu M$ . The results suggest the presence of multiple  $GABA_A$  receptors with different affinities for GABA and desensitization kinetics in rat AM cells, possibly reflecting  $\delta$ - and  $\gamma$ - $GABA_A$  receptors. Therefore, the effects of  $10 \mu M$   $Zn^{2+}$  on GABA  $I_{Cl}$  were examined, since the  $\delta$  and  $\gamma$  isoforms were and were not susceptible to the inhibition by  $10 \mu M$   $Zn^{2+}$ , respectively (Nagaya & Macdonald, 2001; Hosie *et al.* 2003). Figure 7 demonstrates that the susceptibility to  $Zn^{2+}$  inhibition of  $GABA_A$  receptors was closely related to their apparent affinity for GABA. The cell shown in Fig. 7A exhibited a low susceptibility to  $10 \mu M$   $Zn^{2+}$  inhibition and a low affinity for GABA, because the  $10 \mu M$  GABA-induced  $I_{Cl}$  was about 18% of

the maximum of  $I_{Cl}$  evoked by  $30 \mu M$  GABA and the peak  $I_{Cl}$  in response to  $30 \mu M$  GABA was suppressed by 29% in the presence of  $10 \mu M$   $Zn^{2+}$ . On the other hand, the cell in Fig. 7B revealed a higher susceptibility to  $Zn^{2+}$  and a higher apparent affinity for GABA, because the  $10 \mu M$  GABA-induced  $I_{Cl}$  was about 67% of the maximum of  $30 \mu M$  GABA-induced  $I_{Cl}$  and addition of  $10 \mu M$   $Zn^{2+}$  produced the complete inhibition of  $30 \mu M$  GABA  $I_{Cl}$ . In Fig. 7C, the extent of inhibition of  $30 \mu M$  GABA-induced  $I_{Cl}$  by  $10 \mu M$   $Zn$  is plotted against the amplitude of  $10 \mu M$  GABA-induced  $I_{Cl}$  expressed as a fraction of  $30 \mu M$  GABA  $I_{Cl}$ . It is evident that the extent of  $Zn^{2+}$  inhibition became larger with an increase in relative amplitude of  $10 \mu M$  GABA-induced  $I_{Cl}$ . Therefore, as the apparent affinity of  $GABA_A$  receptors became higher, more  $GABA_A$   $I_{Cl}$  was inhibited by  $10 \mu M$   $Zn^{2+}$ . In eight cells the exposure to  $10 \mu M$   $Zn^{2+}$  resulted in a reversible inhibition of  $30 \mu M$  GABA-induced  $I_{Cl}$  by  $50.4 \pm 13.5\%$  (from 0 to 100%).



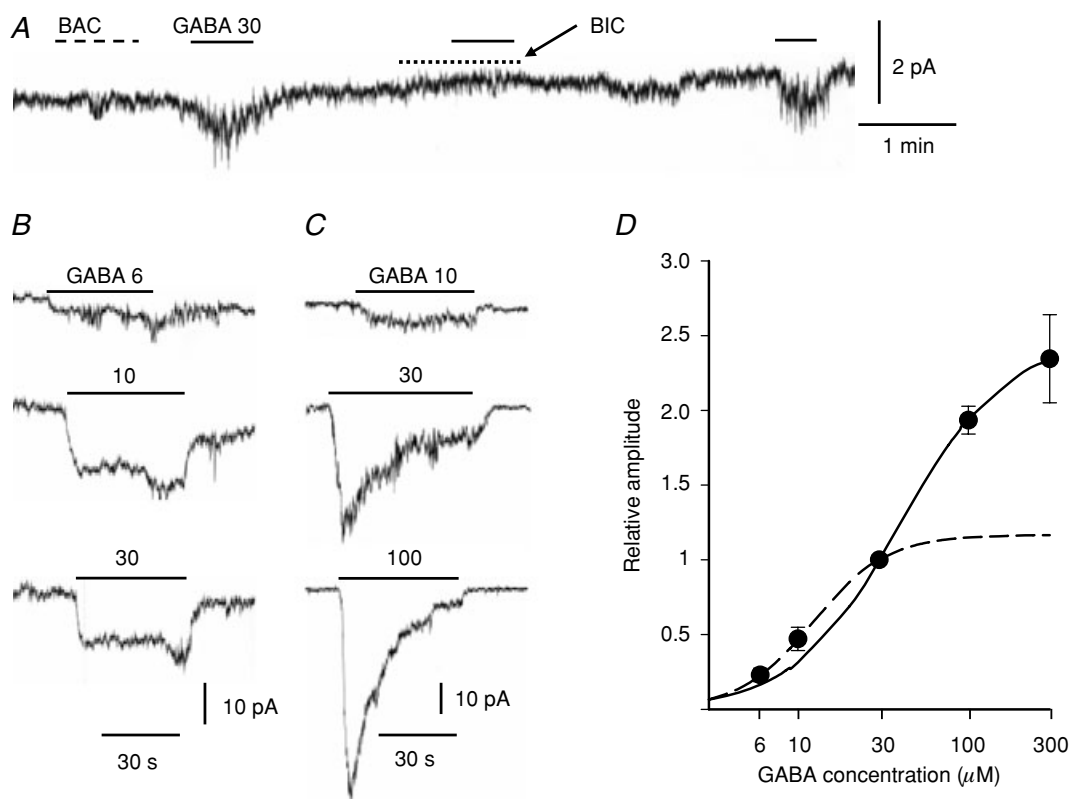
**Figure 5. Immunoprecipitation and immunocytochemistry for  $GABA_A$  receptors**

A, immunoblots for  $\alpha 1$ ,  $\alpha 3$ ,  $\delta$  and  $\beta 2/3$  of immunocomplexes precipitated with the mouse anti- $\beta 2/3$  mAb (62-3G1) (IP:  $\beta$ ), rabbit anti- $\delta$  Ab (Santa Cruz) (IP:  $\delta$ ), or non-immune rabbit IgG (IgG). Immunocomplexes were precipitated from lysates of rat brain (b) and adrenal medulla (m).  $\alpha 1$ ,  $\alpha 3$ ,  $\delta$  and  $\beta 2/3$  were detected by rabbit anti- $\alpha 1$  Ab, rabbit anti- $\alpha 3$  Ab (provided by W. Sieghart), rabbit anti- $\delta$  Ab (Chemicon), and 62-3G1, respectively. B, immunoblots for  $\alpha 1$  and  $\alpha 3$  of immunocomplexes precipitated with the anti- $\delta$  Ab. The immunoprecipitates from lysates of rat medulla (m) were subjected to cross-linking (see Methods). Note that the band of about 50 kDa was not detected with a secondary anti-rabbit IgG Ab alone (Con), indicating that heavy chains of IgG were not eluted from the immunoprecipitates. C, representative fluorescence images of immunostainings for  $\alpha 1$ ,  $\alpha 3$ ,  $\beta 2/3$ ,  $\gamma 2$  and  $\delta$  subunits in dissociated rat AM cells. Arrows indicate immunoreactivity at cell periphery. Immunostaining was repeated at least three times.

### Suppression of GAT expression

Immunohistochemistry suggests that GABA probably functions as a paracrine and not as an inhibitory neurotransmitter in the adrenal medulla. If that is the case, then GAT, which is involved in the termination of rapid inhibitory neurotransmission, may not be expressed in the adrenal medulla. There are four molecules involved in GABA transport into rat cells, and GAT1 and GAT3 are thought to be responsible for clearance of GABA in the vicinity of a GABAergic synapse (Dalby, 2003). Therefore, the expression levels of GAT1 and GAT3 were examined by RT-PCR (Fig. 8A, B and C). It is evident that GAT1 and GAT3 were clearly amplified with each specific set of primers in rat brain cDNA samples, whereas none of the GATs were evidently detected in the rat adrenal medulla. This finding was confirmed at the protein level:

neither GAT1 nor GAT3 was immunologically detected in the homogenates of rat adrenal medullae (Fig. 8D). To examine quantitatively the expression levels of the GATs, the summation of the relative amounts of PCR products for GAT1 and GAT3, which were expressed as fractions of signal levels for the  $\beta$ -actin, was compared with that of the relative amounts of PCR products for  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  subunits expressed similarly. The summation of the relative amounts of the  $\beta$  subunit PCR products would be assumed to parallel the total amount of GABA<sub>A</sub> receptors, since the majority of GABA<sub>A</sub> receptors contain one of the  $\beta$  subunits (McKernan & Whiting, 1996). Similarly, the summation of the relative amounts of the GAT PCR products may reflect the total amount of GATs. The ratio of the GAT to the  $\beta$  subunit PCR product amount in the rat adrenal medulla was conspicuously smaller than that in the brain. This quantitative analysis indicates that the



### Figure 6. Dose dependence of GABA<sub>A</sub> receptor Cl<sup>-</sup> channel activation

A, recording of whole-cell current at  $-70$  mV with the nystatin method. Agents ( $30 \mu\text{M}$  baclofen (BAC),  $30 \mu\text{M}$  GABA and  $10 \mu\text{M}$  bicuculline (BIC)) were bath applied during the indicated periods. B and C, recording traces of whole-cell currents in response to GABA at several concentrations. B and C were recorded at  $-70$  mV from different AM cells with the nystatin method. GABA was bath applied during the periods indicated by the bars (values in  $\mu\text{M}$ ). D, peak amplitudes of GABA-induced  $I_{\text{Cl}}$  are plotted against GABA concentrations. Peak  $I_{\text{Cl}}$ s in response to GABA at several concentrations were expressed as fractions of peak  $I_{\text{Cl}}$ s evoked by  $30 \mu\text{M}$  GABA in the same AM cells (see Methods). The line represents the logistic equation with an  $\text{EC}_{50}$  of  $39.49 \mu\text{M}$ , a Hill coefficient of 1.4, and  $I_{\text{max}}$  of 2.47, a equation which best approximated a set of data from 30 to  $300 \mu\text{M}$ . The dashed line represents the logistic equation with an  $\text{EC}_{50}$  of  $12.25 \mu\text{M}$ , Hill coefficient of 2.0, and  $I_{\text{max}}$  of 1.17, a equation which was obtained in the best fitting of a set of data from 6 to  $30 \mu\text{M}$ . Data represent mean  $\pm$  S.E.M. (4–15 observations for each point).

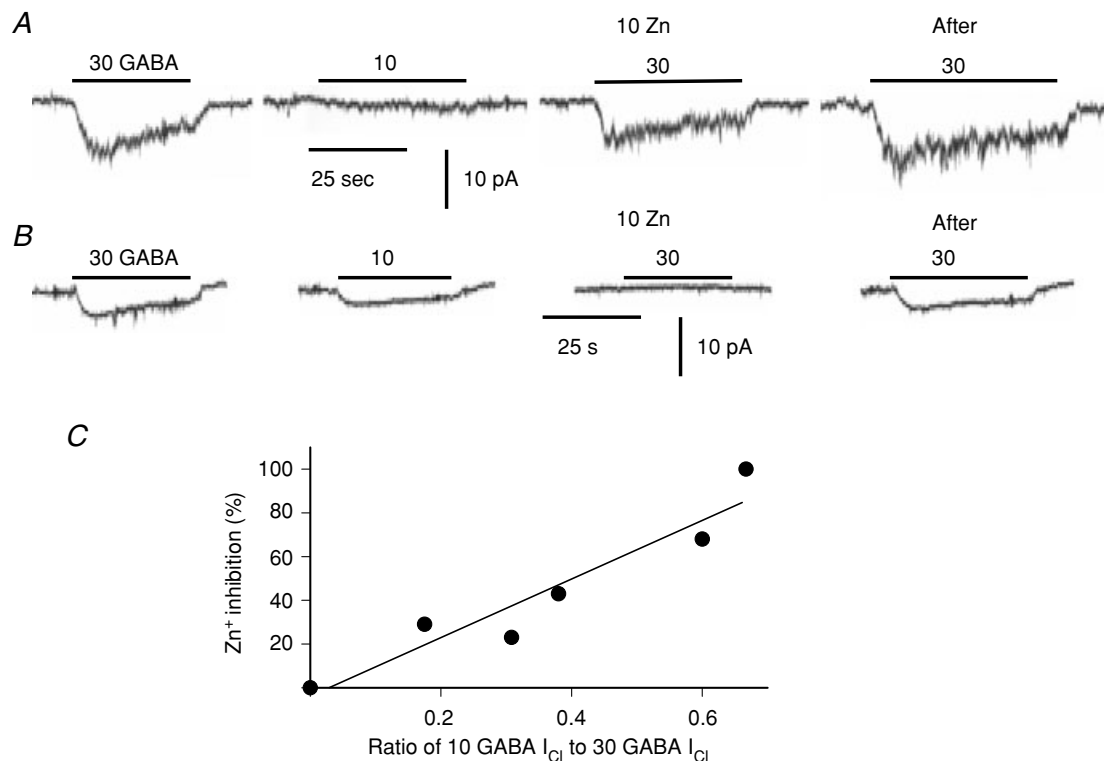
expression of GAT is clearly suppressed in the adrenal medulla, compared with the amount of GABA<sub>A</sub> receptors, which receive the GABA signal.

### Effect of GABA on trans-synaptically evoked excitation

Although GABA has been shown to induce catecholamine secretion in various species of mammals, the effects of GABA on secretion evoked trans-synaptically or by secretagogues have been controversial. When the rat adrenal medulla loaded with fluo-4 AM was electrically stimulated at 10 Hz with a 60 V pulse of 1.5 ms duration for 30 s, nerve fibres in the preparation were excited and consequently the majority of AM cells were activated trans-synaptically (Fig. 9A). On the other hand, when 30  $\mu$ M GABA was applied retrogradely via the adrenal vein, part ( $41.1 \pm 5.5\%$ ,  $n = 5$ ) of the AM cells, which were responsive to the electrical stimulation, showed an increase in fluorescence (compare the responses in the areas y and x in Fig. 9A). Figure 9B clearly shows that application of GABA suppressed the amplitude of the Ca<sup>2+</sup>

response evoked by the electrical stimulation in AM cells responsive to GABA (x in Fig. 9B), but not in unresponsive cells (y in Fig. 9B). Furthermore, the overall amplitude of Ca<sup>2+</sup> response comprising the response to the electrical stimulation and that to GABA was smaller than that of Ca<sup>2+</sup> signal in response to the electrical stimulation alone. These effects of GABA were concluded to be mediated by GABA<sub>A</sub>, but not GABA<sub>B</sub> receptors, because application of 30  $\mu$ M baclofen did not have any effect on the resting or trans-synaptically evoked Ca<sup>2+</sup> signal ( $n = 3$ ; not shown). The results suggest that GABA<sub>A</sub> receptor stimulation diminishes the total amount of catecholamine secretion evoked by the electrical stimulation and GABA, even though GABA has a facilitatory effect on secretion. This notion was confirmed by an on-line measurement of catecholamine secretion evoked by electrical stimulation with or without application of 20  $\mu$ M GABA (see online Supplementary figure).

We next examined whether this inhibitory effect of GABA was due to a shunt effect or not (Fig. 10). The electrical stimulation during the GABA application was found to result in smaller Ca<sup>2+</sup> signals (Fig. 10A). The



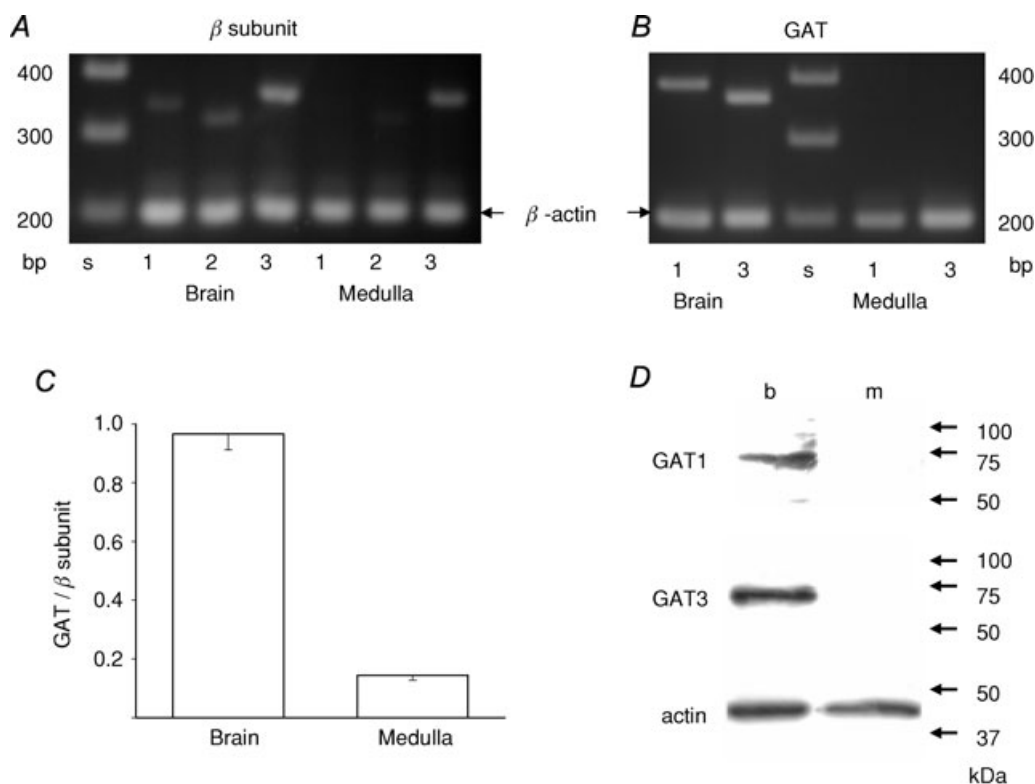
**Figure 7. Zn<sup>2+</sup> inhibition of GABA<sub>A</sub> receptor I<sub>Cl</sub>**

A and B, current traces of whole-cell recordings at  $-70$  mV in different AM cells. GABA at 10 or 30  $\mu$ M was bath applied during the periods indicated by the bars in the presence (10 Zn) and absence of 10  $\mu$ M Zn<sup>2+</sup> ions. Note that 30  $\mu$ M GABA-induced I<sub>Cl</sub> was restored after washout of 10  $\mu$ M Zn<sup>2+</sup> (After). C, the extent of Zn<sup>2+</sup> inhibition of 30  $\mu$ M GABA-induced I<sub>Cl</sub> is plotted against ratio of 10  $\mu$ M GABA-induced I<sub>Cl</sub> to 30  $\mu$ M GABA-induced I<sub>Cl</sub>. The peak amplitudes of currents in response to 10 and 30  $\mu$ M GABA were measured in the same cells. The line represents a regression line ( $r = 0.9505$ ).

extent of reduction of the  $\text{Ca}^{2+}$  signal in response to electrical stimulation depended on the amplitude of GABA-induced  $\text{Ca}^{2+}$  signals at the electrical stimulation, but not on the frequency of stimulation (Fig. 10C). As the amplitude of GABA-induced signals increased, more of the  $\text{Ca}^{2+}$  signals evoked by the electrical stimulation were suppressed. On the other hand,  $\text{Ca}^{2+}$  signals in response to electrical stimulation, especially at low frequencies (0.5 and 1 Hz), did not diminish apparently during application of  $30 \mu\text{M}$  muscarine, although the peak of the muscarine-induced  $\text{Ca}^{2+}$  signal (Inoue *et al.* 2008) was about 67% larger than the GABA-induced signal (Fig. 10B). Figure 10D represents a summary of decreases in the  $\text{Ca}^{2+}$  signal in response to the electrical stimulation at 0.5 Hz to 10 Hz during the application of GABA or muscarine. The extent ( $38.5 \pm 3.8\%$ ) of decreases in the  $\text{Ca}^{2+}$  signal in response to electrical stimulations in the presence of GABA was significantly larger than that ( $18.8 \pm 3.5\%$ ) in the presence of muscarine, although the peak amplitude of GABA-induced  $\text{Ca}^{2+}$  signals was about 64% of that of muscarine-induced  $\text{Ca}^{2+}$  signals. These

results indicate that the GABA-induced decline in  $\text{Ca}^{2+}$  signal increase evoked by electrical stimulation may be mainly attributed to the shunt effect, but not to other factors, such as the saturation of  $\text{Ca}^{2+}$  signals.

The next issue is how the overall  $\text{Ca}^{2+}$  response is altered by the strength of electrical stimulation. If GABA-induced  $\text{Ca}^{2+}$  responses exceed the decrease due to the shunt effect, the overall amplitude of the  $\text{Ca}^{2+}$  signal comprising a  $\text{Ca}^{2+}$  response to electrical stimulation and that to GABA will be greater than the amplitude of  $\text{Ca}^{2+}$  signal to electrical stimulation alone. Figure 10E demonstrates that our notion is the case: the overall amplitude of  $\text{Ca}^{2+}$  responses to GABA and the electrical stimulation at 0.5 and 1 Hz, but not at 5 or 10 Hz, were greater than that of  $\text{Ca}^{2+}$  responses to the electrical stimulation alone. These results clearly indicate that GABA produces facilitatory effects on the  $\text{Ca}^{2+}$  signal in rat AM cells in response to GABA and the electrical stimulation at low frequencies, whereas it induces inhibition of the  $\text{Ca}^{2+}$  signal evoked by GABA and high frequency stimulation.



**Figure 8. Absence of GAT expression in rat adrenal medulla**

A, electrophoresis of the PCR products of rat brain and adrenal medulla cDNAs for  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  subunits, and  $\beta$ -actin. B, electrophoresis of PCR products for GATs and  $\beta$ -actin. C, summary of ratios of totals of relative amounts of PCR products for GAT1 and GAT3 to those of relative amounts of PCR products for  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  subunits. The amounts of PCR products for GATs and  $\beta$  subunits were expressed as fractions of the amounts of PCR products for  $\beta$ -actin (see Methods). Data represent mean  $\pm$  s.e.m. ( $n = 3$ ). D, immunoblot for GAT1 and GAT3 of homogenates of rat brain (b) and adrenal medulla (m). Immunoblotting for actin was performed to confirm that the same amount of proteins ( $6 \mu\text{g}$ ) was loaded for each lane.

## Discussion

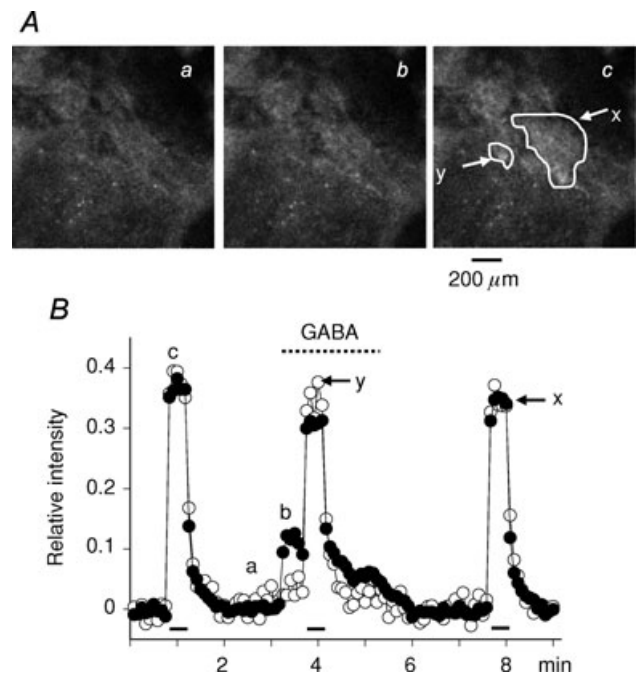
### Origin of GABA in the adrenal medulla

In mouse (Iwasa *et al.* 1998) and canine adrenal medulla (Kataoka *et al.* 1986) nerve fibre-like structures and AM cells were stained with anti-GABA Abs. On the other hand, AC cells, but not AM cells, in human and rat adrenal glands and a human AC cell line were stained with an anti-GAD Ab and anti-VGAT Ab; however, the specificity of the antibodies used had not been examined in details (Metzeler *et al.* 2004). In the present experiment, a PCR product for GAD67 was detected in the adrenal medulla cDNAs, but not in the adrenal cortex, and that for GAD65 was not amplified in either the medulla or the cortex. Furthermore, immunohistochemistry with the anti-GAD Ab, whose specificity was elucidated in immunoblotting, revealed that in the rat adrenal gland, AM, but not AC, cells were immunoreactive to the anti-GAD Ab, and nerve fibre-like structures or nerve terminals surrounding AM cells were not stained with the Ab. The anti-VGAT Ab used identified the VGAT of about 60 kDa in immunoblots of homogenates of the rat adrenal medulla, but not the adrenal cortex. The VGAT-like IR was localized in immunologically identified chromaffin granules, but not SLMVs (Cutler & Cramer, 1990; Annaert *et al.* 1993). This immunocytochemical staining contrasts with the finding that GABA is stored in SLMVs in pancreatic  $\beta$  cells (Thomas-Reetz & De Camilli, 1994). RT-PCR, immunoblotting and immunochemistry for GAD and VGAT all indicate that there is no GABAergic input in the adrenal medulla and GABA is produced and stored in AM cells. This idea is consistent with the finding that adrenaline secretion in a perfused rat adrenal gland in response to electrical stimulation was almost completely suppressed by  $100 \mu\text{M}$  hexamethonium, a nicotinic ACh receptor antagonist (Nagayama *et al.* 1999). In cultured bovine AM cells, GAD was immunodetected in 30% of the adrenaline cells, whereas all of the dissociated rat AM cells were found to have GAD-like immunoreactivity and the section of adrenal medulla exhibited a diffuse staining for GAD. Thus, GABA may be produced in the majority of rat AM cells, as differs from the case in the bovine AM cells.

### Subunit composition of GABA<sub>A</sub> receptor

The results obtained with RT-PCR and immunological methods clearly revealed that rat AM cells express  $\alpha 3$ ,  $\beta 2/3$  and  $\delta$  subunits in addition to the  $\alpha 1$  and  $\gamma 2$  reported in bovine adrenal medullae (Parramón *et al.* 1994). Since  $\gamma 2$  and  $\delta$  subunits have been known to constitute GABA<sub>A</sub> receptors in a mutually exclusive manner (Shivers *et al.* 1989; Mckernan & Whiting, 1996; Araujo *et al.* 1998), these results suggest the expression of  $\delta$ - and  $\gamma 2$ -GABA<sub>A</sub> receptors in rat AM cells. As compared to

$\gamma$ -GABA<sub>A</sub> receptors,  $\delta$ -GABA<sub>A</sub> receptors have a higher affinity for GABA (Quirk *et al.* 1995), desensitize to a smaller extent, and are highly sensitive to  $\text{Zn}^{2+}$  inhibition (Saxena & Macdonald, 1994, 1996; Fisher & Macdonald, 1997; Nagaya & Macdonald, 2001). The whole-cell current experiment revealed that the higher the affinity for GABA is, more GABA  $I_{\text{Cl}}$  is suppressed by  $10 \mu\text{M}$   $\text{Zn}^{2+}$ . This finding may not be accounted for by the expression of  $\alpha\beta$  combination without the  $\gamma 2$  subunit (Hosie *et al.* 2003), because the  $\alpha 1\beta 3$  subtype expressed in HEK cells has an  $\text{EC}_{50}$  of  $3 \mu\text{M}$  for GABA (Wohlfarth *et al.* 2002; Hosie *et al.* 2003), a value which is much smaller than the  $\text{EC}_{50}$  of  $10$ – $40 \mu\text{M}$  found in rat AM cells. This difference in  $\text{EC}_{50}$  suggests that  $\delta$ -GABA<sub>A</sub> receptors, but not the  $\alpha\beta$  combination, are responsible for the inhibition of GABA  $I_{\text{Cl}}$  by  $10 \mu\text{M}$   $\text{Zn}^{2+}$ . The fact that  $\delta$ -GABA<sub>A</sub> receptors are predominantly or exclusively localized at the extrasynaptic



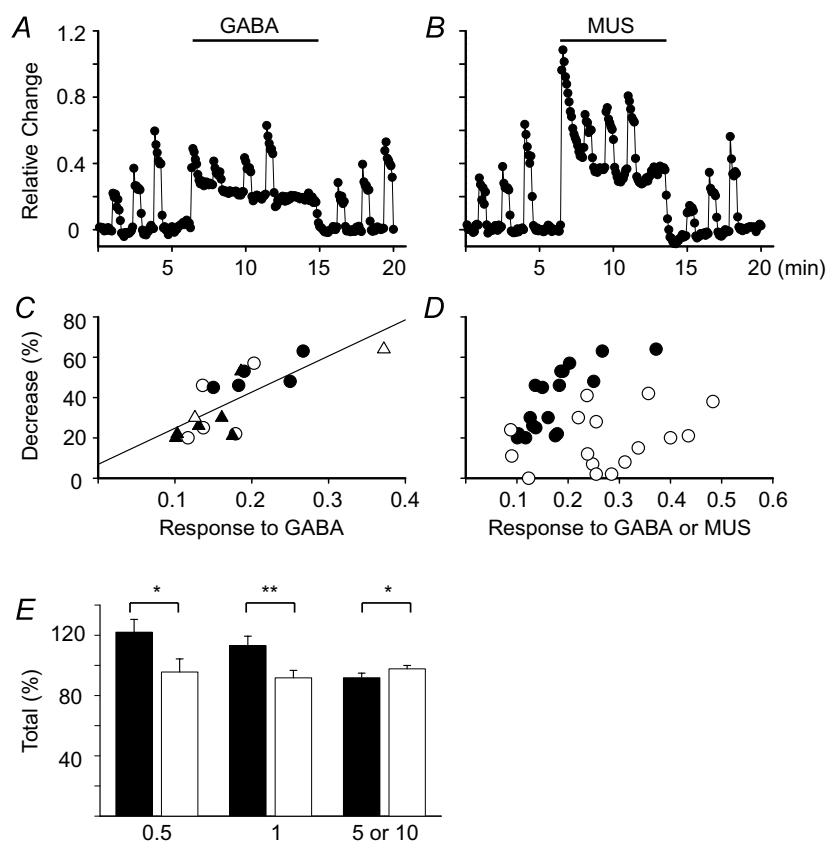
**Figure 9. Inhibitory effects of GABA on trans-synaptically evoked  $\text{Ca}^{2+}$  response in rat AM cells**

A, confocal images of fluo-4 fluorescence in rat adrenal medulla. The adrenal gland was retrogradely perfused through the adrenal vein with saline (see Methods). GABA at  $30 \mu\text{M}$  was added to the perfusion solution during the indicated period (interrupted line). Nerve fibres remaining in the gland were electrically stimulated with 60 V pulses of 1.5 ms duration at 10 Hz for 30 s during the indicated periods (bars). The adrenal medulla was illuminated with 488 laser and emission of above 510 nm was observed every 5 s. B, relative values of change in fluorescence intensity in the presence and absence of GABA are plotted against time. Fluorescence intensities in the areas (x and y) indicated in A were measured and presented as filled (x) and open (y) symbols, respectively. After correction for the decline due to photobleaching, an increase in fluorescence intensity in response to electrical stimulation and GABA was expressed as a fraction of the resting level (see Methods). a, b and c in A correspond to a, b and c in B.

site in CG and DGG cells also favours the notion that AM cells lacking the GABAergic synapse express  $\delta$ -GABA<sub>A</sub> receptors.

The  $\delta$ ,  $\alpha 1$  and  $\alpha 3$  subunits were identified in immunocomplexes precipitated with an anti- $\beta 2/3$  mAb, whereas

the  $\alpha 1$  and  $\alpha 3$  subunits were detected in immunoprecipitates with an anti- $\delta$  Ab. These results indicate that the  $\delta$  subunit constitutes GABA<sub>A</sub> receptors with  $\alpha 1$ ,  $\alpha 3$  or both subunits in rat AM cells. It has recently been reported that the  $\delta$  subunit forms a complex with the



**Figure 10.** Effects of GABA and muscarine on  $\text{Ca}^{2+}$  response to electrical stimulation at various frequencies in rat adrenal medulla

A and B, relative values of change in fluorescence intensity in the presence and absence of  $30 \mu\text{M}$  GABA and muscarine (MUS) are plotted against time, respectively. Changes in fluorescence intensity in several areas were calculated in the same manner as that explained for Fig. 9. Nerve fibres remaining in the gland were electrically stimulated with 60 V pulses of 1.5 ms duration at 0.5, 1 and 5 Hz for 30 s in sequence before, during and after drug application. A and B were obtained from the same preparation. C, extents of decrease in  $\text{Ca}^{2+}$  response to electrical stimulation are plotted against values of responses to  $30 \mu\text{M}$  GABA just before electrical stimulation. Electrical stimulation was applied at 0.5 ( $\circ$ ), 1 ( $\bullet$ ), 5 ( $\blacktriangle$ ) and 10 Hz ( $\triangle$ ). The line represents a regression line ( $r = 0.8241$ ). D, extent of decrease in  $\text{Ca}^{2+}$  response to electrical stimulation at 0.5–10 Hz during application of GABA ( $\bullet$ ,  $n = 6$  preparations) or muscarine ( $\circ$ ,  $n = 7$ ) are plotted against values of response to GABA or muscarine just before electrical stimulation. E, summary of overall  $\text{Ca}^{2+}$  responses to electrical stimulation with and without GABA application. The filled columns represent the maximum of overall  $\text{Ca}^{2+}$  response to electrical stimulation and GABA. The open columns represent the maximum of putative  $\text{Ca}^{2+}$  response to the electrical stimulation without GABA, a value which is estimated by averaging the maximums of the electrically evoked  $\text{Ca}^{2+}$  responses before and after GABA application. The maximums of such  $\text{Ca}^{2+}$  responses with and without GABA application are expressed as a fraction of  $\text{Ca}^{2+}$  response to electrical stimulation at each frequency before GABA application. \* and \*\* represent statistical significance (Student's paired *t* test) of  $P < 0.05$  and  $P < 0.005$ , respectively. Data represent means  $\pm$  S.E.M. ( $n = 6$  for 0.5 Hz;  $n = 7$ , for 1 Hz;  $n = 8$ , for 5 or 10 Hz).

$\alpha 1$  in dentate gyrus interneurons (Glykys *et al.* 2007). On the other hand, the deletion of  $\alpha 6$  subunits in mice results in a decrease in  $\delta$  subunit expression in cerebellum without facilitating  $\alpha 1\delta$  combination (Jones *et al.* 1997), even though the  $\alpha 1$  subunit is expressed at subsynaptic sites of CG cells (Nusser *et al.* 1998). The finding that the recombinant  $\alpha 1\beta 3\delta$  subtype has an  $EC_{50}$  of 2–6  $\mu M$  for GABA (Fisher & Macdonald, 1997; Wohlfarth *et al.* 2002) might not be compatible with the dose dependence of  $I_{Cl}$  evoked by low concentrations of GABA in rat AM cells, whereas the dose dependence of  $I_{Cl}$  evoked by high concentrations of GABA is consistent with the property of the recombinant  $\alpha 3\beta 3\gamma 2$ , but not  $\alpha 1\beta 3\gamma 2$ , subtype expressed in HEK cells: the  $EC_{50}$ s for GABA in the former and latter have been found to be 48 and 3  $\mu M$ , respectively (Böhme *et al.* 2004).

The  $IC_{50}$ s for  $Zn^{2+}$  of the recombinant  $\alpha 1\beta 3\gamma 2$  and  $\alpha 1\beta 3\delta$  subtypes have been reported to be 250 and 5  $\mu M$ , respectively. On the other hand, as far as we know, there are no experiments in which the sensitivity to  $Zn^{2+}$  inhibition in the recombinant  $\alpha 3\beta 2/3\delta$  was examined. Since the  $IC_{50}$  for  $Zn^{2+}$  of the  $\alpha 1\beta 3\gamma 2$  is 250  $\mu M$ , 10  $\mu M$   $Zn^{2+}$  ions are not expected to produce a noticeable inhibition of the  $\alpha 1\beta 3\gamma 2$ -mediated  $I_{Cl}$ . As explained before, the  $\alpha 1\beta 3$  subtype, which has a high sensitivity to  $Zn^{2+}$ , may not contribute significantly to the production of  $I_{Cl}$  in response to 30  $\mu M$  GABA. The present result that 10  $\mu M$   $Zn^{2+}$  ions induced 50% inhibition of 30  $\mu M$  GABA-induced  $I_{Cl}$ , taken together with the findings in the literature, raises the possibility that  $\delta$ -GABA<sub>A</sub> receptors contribute to as much as 50% of 30  $\mu M$  GABA-induced  $I_{Cl}$ . We will need a further study to clarify to what extent  $\delta$ -GABA<sub>A</sub> receptors are involved in production of  $I_{Cl}$  in response to GABA.

The expression of  $\delta$ -GABA<sub>A</sub> receptors in AM cells is important in the functional point of view. Adrenal cortical cells in zona reticularis have been reported to secrete neurosteroids, such as 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (allopregnanolone) (Holzbauer *et al.* 1985; Endoh *et al.* 1996; Mellon & Griffin, 2002). Therefore, AM cells, where GABA  $I_{Cl}$  has been shown to be markedly enhanced by allopregnanolone (Peters *et al.* 1989), might receive humoral information from adrenal cortical cells via  $\delta$ -GABA<sub>A</sub> receptors, which are highly sensitive to neurosteroids (Mihalek *et al.* 1999; Belelli *et al.* 2002; Belelli & Lambert, 2005). The expression level of  $\delta$  subunit in brain has been reported to change in the menstrual cycle in mice (Maguire *et al.* 2005): the expression level of the  $\delta$  subunit increases in late dioestrus (high progesterone phase), whereas that of the  $\gamma 2$  decreases. This circular change in  $\delta$  expression has been assumed to be a cause for premenstrual syndrome, which is associated with not only mental symptoms, such as irritability, but also physical symptoms, such as headache. The expression of

$\delta$ -GABA<sub>A</sub> receptors in AM cells might account in part for such physical symptoms. The activity of  $\delta$ -GABA<sub>A</sub> receptors is also known to be enhanced by ethanol at concentrations that are reached with moderate, social ethanol consumption (Wallner *et al.* 2003; Wei *et al.* 2004), and thus they may be involved in adrenaline secretion in response to ethanol intake in mammals including humans (Adachi & Mizoi, 1983; Thiagarajan *et al.* 1989). Finally, it would be worth noting that the  $\alpha 3$  subunit, which is implicated in the anxiolytic effects of benzodiazepine (Dias *et al.* 2005) is predominantly or significantly expressed in not only AM cells, but also brain catecholaminergic neurones (Luque *et al.* 1994; Guyon *et al.* 1999).

### Function of GABA in adrenal medulla

Three lines of evidence support the idea that GABA functions as a paracrine in the rat adrenal medulla: (i) GABA is produced and stored in AM cells; (ii) AM cells express  $\delta$ -GABA<sub>A</sub> receptors; (iii) the expression of GATs, which are responsible for the termination of the inhibitory synaptic transmission (Farrant & Nusser, 2005), is suppressed. What, then, is the physiological role for GABA in the adrenal medulla? The experiment with the perfused adrenal medulla clearly indicates that GABA has an inhibitory effect on trans-synaptically evoked increase in excitability in AM cells. This inhibition may be due to the shunt effect of GABA on membrane excitability of AM cells (Rudomin & Schmidt, 1999), but not to other factors. First, the  $Ca^{2+}$  signals induced with electrical stimulation in the presence of GABA diminished more conspicuously than that in the presence of muscarine, although the GABA-induced  $Ca^{2+}$  signals were smaller than the muscarine-induced ones. This result indicates that the GABA-induced decrease in the trans-synaptically induced  $Ca^{2+}$  signal was not due to saturation of the  $Ca^{2+}$  signal. Second, the diminution of  $Ca^{2+}$  signal in response to electrical stimulation depended on the amplitude of the  $Ca^{2+}$  signal evoked by GABA, but not on the frequency of electrical stimulations, suggesting that the decrease is due to the shunt effect in AM cells, but not to a pre-synaptic mechanism, such as GABA<sub>B</sub> receptor-mediated inhibition (Inoue *et al.* 1985a). In addition to this inhibitory action of GABA on neuronally evoked increase in excitability, GABA itself produces depolarization in AM cells with the consequent increase in  $[Ca^{2+}]_i$ . The present experiment showed that this direct action of GABA surpassed the shunt effect on the increase in  $[Ca^{2+}]_i$  in response to electrical stimulation at a low frequency. Thus, the overall effect of GABA on excitability in AM cells was facilitatory when AM cells were trans-synaptically stimulated at a low frequency. When

cells were stimulated at a high frequency, however, the inhibition due to the shunting effect became dominant and thus GABA functioned as a brake on the secretion. The present findings extend the previous results on nicotine-induced increase in  $[Ca^{2+}]_i$  in bovine AM cells (González *et al.* 1992) and unambiguously resolve the controversy over the function of GABA in AM cells: the overall action of GABA depends upon the extent of the trans-synaptically evoked increase in excitability in AM cells, and thus GABA plays a modulatory role for the catecholamine secretion.

## References

- Achilles K, Okabe A, Ikeda M, Shimizu-Okabe C, Yamada J, Fukuda A, Luhmann HJ & Kilb W (2007). Kinetic properties of  $Cl^-$  uptake mediated by  $Na^+$ -dependent  $K^+-2Cl^-$  cotransport in immature rat neocortical neurons. *J Neurosci* **27**, 8616–8627.
- Adachi J & Mizoi Y (1983). Acetaldehyde-mediated alcohol sensitivity and elevation of plasma catecholamine in man. *Jpn J Pharmacol* **33**, 531–539.
- Akinci MK & Schofield PR (1999). Widespread expression of GABA<sub>A</sub> receptor subunits in peripheral tissues. *Neurosci Res* **35**, 145–153.
- Annaert WG, Llona I, Backer AC, Jacob WA & De Potter WP (1993). Catecholamines are present in a synaptic-like microvesicle-enriched fraction from bovine adrenal medulla. *J Neurochem* **60**, 1746–1754.
- Araujo F, Ruano D & Vitorica J (1998). Absence of association between  $\delta$  and  $\gamma 2$  subunits in native GABA<sub>A</sub> receptors from rat brain. *Eur J Pharmacol* **347**, 347–353.
- Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN & Langer SZ (1998). International Union of Pharmacology. XV. Subtypes of  $\gamma$ -aminobutyric acid<sub>A</sub> receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50**, 291–313.
- Belelli D, Casula A, Ling A & Lambert JJ (2002). The influence of subunit composition on the interaction of neurosteroids with GABA<sub>A</sub> receptors. *Neuropharmacology* **43**, 651–661.
- Belelli D & Lambert JJ (2005). Neurosteroids: endogenous regulators of the GABA<sub>A</sub> receptor. *Nat Rev Neurosci* **6**, 565–575.
- Bianchi MT & Macdonald RL (2003). Neurosteroids shift partial agonist activation of GABA<sub>A</sub> receptor channels from low- to high-efficacy gating patterns. *J Neurosci* **26**, 10934–10943.
- Blein S, Hawrot E & Barlow P (2000). The metabotropic GABA receptor: molecular insights and their functional consequences. *Cell Mol Life Sci* **57**, 635–650.
- Böhme I, Rabe H & Lüddens H (2004). Four amino acids in the  $\alpha$  subunits determine the  $\gamma$ -aminobutyric acids sensitivities of GABA<sub>A</sub> receptor subtypes. *J Biol Chem* **279**, 35193–35200.
- Castro E, González MP & Oset-Gasque MJ (2003). Distribution of  $\gamma$ -aminobutyric acid receptors in cultured adrenergic and noradrenergic bovine chromaffin cells. *J Neurosci Res* **71**, 375–382.
- Chessler SD, Simonson WT, Sweet IR & Hammerle LP (2002). Expression of the vesicular inhibitory amino acid transporter in pancreatic islet cells: distribution of the transporter within rat islets. *Diabetes* **51**, 1763–1771.
- Cutler DF & Cramer LP (1990). Sorting during transport to the surface of PC12 cells: divergence of synaptic vesicle and secretory granule proteins. *J Cell Biol* **110**, 721–730.
- Dalby NO (2003). Inhibition of  $\gamma$ -aminobutyric acid uptake: anatomy, physiology and effects against epileptic seizures. *Eur J Pharmacol* **479**, 127–137.
- Dias R, Sheppard WFA, Fradley RL, Garrett EM, Stanley JL, Tye SJ *et al.* (2005). Evidence for a significant role of  $\alpha 3$ -containing GABA<sub>A</sub> receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* **25**, 10682–10688.
- Ebihara S, Obata K & Yanagawa Y (2003). Mouse vesicular GABA transporter gene: genomic organization, transcriptional regulation and chromosomal localization. *Brain Res Mol Brain Res* **110**, 126–139.
- Endo Y, Harada K, Fujishiro N, Funahashi H, Shioda S, Prestwich GD, Mikoshiba K & Inoue M (2006). Organelles containing inositol trisphosphate receptor type 2 in adrenal medullary cells. *J Physiol Sci* **56**, 415–423.
- Endoh A, Kristiansen SB, Casson PR, Buster JE & Hornsby PJ (1996). The zona reticularis is the site of biosynthesis of dehydroepiandrosterone and dehydroepiandrosterone sulfate in the adult human adrenal cortex resulting from its low expression of  $3\beta$ -hydroxysteroid dehydrogenase. *J Clin Endocrinol* **81**, 3558–3565.
- Farrant M & Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA<sub>A</sub> receptor. *Nat Rev Neurosci* **6**, 215–229.
- Fisher JL & Macdonald RL (1997). Single channel properties of recombinant GABA<sub>A</sub> receptors containing  $\gamma 2$  or  $\delta$  subtypes expressed with  $\alpha 1$  and  $\beta 3$  subtypes in mouse L929 cells. *J Physiol* **505**, 283–297.
- Geigerseder C, Doepner R, Thalhammer A, Frungieri MB, Gamel-Didelon K, Calandra RS, Köhn F & Mayerhofer A (2003). Evidence for a GABAergic system in rodent and human testis: local GABA production and GABA receptors. *Neuroendocrinology* **77**, 314–323.
- Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR & Mody I (2007). A new naturally occurring GABA<sub>A</sub> receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci* **10**, 40–48.
- González MP, Oset-Gasque MJ, Castro E, Bugada J, Arce C & Parramón M (1992). Mechanism through which GABA<sub>A</sub> receptor modulates catecholamine secretion from bovine chromaffin cells. *Neuroscience* **47**, 487–494.
- Guyon A, Laurent S, Paupardin-Tritsch D, Rossier J & Eugène D (1999). Incremental conductance levels of GABA<sub>A</sub> receptors in dopaminergic neurones of the rat substantia nigra pars compacta. *J Physiol* **516**, 719–737.
- Holzbauer M, Birmingham MK, De Nicola AF & Oliver JT (1985). *In vivo* secretion of  $3\alpha$ -hydroxy- $5\alpha$ -pregnan-20-one, a potent anaesthetic steroid, by the adrenal gland of the rat. *J Steroid Biochem* **22**, 97–102.
- Hosie AM, Dunne EL, Harvey RJ & Smart TG (2003). Zinc-mediated inhibition of GABA<sub>A</sub> receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* **6**, 362–369.



- Inoue M, Fujishiro N, Ogawa K, Muroi M, Sakamoto Y, Imanaga I & Shioda S (2000). Pituitary adenylate cyclase-activating polypeptide may function as a neuromodulator in guinea-pig adrenal medulla. *J Physiol* **528**, 473–487.
- Inoue M, Harada K, Matsuoka H, Sata T & Warashina A (2008). Inhibition of TASK1-like channels by muscarinic receptor stimulation in rat adrenal medullary cells. *J Neurochem* **106**, 1804–1814.
- Inoue M & Imanaga I (1995). Mechanism of activation of nonselective cation channels by putative  $M_4$  muscarinic receptor in guinea-pig chromaffin cells. *Br J Pharmacol* **114**, 419–427.
- Inoue M, Matsuo T & Ogata N (1985a). Characterization of pre- and postsynaptic actions of (–)-baclofen in the guinea-pig hippocampus *in vitro*. *Br J Pharmacol* **84**, 843–851.
- Inoue M, Matsuo T & Ogata N (1985b). Possible involvement of  $K^+$ -conductance in the action of  $\gamma$ -aminobutyric acid in the guinea-pig hippocampus. *Br J Pharmacol* **86**, 515–524.
- Inoue M, Oomura Y, Yakushiji T & Akaike N (1986). Intracellular calcium ions decrease the affinity of the GABA receptor. *Nature* **324**, 156–158.
- Iwasa K, Oomori Y & Tanaka H (1998). Gamma aminobutyric acid immunoreactivity in the mouse adrenal gland during postnatal development. *Arch Histol Cytol* **61**, 373–382.
- Ji F, Kanbara N & Obata K (1999). GABA and histogenesis in fetal and neonatal mouse brain lacking both the isoforms of glutamic acid decarboxylase. *Neurosci Res* **33**, 187–194.
- Jones A, Korpi ER, Mckernan RM, Pelz R, Nusser Z, Mäkelä R, Mellor JR, Pollard S, Bahn S, Stephenson FA, Randall AD, Sieghart W, Somogyi P, Smith AJH & Wisden W (1997). Ligand-gated ion channel subunit partnerships: GABA<sub>A</sub> receptor  $\alpha_6$  subunit gene inactivation inhibits  $\delta$  subunit expression. *J Neurosci* **17**, 1350–1362.
- Kataoka Y, Fujimoto M, Alho H, Guidotti A, Geffard M, Kelly GD & Hanbauer I (1986). Intrinsic gamma aminobutyric acid receptors modulate the release of catecholamine from canine adrenal gland *in situ*. *J Pharmacol Exp Ther* **239**, 584–590.
- Kataoka Y, Gutman Y, Guidotti A, Panula P, Wroblewski J, Cosenza-Murphy D, Wu JY & Costa E (1984). Intrinsic GABAergic system of adrenal chromaffin cells. *Proc Natl Acad Sci U S A* **81**, 3218–3222.
- Kitayama S, Morita K, Dohi T & Tsujimoto A (1990). Enhancement by GABA of the stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **341**, 414–418.
- Lin H, Ozaki S, Fujishiro N, Takeda K, Imanaga I, Prestwich GD & Inoue M (2005). Subunit composition and role of  $Na^+$ ,  $K^+$ -ATPases in adrenal chromaffin cells. *J Physiol* **564**, 161–172.
- Luque JM, Malherbe P & Richards JG (1994). Localization of GABA<sub>A</sub> receptor subunit mRNAs in the rat locus coeruleus. *Brain Res Mol Brain Res* **24**, 219–226.
- McKernan RM & Whiting PJ (1996). Which GABA<sub>A</sub>-receptors subtypes really occur in the brain?. *Trends Neurosci* **19**, 139–143.
- Maguire JL, Stell BM, Rafizadeh M & Mody I (2005). Ovarian cycle-linked changes in GABA<sub>A</sub> receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nature Neurosci* **8**, 797–804.
- Mellon SH & Griffin LD (2002). Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol Metab* **13**, 35–43.
- Metzler K, Agoston A & Gratzl M (2004). An intrinsic  $\gamma$ -aminobutyric acid (GABA) ergic system in the adrenal cortex: findings from human and rat adrenal glands and the NCI-H295R cell line. *Endocrinology* **145**, 2402–2411.
- Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP et al (1999). Attenuated sensitivity to neuroactive steroids in  $\gamma$ -aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci U S A* **96**, 12905–12910.
- Nagaya N & Macdonald RL (2001). Two  $\gamma$ 2L subunit domains confer low  $Zn^{2+}$  sensitivity to ternary GABA<sub>A</sub> receptors. *J Physiol* **532**, 17–30.
- Nagayama T, Matsumoto T, Kuwakubo F, Fukushima Y, Yoshida M, Suzuki-Kusaba M, Hisa H, Kimura T & Satoh S (1999). Role of calcium channels in catecholamine secretion in the rat adrenal gland. *J Physiol* **520**, 503–512.
- Nusser Z, Sieghart W & Somogyi P (1998). Segregation of different GABA<sub>A</sub> receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* **18**, 1693–1703.
- Parramón M, Oset-Gasque MJ, González MP & Stephenson FA (1994). Identification of GABA<sub>A</sub> receptor subunits expressed in bovine adrenal medulla. *Neurosci Lett* **168**, 243–246.
- Peters JA, Lambert JJ & Cottrell GA (1989). An electrophysiological investigation of the characteristics and function of GABA<sub>A</sub> receptors on bovine adrenomedullary chromaffin cells. *Pflugers Arch* **415**, 95–103.
- Quirk K, Whiting PJ, Ragan CI & McKernan RM (1995). Characterisation of  $\delta$ -subunit containing GABA<sub>A</sub> receptors from rat brain. *Eur J Pharmacol* **290**, 175–181.
- Ribak CE, Tong WMY & Brecha NC (1996). GABA plasma membrane transporters, GAT-1 and GAT-3, display different distributions in the rat hippocampus. *J Comp Neurol* **367**, 595–606.
- Rudomin P & Schmidt RF (1999). Presynaptic inhibition in the vertebrate spinal cord revisited. *Exp Brain Res* **129**, 1–37.
- Saxena NC & Macdonald RL (1994). Assembly of GABA<sub>A</sub> receptor subunits: role of the  $\delta$  subunit. *J Neurosci* **14**, 7077–7086.
- Saxena NC & Macdonald RL (1996). Properties of putative cerebellar  $\gamma$ -aminobutyric acid<sub>A</sub> receptor isoforms. *Mol Pharmacol* **49**, 567–579.
- Semyanov A, Walker MC, Kullmann DM & Silver RA (2004). Tonically active GABA<sub>A</sub> receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27**, 262–269.
- Shivers BD, Killisch I, Sprengel R, Sontheimer H, Köhler M, Schofield PR & Seeburg PH (1989). Two novel GABA<sub>A</sub> receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**, 327–337.
- Soghomonian JJ & Martin DL (1998). Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol Sci* **19**, 500–505.
- Thiagarajan AB, Mefford IN & Eskay RL (1989). Single-dose ethanol administration activates the hypothalamic-pituitary-adrenal axis: exploration of the mechanism of action. *Neuroendocrinology* **50**, 427–342.

- Thomas-Reetz AC & De Camilli P (1994). A role for synaptic vesicles in non-neuronal cells: clues from pancreatic  $\beta$  cells and from chromaffin cells. *FASEB J* **8**, 209–216.
- Wallner M, Hancher HJ & Olsen RW (2003). Ethanol enhances  $\alpha_4\beta_3\delta$  and  $\alpha_6\beta_3\delta\gamma$ -aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A* **100**, 15218–15223.
- Warashina A & Satoh Y (2001). Modes of secretagogue-induced  $[Ca^{2+}]_i$  responses in individual chromaffin cells of the perfused rat adrenal medulla. *Cell Calcium* **30**, 395–401.
- Wei W, Faria LC & Mody I (2004). Low ethanol concentrations selectively augment the tonic inhibition mediated by  $\delta$  subunit-containing GABA<sub>A</sub> receptors in hippocampal neurons. *J Neurosci* **24**, 8379–8382.
- Whiting P, McKernan RM & Iversen LL (1990). Another mechanism for creating diversity in  $\gamma$ -aminobutyrate type A receptors: RNA splicing directs expression of two forms of  $\gamma 2$  phosphorylation site. *Proc Natl Acad Sci U S A* **87**, 9966–9970.
- Wisden W, Laurie DJ, Monyer H & Seeburg PH (1992). The distribution of 13 GABA<sub>A</sub> receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* **12**, 1040–1062.
- Wohlfarth KM, Bianchi MT & Macdonald RL (2002). Enhanced neurosteroid potentiation of ternary GABA<sub>A</sub> receptors containing the  $\delta$  subunit. *J Neurosci* **22**, 1541–1549.
- Xie Z, Currie KPM, Cahill AL & Fox AP (2003). Role of Cl<sup>-</sup> co-transporters in the excitation produced by GABA<sub>A</sub> receptors in juvenile bovine adrenal chromaffin cells. *J Neurophysiol* **90**, 3828–3837.

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### Supplemental material

Online supplemental material for this paper can be accessed at: <http://jp.physoc.org/cgi/content/full/jphysiol.2008.158709/DC1>