DISTRIBUTION AND NEUROCHEMICAL CHARACTERIZATION OF NEURONS EXPRESSING GIRK CHANNELS IN THE RAT BRAIN

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ABSTRACT

G-protein inwardly rectifying potassium (GIRK) channels mediate the synaptic actions of numerous neurotransmitters in the mammalian brain and play an important role in the regulation of neuronal excitability in most brain regions through activation of various G-protein coupled receptors such as the serotonin 5-HT$_{1A}$ receptor. In this report we describe the localization of GIRK1, GIRK2 and GIRK3 subunits and 5-HT$_{1A}$ receptor in the rat brain, as assessed by immunohistochemistry and *in situ* hybridization. We also analyze the co-expression of GIRK subunits with the 5-HT$_{1A}$ receptor and cell markers of glutamatergic, GABAergic, cholinergic and serotonergic neurons in different brain areas by double-label *in situ* hybridization. The three GIRK subunits are widely distributed throughout the brain, with an overlapping expression in cerebral cortex, hippocampus, paraventricular nucleus, supraoptic nucleus, thalamic nuclei, pontine nuclei and granular layer of the cerebellum. Double-labeling experiments show that GIRK subunits are present in most of the 5-HT$_{1A}$ receptor-expressing cells in hippocampus, cerebral cortex, septum and dorsal raphe nucleus. Similarly, GIRK mRNA subunits are found in glutamatergic and GABAergic neurons in hippocampus, cerebral cortex and thalamus, in cholinergic cells in the nucleus of vertical limb of the diagonal band and in serotonergic cells in the dorsal raphe nucleus. These results provide a deeper knowledge of the distribution of GIRK channels in different cell subtypes in the rat brain and might help to elucidate their physiological roles and to evaluate their potential involvement in human diseases.

INTRODUCTION

Inwardly rectifying potassium channels have dominant control of the membrane potential of mammalian neurons, and modulation of their activity can profoundly influence neuronal excitability (Jan and Jan, 1997). A major signal transduction mechanism in neurobiology is the direct coupling of metabotropic receptors to ion channels by a membrane-delimited pathway that does not involve cytoplasmic intermediates (Nicoll, 1988; Neer, 1995). Inwardly rectifying potassium channels fall into seven subfamilies, and include a subfamily designated as Kir3 that is directly coupled to heterotrimeric G-proteins and mediates the effect of activated metabotropic receptors (Dascal, 1997; Yamada et al., 1998). Activation of metabotropic receptors coupled to $G_{i/o}$ proteins catalyses the release of Gβγ subunits and directly interact with Kir3 channels to increase their open probability (Logothetis et al., 1987; Krapivinsky et al., 1995; Wickman and Clapham, 1995). This subfamily of inward rectifiers, also known as the G protein-regulated inwardly rectifying potassium (GIRK) channels, is composed of four members designated GIRK1-4 (Dascal, 1997). GIRK channels are tetramers and hence could exist as homo- or heteromeric complexes (Yang et al., 1995). Although the different GIRK subunits are regulated differentially by a variety of intracellular factors, reports indicate that these channels require membrane phosphatidylinositol-4,5-bisphosphate (PIP$_2$) for activity (Sui et al., 1998; Huang et al., 1998; Ho and Murrell-Lagnado, 1999). The levels of PIP$_2$ can be changed and *per se* acts as a novel signal to regulate GIRK channels. Moreover, the functional dependence of Gβγ signalling on PIP$_2$ predicts the possible cross-talk of different metabotropic receptors. In fact, GIRK channels are inhibited by activation of metabotropic receptors coupled to $G_{q/11}$ proteins and fosfolipase C-β via local PIP$_2$ depletion (Cho et al., 2005).

In addition to their major physiological role in synaptic transmission and regulation of membrane potential, GIRK proteins have been shown to be potential sites for genetic mutations inducing neuronal cell death. Our understanding of the development, maintenance and degeneration of mammalian nervous systems has been greatly facilitated by examination of naturally occurring ("weaver mouse") and engineered ("Ts65Dn mouse") neurological mouse mutants at chromosome 16 (Davisson et al., 1990; Patil et al., 1995). Homozygous weaver (wv) mutations result in aberrant postnatal development and death of several classes of neurons. Compelling evidence indicates that the weaver phenotype results from a point mutation in the

sequence of the gene coding for the GIRK2 protein (Patil et al., 1995). The expression of GIRK2wv in heterologous systems has electrophysiological consequences (Slesinger et al., 1997; Rossi et al., 1998). A hypothesis hold that the mutated channel is inactive and that subsequent lack of regulation of membrane potential leads to excessive Ca^{+2} entry and death (Rossi et al., 1998). Mice with segmental trisomy 16 (Ts65Dn) which have triplication of a region of mouse chromosome 16 homologous to the Down syndrome critical region in human chromosome 21, demonstrate behavioural abnormalities related to spatial learning and memory (Davisson et al., 1990). Evidence demonstrating alterations in signal transduction pathways through adenylyl cyclases and phospholipase C-β have been reported and interestingly, all GIRK2 splicing isoforms examined are expressed at higher levels in the Ts65Dn in comparison to the diploid brain mouse (Dierssen et al., 1997; Ruiz de Azua et al., 2001; Harashima et al., 2006). Importantly, elevated levels of GIRK2 protein correlated with elevated levels of GIRK1 in several regions of the Ts65Dn mouse (Harashima et al., 2006). These results hold the current view of the GIRK2 subunits as an essential component that determines the surface localization of GIRK1 subunits. Therefore, a better knowledge of the co-expression pattern of GIRK proteins in the brain could help to elucidate their potential role in the genesis of or susceptibility to various neurodegenerative and developmental disorders.

Three GIRK channel subunits (GIRK1-GIRK3) exhibit broad and overlapping distributions in the central nervous system (CNS), whereas the fourth subunit (GIRK4) is found primarily in the heart (Kobayashi et al., 1995; Krapivinsky et al., 1995; Karschin et al., 1996; Chan et al., 1997). Formerly, the overlapping distributions of GIRK subunits in the CNS suggested that channels formed by multiple subunit combinations could contribute to neuronal channel content. An area of research now becoming appreciated is the process by which ion-channel proteins are translocated to the cell surface (Ma and Jan, 2002). Several groups have reported specific trafficking signals embedded within the ion channels, that is, aminoacid sequence motifs that are crucial for channel membrane-targetting and coassembly (Deutsch, 2002). Studies on GIRK channels have provided some clues to the complexity with which ion channels are processed and targeted to the membrane (Woodward et al., 1997; Ma et al., 2001). GIRK1 does not form functional channels when expressed alone, due to defects in membrane targeting (Chan et al., 1996; Woodward et al., 1997; Mirshahi and Logothetis, 2004). GIRK2 and GIRK4, both of which can function as homomers contain

endoplasmic reticulum (ER) export signals and reach the surface efficiently (Kennedy et al., 1999). However, although homomeric GIRK2 and GIRK4 channels have been found in native tissues exogenously expressed homomers of these channels give very brief and poorly resolved open-time kinetics that are different from native channels (Chan et al., 1996; Corey and Clapham, 1998). Importantly, both GIRK2 and GIRK4 can recruit GIRK1, greatly increasing its surface expression and those results in highly active channels that are indistinguishable from native channels (Krapivinsky et al., 1995; Chan et al., 1997; Woodward et al., 1997; Kennedy et al., 1999). Interestingly, it has been showed that GIRK2/GIRK3 heteromers have some activity, but showing reduced Gβγ sensitivity compared to other heteromers containing GIRK1 subunits (Jelacic et al., 2000). Comparison of the sequences of GIRK3 and other GIRK family members suggests that other differences might have functional consequences as well. For example, Glu^{35} in GIRK3 is replaced by His^{64} (the equivalent position) in GIRK4, and His^{64} interacts with Gβγ (He et al., 2002). Whereas GIRK3 has Asp^{165}, other inward rectifiers contain a lysine residue that is involved in interacting with PIP_2 (Lopes et al., 2002). A most intriguing finding is that GIRK3, not only lacks an ER export signal, but in addition, GIRK3 contains a lysosomal targeting signal (Ma et al., 2002). Co-expression of GIRK3 with GIRK1 and GIRK2 leads to reduced expression of GIRK1 at the cell surface, and it has been proposed that GIRK3 target functional neuronal GIRK channels (GIRK1/GIRK2 heteromers and GIRK2 homomers) to lysosomal degradation pathways (Ma et al., 2002). Therefore, although the current view is that GIRK1/GIRK2 heteromers are considered the prototypical GIRK channel in the CNS, the functional differences along with the unique trafficking characteristics of GIRK3, could open a new scenario for regulating the density of functional GIRK1/GIRK2 (or GIRK1/GIRK4) channels on cell surface simply controlling the amount of GIRK3 expressed, and therefore, the number of GIRK1/GIRK2 channels on the plasma membrane (Leaney, 2003).

Despite the importance of GIRK3, and in contrast to the extensive studies of GIRK mRNA distribution, little is known about the co-localization of GIRK subunits mRNA at cellular level in the brain. In this report we describe the distribution (protein and mRNA) and co-localization (mRNA) of GIRK(1-3) subunits and 5-HT$_{1A}$ serotonin receptors in the adult rat brain. We choose the 5-HT$_{1A}$ receptor for further anatomical co-localization with GIRK channels because extensive functional studies of GIRK-mediated functions of 5-HT$_{1A}$ receptors have been performed. In fact, it has been long

known that the 5-HT$_{1A}$ serotonin receptor can stimulate the opening of GIRK channels in neurons (Andrade and Nicoll, 1987; Colino and Halliwell, 1987; Zgombick et al., 1989; Penington et al., 1993). Again, there exists limited knowledge on the co-expression of 5-HT$_{1A}$ receptor and GIRK subunits on defined cell subtypes of rat brain. The second aim of the study was to examine in detail the relationships of GIRK(1-3) mRNA positive neurons to glutamatergic, GABAergic and serotonergic neurons by *in situ* hybridization.

MATERIALS AND METHODS

ANIMALS

Male albine Sprague-Dawley rats weigh 250-300 g were obtained from Iffa Credo (Lyon, France). Animals were kept in a controlled environment (12 h light-dark cycle, lights on at 08:00 h, and 22 ± 2 °C room temperature) with food and water provided *ad libitum*. Rats were carefully handled following the European Union regulations (O.J. of E.C. L358/1 18/12/1986).

WESTERN BLOT STUDIES OF GIRK SUBUNITS

After killing animals by decapitation, their brains were immediately removed and cerebral cortices were dissected out on ice and stored at -80ºC. The preparation of membranes from rat cerebral cortex samples was essentially as previously described for rat and human brain tissues (Garro et al., 2001; Salles et al., 2001). Western blot studies were performed as previously reported for GIRK subunits in rodent brain with minor modifications (Liao et al., 1996; Inanobe et al., 1999; Torrecilla et al., 2002). Briefly, plasmatic membrane proteins were solubilized in a sample buffer (2.2% (w/v) sodium dodecylsulphate [SDS], 6.6% (v/v) 2-mercaptoethanol, 11% (v/v) glycerol and 125mM Tris-HCl, pH 7.5), resolved by electrophoresis in 10% SDS–polyacrylamide gels and then transferred to polyvinylidine difluoride membranes (Amersham Pharmacia, Madrid, Spain). Blots were blocked in 5% non-fat dry milk/phosphate-buffered saline for 1 h, and incubated overnight with anti-GIRK1, GIRK2 or GIRK3 antibodies. GIRK1 protein was detected using a rabbit polyclonal antiserum (1:7000; Alomone Labs, Jerusalem, Israel; cat. No. APC-005) raised against the synthetic peptide corresponding to 437-501 residues of mouse GIRK1 protein (acc. No. P35562). GIRK2 protein was detected using a rabbit polyclonal antiserum (1:250; Alomone Labs; cat. No. APC-006) raised against the synthetic peptide corresponding to 374-414 residues of mouse GIRK2 protein (acc. No. P48542). GIRK3 protein was detected using a rabbit polyclonal antiserum (1:250; Alomone Labs; cat. No. APC-038) raised against the synthetic peptide corresponding to 344-361 residues of mouse GIRK3 protein (acc. No. Q63511). Blots were washed and incubated with secondary antibodies (donkey anti-rabbit IgG; Amersham Pharmacia, product code NA934) conjugated to horseradish peroxidase diluted to 1:4000 in blocking buffer for 2 h at

room temperature. Immunoreactive bands were visualized with the enhanced chemiluminiscence (ECL) system (Amersham Pharmacia). Film analysis was conducted by quantitative densitometry. Each gel contained a prestained broad-range protein ladder to measure molecular masses of individual bands.

IMMUNOHISTOCHEMISTRY

Tissue preparation

Animals were anaesthetized intraperitoneally with 15% choral hydrate (Sigma) and perfused transcardially under deep anaesthesia with saline in 50 mM phosphate buffer (pH 7.4), followed by 4% paraformaldehyde (Sigma). Brains were removed, kept immersed in the same fixative medium overnight and stored at 4 ºC for 2 days in 0.1 M phosphate buffer containing 30% sucrose. Tissue sections, 30 µm thick, were cut using a microtome-cryostat (Cryocut 3.000, Leica , Germany) with a stereotaxic atlas guide (Paxinos and Watson, 1998).

Immunohistochemistry procedure

The antigens, GIRK1, GIRK2, GIRK3 subunits and 5-HT_{1A} receptor, were detected by immunohistochemistry using the avidin-peroxidase method with 3,3´-diaminobenzidine (DAB) as chromogen. Following reduction of endogenous peroxidases with 1% hydrogen peroxide (Sigma) and blocking of nonspecific background staining with 5% normal goat serum (NGS) (Sigma), the sections were incubated with the following immunoreagents: 1) primary antisera: commercially obtained polyclonal antibodies used were rabbit anti-GIRK1, GIRK2 or GIRK3 channels (diluted to 1:100, 1:300 and 1:300, respectively; Alomone Labs; cat. No.s APC-005, APC-006, APC-038) or guinea pig anti-5-HT_{1A} receptor (1:1000, raised against a synthetic peptide –PPPKKSLNGQPGSGD– corresponding to a region located in the large third intracellular loop of the rat and mouse 5-HT_{1A} receptor protein; Chemicon Intl. Inc., Temula, CA; cat. No. AB5406); 2) goat anti-rabbit biotin-conjugated immunoglobulines (1:200; Chemicon Intl. Inc.; cat. No. AP132B) for GIRK1, GIRK2 and GIRK3, or goat anti-guinea pig biotin-conjugated immunoglobulines (1:200, Chemicon Intl. Inc.; cat. No. AP108B) for the 5-HT_{1A} receptor; 3) ABC-peroxidase complex: streptavidin-ABC complex/HRP (Dako A/S, Glostrup, Denmark); 4) chromogen: 3,3´-DAB (Sigma) 0.2 mg/mL in 0.2 M Tris HCl buffer containing 0.03% hydrogen peroxide.

Each step was followed by an appropriate wash per triplicate in phosphate-buffer saline. Sections were carefully extended and mounted into gelatine-coated slides, dehydrated, mounted (DPX mountant for histology, Fluka Chemie AG, Buchs, Switzerland), and examined in an Olympus BX50F optic microscope (Olympus Optical Co. Ltd., Japan).

Controls for specificity of the antibodies

The specificity of the staining obtained in immunohistochemistry experiments was confirmed by preadsorption of the antibodies (anti-GIRK1, anti-GIRK2, anti-GIRK3 and anti-5-HT$_{1A}$ receptor) with the addition of an excess of the corresponding control peptide (3:1), which resulted in the complete abolition of immunostaining. The remaining signal was not different from background.

We used a second method to further control the specificity of GIRK1, GIRK2 and GIRK3 antibodies. As described above, western blot experiments were carried out on membrane preparations of cerebral cortex and we corroborated that the autoradiographic signal was abolished when we incubated the antibodies with an excess of the corresponding control peptide (3:1) (see also "Results, Evaluation of GIRK subunit specific antibodies"). We did not use this second method for the 5-HT$_{1A}$ receptor antibody as it was no useful for Western blot analysis.

Analysis of the results

Cell counting was performed directly at the microscope with the help of Quantimet 500 MC software. For each region, immunolabeled cells present in a predetermined area (32383.2 μm^2, or 2030.5 μm^2 for the habenula) were counted manually in 8 different samples obtained from the 2 hemispheres, out of 2 sections from 2 rats. In the case of the DR 4 different sections from each animal were examined. For a given structure, tissue sections from virtually identical anteroposterior levels were carefully selected for counting. The actual field to count was chosen at random in the first section and the same zone was systematically explored in the following sections. The standard deviations obtained ranged between 5-10% of the cell counts value. Table 1 contains the results of cell counting as: "++++" very abundant (more than 2500 labeled elements/mm^2), "+++" abundant (1500-2500 labeled elements /mm^2), "++" moderate expression (1000-1500 labeled elements /mm^2), "+" low expression (less than 1000 labeled elements /mm^2), "-" indicates the consistent absence of detectable

staining and "n.d" indicates that the presence of specific cellular staining could not be determined.

IN SITU HYBRIDIZATION

Tissue preparation

Animals were killed by decapitation and the brains rapidly removed, frozen on dry ice and stored at -20ºC. Tissue sections, 14 µm thick, were cut using a microtome-cryostat (HM500 OM; Microtom, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane; Sigma) coated slides and kept at -20ºC until use.

Hybridization probes

The oligodeoxyribonucleotide probes used were as follows. For the mRNA encoding for GIRK1 channel the five oligonucleotides used were complementary to bases 4-48, 8-31, 137-181, 352-396 and 1504-1548 (GenBank acc. no. U01071). For GIRK2 mRNA four oligonucleotides were used, complementary to bases 219-263, 947-991, 1326-1370 and 1401-1446 (GenBank acc. no AB073755). For the mRNA encoding for GIRK3 channel, three oligonucleotides were used, complementary to bases 276-320, 381-425 and 1398-1442 (Gen Bank acc. no L77929). For 5-HT$_{1A}$ receptor mRNA three oligonucleotides were simultaneously used, complementary to bases 1-48, 763-810 and 1219-1266 (Gen Bank acc. no NM_012585).

Glutamatergic cells were identified by the presence of the vesicular glutamate transporters (vGluT) vGluT1 and vGluT2 mRNAs with two oligonucleotides complementary to bases 127-172 and 1756-1800 for vGluT1 (Gen Bank acc. no U07609) and another two oligonucleotides complementary to bases 466-510 and 2156-2200 for vGluT2 (Gen Bank acc. no AF271235). The distributions of vGluT1 and vGluT2 mRNAs in rat brain show a complementary pattern that agrees with the localization of glutamatergic neurons (Kaneko et al., 2002). GABAergic cells were identified by the presence of the enzyme synthesizing GABA, glutamic acid descarboxylase (GAD), that in adult brain exist as two major isoforms, GAD65 and GAD67 (Soghomonian and Martin, 1998). Immunohistochemical and *in situ* hybridization histochemistry indicate that the majority of GABA-containing neurons in the brain co-express the genes encoding the two GAD isoforms. Two oligonucleotides for GAD65 isoform were used: bp 159-213 and 514-558 (Gen Bank acc. no

NM_012563) and another two oligonucleotides were used for GAD67 isoform: bp 191-235 and 1600-1653 (Gen Bank acc. no NM_017007). Cholinergic cells were distinguished by the presence of the enzyme choline acetyl-transferase (ChAT) mRNA with two oligonucleotides complementary to bases 880-927 and 1669-1716 of the rat ChAT cDNA sequence (Gen Bank acc. no XM_224626). Since it has been shown that the serotonin transporter is exclusively expressed by serotonergic neurons, we have taken the serotonin transporter (ST) mRNA as indicator of serotonergic phenotype (Fujita et al., 1993). Serotonergic cells were identified by the presence of the serotonin transporter using one oligonucleotide complementary to bases 865-912 (Gen Bank acc. no m79450).

The oligonucleotides were all synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Evaluation of the oligonucleotide sequences with basic local alignment search tool of EMBL and Gen-Bank databases indicated that the probes do not show any significant similarity with mRNAs other than their corresponding targets in the rat.

Oligonucleotides for GIRK1, GIRK2, GIRK3 and 5-HT$_{1A}$ mRNAs were labeled at their 3´-end using [α-^{33}P]dATP (3000 Ci/mmol; New England Nuclear, Boston, MA, USA) for the *in situ* hybridization histochemistry experiments and terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA, USA), purified using QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany). For the co-localization studies GIRK, vGluT, GAD, ChAT and ST oligonucleotides (100 pmol) were non-radioactively labeled with the same enzyme and Dig-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) according to a previously described procedure (Schmitz et al., 1991).

In situ hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization histochemistry were based on a previously described procedure and have been already published (Tomiyama et al., 1997; Landry et al., 2000; Serrats et al., 2003). Briefly, frozen tissue sections were brought to room temperature, fixed for 20 min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1X PBS: 8 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3X PBS at room temperature, twice for 5 min each in 1X PBS, and incubated for 2 min at 21ºC in a solution of predigested pronase (Calbiochem, San Diego, CA, USA) at a final

concentration of 24 U/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA. The enzymatic activity was stopped by immersion for 30 seconds in 2 mg/ml glycine in 1X PBS. Tissues were finally rinsed in 1X PBS and dehydrated through a graded series of ethanol. For hybridization, radioactively labeled and non-radioactively labeled probes were diluted in a solution containing 50% formamide, 4X SSC (1X SSC: 150 mM NaCl, 15 mM sodium citrate), 1X Denhardt´s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg/ml yeast tRNA and 500 µg/ml salmon sperm DNA. The final concentrations of radioactive and Dig-labeled probes in the hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probe/s, overlaid with Nescofilm coverslips (Bando Chemical Inc., Kobe, Japan) and incubated overnight at 42ºC in humid boxes. Sections were washed four times (45 minutes each) in 0.6 M NaCl, 10 mM Tris-HCl pH 7.5 at 60ºC, and once in the same buffer at room temperature for 10 min. For single *in situ* hybridization experiments, tissue sections were dehydrated in a graduate series of ethanol.

Development of radioactive and non-radioactive hybridization signal

Hybridized sections were treated as described by (Landry et al., 2000). Briefly, after washing, the slides were immersed for 30 min in a buffer containing 0.1 M Tris-HCl pH 7.5, 1M NaCl, 2 mM $MgCl_2$ and 0.5% bovine serum albumin (Sigma) and incubated overnight at 4 ºC in the same solution with alkaline-phosphatase-conjugated anti-digoxigenin-F(ab) fragments (1:5000; Roche Diagnostics GmbH). Afterward, they were washed three times (10 min each) in the same buffer (without antibody), and twice in an alkaline buffer containing 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Diagnostics GmbH) diluted in 10 ml of alkaline buffer. The enzymatic reaction was blocked by extensive rinsing in the alkaline buffer containing 1 mM EDTA. The sections were then briefly dipped in 70% and 100% ethanol, air-dried and dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Chesire, UK) diluted 1:1 with destilled water. They were exposed in the dark at 4 ºC for 5-6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

For film autoradiography, some hybridized sections were exposed to Biomax-MR

(Kodak) films for 4 to 6 days at -80 ºC with intensifying screens.

Analysis of the results

The average densities of each mRNA in different brain regions were evaluated semi quantitatively on film autoradiograms with the aid of an image analysis system (MCID M4; Imaging Research, St. Catherines, Ontario, Canada). In table 1 we show these results as: "+++" very strong (relative optical density higher than 0.5), "++" moderate (relative optical density from 0.35 to 0.5), "+" low (relative optical density lower than 0.35), "-" indicates that relative optical density was not detected and "n.d" indicates that relative optical density could not be determined.

Tissue sections were examined in bright- and dark-field in a Wild 420 macroscope (Leica, Heerbrugg, Germany) and in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). A Darklite iluminator (Micro Video Instruments, Avon, MA, USA) was used to improve the visualization of autoradioghaphic silver grains and capture of bright and dark-field images.

GIRK and glutamatergic, GABAergic, cholinergic and serotonergic neurons were identified as cellular profiles exhibiting a dark precipitate (alkaline phosphatase reaction product) surrounding or covering the nucleus. GIRK1, GIRK2, GIRK3 and 5-HT$_{1A}$ radioactive-hybridization signal was considered positive when accumulation of silver grains over the stained cellular profiles was at least two- to three-fold higher than the background levels.

Only cellular profiles showing abundance of GIRK1, GIRK2, GIRK3 or 5-HT$_{1A}$ mRNAs and the other GIRK subunits or cell type identifier (vGluT1, vGluT2, GAD, ChAT or ST mRNAs) were considered double labeled.

Controls for specificity of the probes

The specificity of the autoradiographic signal obtained in the *in situ* hybridization histochemistry experiments was confirmed by performing a series of routine controls (Pompeiano et al., 1992). For each mRNA under study, at least two different oligonucleotide probes complementary to different regions of the same mRNA were used independently as hybridization probes in consecutive sections of the same animal showing identical hybridization patterns. For a given oligonucleotide probe, addition in the hybridization solution of an excess of the same unlabeled oligonucleotide resulted in the complete abolition of the specific hybridization signal. The remaining

autoradiographic signal was considered background. The thermal stability of the hybrids was examined by washing at increasing temperatures: a sharp decrease in the hybridization signal was observed at a temperature consistent with the T_m of the hybrids (Figure 1).

PREPARATION OF THE FIGURES

Photomicrography was performed in a Wild macroscope M420 (Leica, Heerbrugg, Switzerland) or a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany), equipped with a digital camera (DXM1200 F, Nikon) and ACT-1 (Nikon) as image capture software. Figures were prepared for publication using Adobe Photoshop software (Adobe Software, San Jose, CA, USA). Contrast and brightness of images were the only variables we adjusted digitally.

RESULTS

Evaluation of GIRK subunit specific antibodies

As a first approach to determine the levels of expression of GIRK1-3 subunits in rat brain, we characterized the specificity of the immunolabeling of these proteins in cerebral cortical membranes with the antibodies employed. As described previously by Krapivinsky et al.(1995), Liao et al.(1996), Torrecilla et al.(2002), and Koyrakh et al.(2005), GIRK1 immunoreactivity in rat brain cortical membranes was visualized as three bands, the lower molecular weight versions thought to represent core (50 KDa) and core-glycosylated species (55 KDa), and the heavily glycosylated (approximately 60 KDa) GIRK1 subunit the functional mature protein (Figure 2). Detection of these polypeptides was completely abolished when the antibody was preincubated with excess of synthetic peptide (see Figure 2, A1). These results indicated that the antibody used specifically recognized GIRK1 subunits in rat brain. Analysis of the standard curves revealed a linear relationship between the amount of total membrane protein (1-6 µg) in each lane and the relative optical density of the 60 KDa GIRK1 subunits band (see legend Figure 2, A2-A3).

There are four different splice variants of GIRK2 subunits that differ in their sequences mainly at the extreme N- and C-termini of the subunit (Lesage et al., 1994; Lesage et al., 1995; Isomoto et al., 1996). GIRK2b, which is truncated at the C-terminus and lacks the acidic cluster, shows reduced surface expression. The primary amino acid sequences of GIRK2a and GIRK2c show that these isoforms diverge only at their carboxyl terminal tails. GIRK2c is 11 amino acid residues longer at its C-terminal end than GIRK2a (Lesage et al., 1995). The GIRK2d isoform is predominantly expressed in testis (Inanobe et al., 1999). It has been established that the antibody employed by us recognizes the GIRK2a, 2c and 2d splicing isoforms, but not the ubiquitously expressed GIRK2b splice variant (Lesage et al., 1994; Lesage et al., 1995; Isomoto et al., 1996; Inanobe et al., 1999). Accordingly, our results show that the antibody recognized a band of approximately 75 KDa and a band of lower molecular weight of around 50 KDa in rat cerebral cortex (Figure 2, B1). To demonstrate antibody specificity, GIRK2 antibodies were tested on western blots containing proteins from rat cerebral cortex with blocking peptides. Antibody against the C terminus of GIRK2 preadsorbed with competitive peptide gave rise to only the 50 KDa immunoreactive

band (Figure 2, B1). Therefore, the possibility that the 50 KDa immunoreactive band was a deglycosylation product of the 75 KDa was not considered further. Potentially, both GIRK2a and GIRK2c splice variants could contribute to the 75 KDa immunoreactive band. Analysis of the standard curves revealed a linear relationship between the amount of total membrane protein (2.5-20 µg) in each lane and the relative optical density of the 75 KDa GIRK2 subunits band (see legend Figure 2, B2-B3).

Figure 2, C shows representative immunoblots of GIRK3 subunits in crude plasmatic membranes from rat brain cortex. One band migrating at expected molecular mass (approximately 40 KDa) is shown. To demonstrate antibody specificity, GIRK3 antibodies preincubated with blocking peptides were tested on western blots containing proteins from rat cerebral cortex. Antibody against the C-terminal domain of GIRK3 subunit preadsorbed with competitive peptide gave rise to no immunoreactive bands (Figure 2, C1). Analysis of the standard curves revealed a linear relationship between the amount of total membrane protein (5-40 µg) in each lane and the relative optical density of the GIRK3 subunits band (see legend Figure 2, C2-C3).

GIRK and 5-HT$_{1A}$ receptor protein and mRNA distribution in rat brain

Immunocytochemistry studies carried out with specific polyclonal antibodies against GIRK1, GIRK2 and GIRK3 subunits and the 5-HT$_{1A}$ serotonin receptor at various coronal levels from adult rat brain confirmed that all GIRK subunits analyzed are very abundant in the rat forebrain and that the 5-HT$_{1A}$ receptor is present notably in certain areas. Moreover, *in situ* hybridization studies also showed that the mRNAs corresponding to these GIRK channel subunits are widely and heterogeneously distributed along the rat brain. Analysis of emulsion-dipped coronal brain sections revealed more details of the hybridization pattern at the cellular level. In all cases, we observed some nuclei with a strong hybridization signal in contrast to some other regions with no detectable signal. The distribution pattern of the three GIRK subunits studied was very similar. However, some differences between the brain expressions of these GIRK subunits can be pointed out.

GIRK1 subunit. Results are summarized in Table 1 and illustrated in Figure 3, A1-G1. In the particular case of the GIRK1, we found a high density of stained cells through all cortical layers with higher density in layer VI. This protein was also found as a stained neuropil in layer IV and in apical dendrites through the different layers. The

mRNA encoding GIRK1 was also widely distributed through all cortical layers although the highest density of this mRNA expression appeared in layer IV (Figure 4, A1-A2). The piriform cortex was the cortical area where both GIRK1-immunoreactive (IR) cells and its mRNA were expressed at highest densities. The dentate gyrus and the pyramidal layer of the hippocampus were among the most prominently labeled brain regions when stained with specific antibodies and also when hybridized with GIRK1 33P-labeled probes (Figure 5, A1-A2). In the septum the number of cells showing GIRK1-IR seamed to be higher than the density of mRNA positive cells that was noticed in this area. Similarly, in the caudate-putamen, abundant small cells containing GIRK1-immunoreactivity (IR) were found, although the staining was quite weak. In contrast, the hybridization signal in this area was very low. It was notable the presence of GIRK1-IR in the medial and lateral habenular nuclei with the highest number of immunostained cells per mm^2 in the medial nucleus. The presence of GIRK1 mRNA was moderate in the medial habenular nucleus and lower in the lateral nucleus (Figure 6, A1-A3). In the thalamus high number of cells showed GIRK1-IR and also, moderate hybridization signal was observed with the *in situ* hybridization experiments (Fig. 7, A1-A3). Interestingly, the arcuate, the paraventricular and the supraoptic nuclei (Figure 8) showed a high number of immunostained cells, although a very low hybridization signal could be detected in these three nuclei of the hypothalamus. In other hypothalamic nuclei, the number of GIRK1-IR cells was abundant in contrast to a low presence of mRNA at this level. In the red nucleus it was possible to observe large stained cells although they were not abundant. Moderate GIRK1-IR was found in the ventral tegmental area as well as in the compact part of the substantia nigra. However, no mRNA expression could be detected in these two areas. With regard to the dorsal raphe nucleus, abundant GIRK1-IR cells were found although low densities of mRNA were observed in this nucleus. In the pontine nucleus, we observed numerous GIRK1-IR cells and moderate densities of mRNA expression. In the locus coeruleus, we found a low presence of GIRK1 mRNA. In contrast, in the granular cell layer of the cerebellum abundant GIRK1-IR cells and a very strong hybridization signal were found. In the cerebellum we found GIRK1 mRNA expressing-cells in the granular layer and there was an absence of mRNA expression in Punkinje cells (Figure 9, A1-A3).

In relation to the subcellular localization, GIRK1-IR was found in cell bodies of immunostained neural cells and dendrites, for example in apical and basal dendrites of the pyramidal layer of the hippocampus as well as in granule cells of the dentate gyrus

(Figure 6, A2) and also in the apical dendrites of cells in the cerebral cortex. GIRK1-IR fibers were also present in the lateral septum (data not shown).

GIRK2 subunit. GIRK2 protein and its mRNA also presented a widespread distribution in the rat brain, as summarized in Table 1 and illustrated in Figure 3, A2-G2. All different neocortical areas examined presented a high density of GIRK2-immunostained cells through all layers. Nevertheless, because of the characteristics of the staining in the cortex, it was not possible to count neuronal cell bodies in every cortical layer. The most important characteristic of GIRK2-IR in layers II to III was the presence of stained fibers oriented perpendicularly to the surface of the cortex with an intense neuropil in layer I. GIRK2-IR cells were found, in particular, in pyramidal cell bodies of layer V. GIRK2 mRNA was also observed through all cortical layers (Figure 4, B1-B2). As in the case of GIRK1 subunit, GIRK2-IR and its mRNA were both enriched in piriform cortex. In the hippocampal formation, a large number of GIRK2-IR cell bodies were found in the pyramidal layer of all CA subfields and in the granule cell layer of the dentate gyrus, surrounded by an intensely stained neuropil. This abundant expression of GIRK2-IR in the hippocampus was paralleled by a very strong hybridization signal obtained for the corresponding mRNA (Figure 5, B1-B2). Similarly to more superficial layers of the cortex, the number of GIRK2-IR cells in the septum could not be determined because of the presence of abundant stained fibers. However, by *in situ* hybridization, we demonstrated a moderate presence of GIRK2 mRNA in the septum. In the caudate-putamen, neither GIRK2-IR nor its mRNA showed a significant signal. In the habenula, high numbers of GIRK2-IR cells and strong signal of GIRK2 mRNA were observed in the medial nucleus. These densities were lower in the lateral habenula which was characterized by the presence of abundant IR-fibers and varicosities (Figure 6, B1-B3). In the thalamus numerous cells expressed GIRK2-IR and showed a moderate hybridization signal (Figure 7, B1-B3). We observed a remarkable presence of GIRK2-IR in the arcuate, the paraventricular and the supraoptic nuclei of the hypothalamus, where large neurons were intensely immunostained. These results correlated with a moderate to very strong hybridization signal. In the rest of the hypothalamic nuclei, GIRK2 mRNA was relatively abundant, although no GIRK2-IR could be detected. Very few large GIRK2-stained cells were observed in the red nucleus (Figure 10). Other areas enriched in GIRK2-IR were the ventral tegmental area, with an important presence of immunostained fibers, and the substantia nigra. In the latter, and specifically in its compact part, the staining of neural cell bodies was very

intense and fibers penetrating into the reticular part were the most important characteristics, together with positively stained dendritic spines (Figure 11). In the dorsal raphe nucleus a few IR-cells were found, and we could determine a moderate intensity in GIRK2 mRNA expression signal. Abundant cells were GIRK2-IR in the pontine nucleus, with a moderate density of mRNA. In the locus coeruleus, intermediate levels of GIRK2 mRNA were found. Intense GIRK2-IR and hybridization signal was found in the granule cell layer of the cerebellum. A high GIRK2 mRNA labeling was also seen in the Punkinje cells (Figure 9, B1-B3).

In general, GIRK2 protein was found at a somatodendritic level, for example in the hippocampus, and we also found IR-fibers in the cortex, the lateral habenular nucleus and the reticular part of the substantia nigra.

GIRK3 subunit. This subunit also showed a wide distribution in the rat brain (Table 1 and Figure 3, A3-G3). GIRK3 was expressed in all cortical layers with higher number of IR-cells per mm^2 in the most superficial layers (II and III). The distribution of GIRK3 mRNA was in agreement with the IR-expression pattern in the different cortical layers with a high presence of mRNA in layers II-III and a little bit stronger density of mRNA in layer IV (Figure 4, C1-C2). Both GIRK3-IR and its mRNA were most abundant in the piriform cortex. In the dentate gyrus and the pyramidal layer of the hippocampus we found a high number of positive immunostained cells. Both structures exhibited only moderate densities of GIRK3 mRNA (Figure 5, C1-C2). A dense plexus of immunostained fibers was found in the septum. It was not possible to determine the density of GIRK3 IR-cells due to the presence of numerous immunostained fibers in this area. Using *in situ* hybridization, we demonstrated a very low presence of this mRNA in the septum. In the caudate-putamen a moderate number of cells were GIRK3-IR. The hybridization signal in this area was very low. It was notable the GIRK3-IR in the habenula, mainly in its medial part. In contrast, the presence of the corresponding mRNA was very low in both parts of the habenula (Figure 6, C1-C3). In the thalamic nuclei GIRK3-IR and mRNA were expressed at moderate levels (Figure 7, C1-C3). GIRK3-IR was found abundantly in cells of the paraventricular and the supraoptic nuclei of the hypothalamus with a distinct and intense staining, although the hybridization signal was not detected in the supraoptic nucleus and was very low in the paraventricular nucleus. Interestingly, GIRK3-IR was abundant in many hypothalamic nuclei but a low presence of hybridization signal was noticed. GIRK3-IR could also be seen in large stained cells in the red nucleus. The staining pattern of GIRK3-IR in the

ventral tegmental area as well as in the reticular part of the substantia nigra was its presence in fibers which did not allow the visualization of stained cell bodies. In the compact part of the substantia nigra, a few neural cells showed GIRK3-IR. In none of these areas the corresponding mRNA could be detected. Regarding the dorsal raphe nucleus, a moderate number of cells positively immunostained for this protein were found and a low density of hybridization signal was observed. Few GIRK3-IR cells and low mRNA densities were observed in the pontine nucleus. In the locus coeruleus, low amounts of GIRK mRNA were detected. Abundant GIRK3-IR cells were found in the granule cell layer of the cerebellum, which showed also a strong hybridization signal. GIRK3 mRNA labeling was also seen in Punkinje cells (Figure 9, C1-C3).

In relation to its subcellular localization, GIRK3-IR was mainly present in cell bodies of neural cells, dendrites and axon-like fibers.

5-HT_{1A} **receptor**. The expression of this serotonin receptor along the rat brain has some similarities with the localization of the studied GIRK subunits (Figure 3, A4-G4). We found 5-HT_{1A}-IR in all cortical layers; especially in the most internal ones (layers V and VI). The density of the hybridization signal was low in all neocortical areas and the highest intensities corresponded to the internal layers (layers V and VI). The hippocampal formation exhibited very high densities of 5-HT_{1A}-IR cells and 5-HT_{1A} mRNA labeling (Figure 3, D4-E4 and Figure 5, D1-D2). Abundant stained fibers were seen in the septum together with 5-HT_{1A}-IR cell bodies. These cells showed a moderate density of mRNA. It was notable the presence of 5-HT_{1A}-IR in the medial habenular nucleus, however, its corresponding mRNA was not detected. In the thalamus the expression of both the protein and its mRNA were quite low. We could emphasize the presence of remarkable levels of mRNA encoding this receptor in the zona incerta (Figure 3, C4). There were abundant 5-HT_{1A}-IR cells in the arcuate, paraventricular and supraoptic nuclei. Nevertheless, no mRNA could be detected in none of these three areas. In the other hypothalamic nuclei, 5-HT_{1A}-IR and its mRNA expression were very low. In the case of the red nucleus there were a low number of intensely stained cells. In the substantia nigra a low expression of the 5-HT_{1A}-IR was found in the compact part but no mRNA was detected in the entire area. In contrast, abundant 5-HT_{1A} receptor-IR cells were found in the dorsal raphe nucleus which showed also very high densities of mRNA (Figure 3, F4). The expression of this receptor in the pontine nucleus was moderate and we noticed a low presence of mRNA. In the locus coeruleus, it was noticed a moderate signal of mRNA.

The 5-HT$_{1A}$ receptor appeared expressed in cell bodies and dendrites of neural cells, for example in the medial septum.

Co-localization of GIRK subunits

In order to analyze the cellular coexistence of the different GIRK mRNAs, we performed double *in situ* hybridization experiments using ^{33}P-labeled and Dig-labeled probes to detect the GIRK subunits. The co-localization of GIRK subunits was examined in dentate gyrus, pyramidal layer of the hippocampus, cortex and thalamic nuclei (Figure 12).

As shown in Figure 12 (A), in the dentate gyrus of the hippocampus GIRK1 mRNA hybridization signal can be clearly seen as autoradiographic grains over GIRK2 mRNA-expressing cell profiles. Within this area, the vast majority of the cells were both GIRK1 and GIRK2 mRNA-positive, although we could also see a few GIRK1 or GIRK2 single-labeled cells. Similarly, in Figure 12 (B) GIRK3 mRNA and GIRK1 mRNA as well as GIRK3 and GIRK2 mRNAs (Figure 12, C), co localized in a large number of cells along the dentate gyrus.

In the pyramidal layer of the hippocampus, the large majority of cells also showed double-hybridization signal of GIRK1 and GIRK2, GIRK1 and GIRK3 and GIRK2 and GIRK3 mRNAs with only few single-labeled cells (Figure 12, D-F).

A high percentage of cells in the cerebral cortex (Figure 12, G-I) and the thalamus (Figure 12, J-L) showed co expression of the pairs of GIRK subunits analyzed, but considerable numbers of single-labeled cells were also found.

GIRK subunits in glutamatergic cells

Glutamatergic cells were identified by the presence of the vesicular glutamate transporters vGluT1 and vGluT2 mRNAs and were detected simultaneously in tissue sections using non-radioactive probes. The presence of the GIRK subunit mRNAs was determined using ^{33}P-labeled oligonucleotides.

In the dentate gyrus (Figure 13, A1-A3) and pyramidal cell layer of the hippocampus (Figure 13, B1-B3), the vast majority of the glutamatergic cells were GIRK mRNA-positive. Only a few vGluT mRNA containing cells lacked GIRK mRNA and a few cells expressing mRNA coding one of the GIRK proteins were not

glutamatergic.

In the cerebral cortex, the vast majority of glutamatergic cells also co-expressed GIRK subunits. In contrast to the hippocampus, many GIRK mRNA-positive cells were devoid of vGluT mRNA (Figure 13, C1-C3).

In the thalamus, the majority of labeled cells were double-positive and showed mRNA encoding vGluT and one of the GIRK subunits, but substantial populations of GIRK-negative glutamatergic cells as well as GIRK-positive non-glutamatergic cells were observed (Figure 13, D1-D3).

GIRK subunits in GABAergic cells

GABAergic cells were identified by the presence of the glutamic acid descarboxylase (GAD65 and GAD67) mRNAs and were detected in tissue sections using non-radioactive probes. The presence of the GIRK subunit mRNAs was determined using ^{33}P-labeled oligonucleotides.

The majority of GIRK mRNA-expressing cells in the dentate gyrus (Figure 14, A1-A3), the pyramidal cell layer of the hippocampus (Figure 14, B1-B3) and the cerebral cortex (Figure 14, C1-C3), did not show GAD mRNA signal. In these structures, most of GABAergic cells were also GIRK mRNA-positive.

Virtually all cells in the thalamus showing GIRK mRNAs were devoid of GAD mRNA. In contrast, in the reticular thalamic nucleus, GIRK mRNAs were always found in GAD mRNA-containing cells. Some GAD mRNA-positive cells that lacked GIRK mRNA were also found in the reticular thalamic nucleus (Figure 14, D1-D3).

GIRK subunits in cholinergic cells

Cholinergic cells were distinguished by the presence of choline acetyl-transferase (ChAT) mRNA. We detected these cells in the nucleus of the vertical limb of the diagonal band using non-radioactive probes. The presence of the GIRK subunit mRNAs was determined using ^{33}P-labeled oligonucleotides. The majority of cholinergic cells expressed GIRK1 or GIRK2 mRNA. Some GIRK1 mRNA- and some GIRK2 mRNA-positive cells devoid of ChAT mRNA were also observed (Figure 15, A-H). GIRK3 mRNA expression was absent in this area.

Co-localization of GIRK subunit mRNAs and 5-HT$_{1A}$ receptor mRNA

We analyzed the cellular co-existence of the 5-HT$_{1A}$ receptor mRNA and GIRK subunits through double *in situ* hybridization. We used ^{33}P-labeled oligonucleotides for the serotonin receptor and Dig-labeled probes to detect GIRK subunits. The co-localization of these three GIRK subunits with the 5-HT$_{1A}$ receptor was examined in dentate gyrus, pyramidal layer of the hippocampus, cortex, septum and dorsal raphe.

In the dentate gyrus (Figure 16, A1-A3) and pyramidal layer of the hippocampus (Figure 16, B1-B3) as well as in the cerebral cortex (Figure 16, C1-C3), the vast majority of 5-HT$_{1A}$ receptor-expressing cells showed GIRK subunit mRNAs. Cells that expressed GIRK mRNA and were 5-HT$_{1A}$ mRNA-negative, were also observed in the dentate gyrus and CA fields of the hippocampus. This type of cells was very abundant in the cerebral cortex.

In contrast, in the septum, GIRK mRNA was mostly found in 5-HT$_{1A}$ receptor mRNA-expressing cells. Nevertheless, numerous 5-HT$_{1A}$ receptor mRNA positive cells lacked GIRK mRNA labeling (Figure 16, D1-D3).

In the dorsal raphe nucleus, the majority of cells expressing the serotonin receptor 5-HT$_{1A}$ mRNA were also GIRK mRNA-positive. Few cells were found that expressed GIRK mRNAs without co-expression of 5-HT$_{1A}$ receptor mRNA (Figure 16, E1-E3). In general, the expression of GIRK3 mRNA in the dorsal raphe nucleus was more limited than GIRK1 and GIRK2 in terms of number of labeled cells.

GIRK subunits in serotonergic cells

Serotonergic cells were identified by the presence of the serotonin transporter (ST) mRNA in the dorsal raphe nucleus. We used ^{33}P-labeled oligonucleotides to detect the ST mRNA and Dig-labeled probes for GIRK subunits. In most of the cells expressing the ST mRNA we also found GIRK mRNA. In addition, only a few cells were found that expressed GIRK mRNA without apparent ST mRNA labeling (Figure 17).

DISCUSSION

In this work we have carried out a comparative analysis of the distribution of the GIRK1, GIRK2, GIRK3 subunits and 5-HT$_{1A}$ receptor in the rat brain. Our study is the first to compare the expression of these proteins and their corresponding mRNAs in different brain areas. In addition, we have examined the co-expression of the mRNAs codifying the GIRK subunits and their presence in glutamatergic, GABAergic, cholinergic, serotonergic and 5-HT$_{1A}$ receptor-containing cells in selected rat brain structures. Moreover, to our knowledge, this is the first detailed report of the distribution of the GIRK3 protein in the adult rat brain.

GIRK1, GIRK2 and GIRK3 protein and mRNA expression

GIRK1 protein-immunoreactivity (IR) showed a wide distribution along the different brain areas analyzed. It is noteworthy its presence in areas such as the cerebral cortex, hippocampal formation, habenular nuclei, thalamic nuclei, some hypothalamic nuclei and the granular layer of the cerebellum. Studies by Bausch et al. (1995) showed a similar distribution of GIRK1, although this group observed this protein in dendrites of granular and pyramidal cells of the hippocampus, while they did not recognize clearly its presence in the somata of these neurons. In contrast, Ponce et al. (1996), Miyashita and Kubo (1997) and us found immunoreactivity in the somata and dendrites of pyramidal cells in the CA1-3 as well as in the granular layer of the dentate gyrus. In fact, the high presence of GIRK1 protein in dendrites and also in cell bodies of the pyramidal layer of the hippocampus has already been described by Drake et al. (1997). Liao et al. (1996) found GIRK1 in the pyramidal cells but not in the granule cells of the dentate gyrus. These discrepancies could be due to technical differences, such as the use of different antibodies with different specificity. In the cerebral cortex, the neuropil was GIRK1-positive as already reported by Liao et al. (1996), Ponce et al. (1996) and Miyashita and Kubo (1997). Apparently, the labeling was stronger in layer IV, which contains thalamocortical projections that, most likely, contribute to the immunostaining in the cortex. This has been demonstrated by Ponce et al. (1996) using unilateral kainate lesions of the thalamus that resulted in a loss of staining on the side ipsilateral to the lesion in cortical layer IV. In addition, some studies have analyzed the localization of mRNA through *in situ* hybridization in the rat brain

and also in the mouse brain (Karschin et al., 1994; DePaoli et al., 1994; Kobayashi et al., 1995; Karschin et al., 1996). In agreement with these authors, we found a very high density of GIRK1 mRNA in the cerebral cortex, hippocampus (dentate gyrus and CA1, CA2 and CA3 fields of hippocampus), thalamic nuclei and the cerebellar granular layer.

The distributions of GIRK1-IR and its mRNA are coincident in the cerebral cortex, hippocampus, amygdala, thalamic nuclei, pontine nuclei and granular layer of the cerebellum. In contrast, this parallelism is not so evident in other structures such as the septum, caudate putamen, habenular nuclei, hypothalamic arcuate, paraventricular and supraoptic nuclei, and substantia nigra, where an important number of cells were immunoreactive but expressed only low mRNA densities.

Our distribution of GIRK2 protein-IR is in accordance with a previous work by Murer et al. (1997), who noticed an intense immunostaining of cell bodies in the septum, hippocampus, compact part of the substantia nigra and granular layer of the cerebellum. We found strongly GIRK2-IR fibres in areas such as the external layers of the cerebral cortex (with stained fibres oriented perpendicularly to the surface of the cortex), septum, lateral habenular nucleus and reticular part of the substantia nigra with remarkably stained dendrites, fibres and spines. This distribution is comparable to that described by Murer et al. (1997) and Liao et al. (1996). In addition, we found a strong staining of GIRK2 protein in the arcuate and the paraventricular nuclei of the hypothalamus, which has not been described before. In contrast to these authors, we found positively stained cells in the red nucleus and the dorsal raphe. As in the case of the GIRK1 subunit, the use of different antibodies can be the reason of these differences between studies. In the present study, the specificity of the immunostaning was assessed by preadsorption of the antisera with the corresponding control peptide antigens.

The distribution of the mRNA encoding GIRK2 protein in the adult rat brain we described in this work is in agreement with previously published localizations in the mouse and rat brain (Kobayashi et al., 1995; Liao et al., 1996). GIRK2 mRNA was substantially expressed in the hippocampus, ventral tegmental area, substantia nigra and the granular layer of the cerebellum. A moderate expression of this subunit was observed in the cerebral cortex, thalamic and hypothalamic nuclei, supraoptic nucleus, pontine nuclei and dorsal raphe nucleus and a lower signal was noticed in the caudate putamen and septum. In general, there was a good correlation between the GIRK2 protein expression and the localization of its corresponding mRNA. It is noteworthy that

in areas such as the superficial layers of the cerebral cortex and the septum, where we described stained fibres and were not able to detect cellular immunostaining, we did find mRNA hybridization signal.

With regard to the GIRK3 subunit, to our knowledge, this is the first study on the distribution of this protein in the adult rat brain apart from the study carried out by Grosse et al. (2003) based on GIRK3-IR in the hippocampus. It is remarkable, the presence of GIRK3-IR in layers II-III of the cerebral cortex, hippocampus, amygdala, medial habenular nucleus, some hypothalamic nuclei such as the paraventricular and supraoptic nuclei and also in the granular layer of the cerebellum. We found this protein not only in cell bodies but also in fibres of the septum, ventral tegmental area and substantia nigra. Using *in situ* technique, we also found high densities of mRNA encoding the GIRK3 subunit in the cerebral cortex, hippocampal formation, amygdala, thalamic nuclei, posterior hypothalamic nuclei and cerebelum. These results are in accordance with those described by Karschin et al. (1996). However, these authors found mRNA-positive cells in the nucleus of the vertical limb of the diagonal band, caudate putamen and globus pallidus, where we noticed only very low hybridization signal. These results show that this GIRK3 subunit is located in areas where GIRK1 and GIRK2 are also expressed and this parallelism is in agreement with the possible formation of heterotetrameric GIRK channels GIRK1/3 and GIRK2/3 as well as homomeric GIRK3 channels.

It is important to highlight that, in general, the anatomical distributions of cell bodies immunoreactive for the three different GIRK subunits are highly comparable to their respective mRNAs, indicating that these proteins are mainly expressed at the somatodendritic level. This subcellular localization is coherent with their function as G protein-regulated channels. However, we also observed some cases of mismatch between mRNA and GIRK-IR. For example, we found relatively strong GIRK1- and GIRK3-IR in the supraoptic nucleus, where mRNA levels were very low.

In synthesis, we report that the three GIRK subunits are widely distributed along the adult rat brain and present similar anatomic localizations (see Table 1). In particular, they have overlapping distributions in the cerebral cortex, hippocampus, thalamic nuclei, paraventricular and supraoptic nuclei, pontine nuclei and the granular layer of the cerebellum among others. However, this is not the case in other areas, for example, the GIRK1 subunit is found in the caudate putamen where the other GIRK subunits are only moderately expressed. In this area, we could expect to find more

GIRK1/2 and GIRK1/3 than any other combination of subunits. It is noteworthy the presence of GIRK2 subunit in the substantia nigra where there are a vast majority of homotetrameric GIRK channels composed by four GIRK2 subunits in dopaminergic cells or the GIRK3 subunit in the amygdala (Inanobe et al., 1999).

GIRK mRNAs co-expression

We used double *in situ* hybridization to analyze the cellular co-expression of different GIRK subunits in brain areas with a high degree of co-localization, such as the dentate gyrus, pyramidal layer of the hippocampus, cortex and thalamic nuclei. Independently of the two GIRK subunits selected, we found a similar pattern of co-expression. For example, we found numerous cells expressing GIRK1 and GIRK2 mRNA subunits, along with a few GIRK1 mRNA positive cells and a few GIRK2 mRNA positive cells. These single labeled cells are likely to contain GIRK1/3 channels and GIRK2/2 or GIRK2/3 channels, respectively. In summary, we demonstrated the co-existence of different subunits in variable combinations in rat brain, suggesting that different subunit organizations of the GIRK channels might occur in different neurons, resulting in diversity of channel functions.

GIRK mRNAs expression in glutamatergic, GABAergic and cholinergic cells

Another important aim of our study was to determine whether these GIRK channels are present in glutamatergic, GABAergic and cholinergic cells. Our work provides evidence showing that the vast majority of glutamatergic and GABAergic cells in the dentate gyrus, pyramidal layer of the hippocampus and the cerebral cortex, express GIRK mRNAs. In the thalamus, however, we found GIRK subunits in a high proportion of glutamatergic cells, but not in GABAergic neurons, with the exception of the reticular thalamic nucleus, where GIRK mRNAs always co-localized with GAD mRNA. Almost all cholinergic cells studied in the nucleus of the vertical limb of the diagonal band showed GIRK1 or GIRK2 mRNA labeling, whereas no GIRK3 mRNA was found. Therefore, GIRK channels in this area are likely to contain GIRK1 and GIRK2 subunits.

5-HT$_{1A}$ receptor distribution and co-localization with GIRK subunits. GIRK mRNAs expression in serotonergic cells of the dorsal raphe nucleus

Similarly to cholinergic cells, we found expression of GIRK subunits in serotonergic cells of the raphe nuclei as identified by the presence of serotonin transporter mRNA. This suggests that GIRK channels are able to mediate inhibitory responses in this area and regulate serotonin release from the raphe nuclei to the hippocampus, septum and other projection areas. Consequently, these channels could have an important role in learning and memory.

On the other hand, it has been long know that the activation of GPCR coupled to Gi/o proteins can cause the opening of GIRK channels through a membrane-delimited pathway. It has already been proved that, in mammalian neurons and in Xenopus oocytes, GIRK channels can be activated by neurotransmitters through GPCR such as the serotonin 5-HT$_{1A}$, muscarinic M$_2$, dopamine D$_2$, D$_3$ and D$_4$, α$_2$-adrenergic, metabotropic mGluR1a, 2 and 7, GABA$_B$, neuropeptide Y1 and Y2, cannabinoid, melatonin, adenosine A$_1$, μ-, δ- and κ-opioid and nociceptin/orphanin receptors (Andrade et al., 1986; North, 1989; Brown and Birnbaumer, 1990; Hille, 1992; Lesage et al., 1995; Kim et al., 1995; Murer et al., 1997; Karschin, 1999). Moreover, GIRK subunit knock-out mice have revealed important roles for GIRK channels in the generation of IPSPs in hippocampal and cerebellar neurons, where they serve as a common effector for various neurotransmitters. For instance, in hippocampal neurons of GIRK2 knock-out mice there is an evident loss of responsiveness both to 5-HT$_{1A}$ and GABA$_B$ agonists (Luscher et al., 1997; Slesinger et al., 1997). Therefore, since GIRK channels could be acting as important effectors of 5-HT$_{1A}$ receptors upon activation by serotonin or serotonin agonists, we carried out co-localization assays in the adult rat brain, in order to determine whether GIRK subunits are expressed in 5-HT$_{1A}$ positive cells and could thus mediate regulatory responses through this receptor. The distribution pattern of the 5-HT$_{1A}$ receptor and its mRNA reported here is in accordance with previous data from Kia et al. (1996) and Chalmers and Watson (1991) and Pompeiano et al. (1992), respectively. In general, there is a good concordance between 5-HT$_{1A}$ protein distribution and the expression of its mRNA, both showing the highest densities in the hippocampus, cerebral cortex, septum and raphe nuclei. It is worth mentioning that clear mismatches are found in nuclei such as the habenula as well as in the hypothalamic supraoptic, paraventricular and arcuate nuclei, where we

and other authors (Collin et al., 2002) find $5-HT_{1A}$ receptor immunoreactive neurons but the corresponding mRNA is not detected (see also Chalmers and Watson 1991). These mismatches might be due to particular turnover rates of $5-HT_{1A}$ receptor protein and/or mRNA in these regions compared to other brain areas, which might result in undetectable levels of this mRNA. We found GIRK subunits co-expressed with both somatodendritic $5-HT_{1A}$ autoreceptors in the raphe nuclei and with postsynaptic $5-HT_{1A}$ heteroreceptors in projection areas. Our results suggest that GIRK channels can mediate inhibitory responses in these areas when serotonin or its agonists activate the serotonin receptor $5-HT_{1A}$.

In summary, this work provides for the first time a useful and until now unknown evidence showing that most glutamatergic, GABAergic, cholinergic and serotonergic cells in the rat brain areas studied show mRNA codifying the GIRK channels forming subunits GIRK1, 2 and 3. Therefore, these channels can be found expressed in the plasmatic membrane of these cells controlling the release of both excitatory and inhibitory neurotransmitters to their projection areas and so, they could play a very important role in the regulation of neuronal excitability of different neurons in a variety of brain regions. Moreover, the close relationship between $5-HT_{1A}$ receptor mRNA and GIRK1-3 channel mRNAs on both GABAergic and glutamatergic neurons of the rat hippocampus, provides an anatomical framework to explain the important role as modulator of hippocampal functions, such as learning/memory and emotional state, of serotonin via $5-HT_{1A}$ receptors using as effectors the GIRK channels coupled to this receptor trough the heterotrimeric $G_{i/o}$ proteins. This may have important clinical implications in aging, dementia and affective disorders.

On the one hand, since there is a high colocalizacion between GIRK subunits and the serotonin receptor, and also with the serotonin transporter (marker of serotonergic neurons) in the dorsal raphe nucleus, we may think that substances that could specifically inhibit these channels in this brain area would be useful in controlling the depressive illness. In fact, with current antidepressant treatments, such as selective serotonin reuptake inhibitors, we look for an increase in the neurotransmitter serotonin levels in areas such as the hippocampus. By inhibiting the activity of these GIRK channels, we could induce a greater depolarization of serotonergic neurons at the raphe nucleus and therefore, achieve higher levels of this neurotransmitter in projection areas.

On the other hand, the fact that these GIRK channels are expressed in a wide variety of brain structures is indicative of their importance as effectors of many different receptors and therefore, their pharmacological control can be a great benefit to the treatment of a wide range of pathologies such as anxiety disorders, prevention of damage in situations of ischemia, treatment of drug addiction, and so forth, as has been pointed out by other authors before (Blednov et al., 2001; Mao et al., 2002; Morgan et al., 2003; Kobayashi et al., 2006).

Therefore, a greater knowledge of the brain location of the GIRK subunits presented in this paper may present a starting point for considering these channels as future therapeutic targets in the control of various pathologies as well as to understand some of the adverse effects caused by drugs currently used in therapeutic such as some antidepressants that acting on GIRK channels at heart can cause bradycardy in overdose (Kobayashi et al., 2003; Kobayashi et al., 2004).

FIGURE LEGENDS

Figure 1.- A-C: Specificity control of the hybridization signal with the GIRK1-1 (137-181) oligonucleotide probe. Rat coronal consecutive sections were hybridized with the ^{33}P-labeled GIRK1-1 probe. Hybridized sections were washed at increasing temperatures of 60ºC (**A**), 70ºC (**B**) and 80ºC (**C**). A large decrease in the hybridization signal could be observed at 80ºC. Black spots in C are non specific staining. **D-F**: Cohybridization experiments. The signal obtained with the GIRK1-1 labeled oligonucleotide (**A**) was completely blocked by competition with a 50-fold excess of the corresponding unlabeled probe (**D**). The same hybridization pattern as with the GIRK1-1 probe was observed with the ^{33}P-labeled GIRK1-2 (352-396) oligonucleotide probe (**E**). The signal obtained with the GIRK1-2 labeled oligonucleotide (**E**) was completely pageed by competition with a 50-fold excess of the corresponding unlabeled probe (**F**). Pictures are digital images taken from film autoradiograms. Scale bar = 3 mm.

Figure 2.- Detection of GIRK1-3 subunits immunoreactivity in rat brain cortical membranes. **A1**. Representative immunoblotting of GIRK1 subunits in samples of cortical membranes without (lanes 1, 2 and 3 on the right) and after preincubation with the synthetic peptide used as the immunogen (lanes 4, 5 and 6 on the left). **A2**. GIRK1 appears as a triplet, with the three bands representing differentially glycosylated versions of the core polypeptide. Standard curves for the 60 KDa version of the GIRK1 subunits were generated with incremental amounts of total membrane protein ranging from 1 to 6 μg. **A3**. The correlation coefficient was obtained by linear regression analysis and was r^2 = 0.9720. **B1**. Representative Western blots of GIRK2 subunit. Note that immunoblots labelled with anti-GIRK2 exhibited two positive bands (right), of which only the 75 KDa band disappeared after preincubation with the synthetic peptide used as the immunogen (left). **B2-B3**. Immunoblotting with incremental amounts of protein ranging from 2.5 to 20 μg, allowed to obtain the corresponding standard curves from which the correlation coefficient (r^2 = 0.9879) was estimated by linear regression analysis. **C1**. Representative Western blots of GIRK3 subunit immunoreactivity in samples of cortical membranes without (right) and after preincubation with the blocking peptide (left). **C2**. Standard curves were generated by immunoblotting using incremental amounts of total membrane protein ranging from 5 to 40 μg. **C3**. The correlation coefficient was obtained by linear regression analysis and was r^2 = 0.9664.

The positions of molecular weight markers are indicated on the left in A1, B1 and C1.

Figure 3.- Regional distribution of the different GIRK subunit mRNAs and 5-HT$_{1A}$ mRNA in consecutive sections of the rat brain at different rostrocaudal levels. Autoradiographic images show GIRK1 (**A1-G1**), GIRK2 (**A2-G2**), GIRK3 (**A3-G3**) and 5-HT$_{1A}$ receptor (**A4-G4**) mRNAs visualized by *in situ* hybridization with the corresponding ^{33}P-labeled oligonucleotides. Pictures are digital photographs from film autoradiograms. **A5-G5** show photographs of a Nissl stained section for each level. Scale bar = 3 mm.

Figure 4.- Laminar distribution of GIRK subunit mRNAs and detail of positively immunostained neural cells in the rat frontal cortex. Dark-field photomicrographs from emulsion-dipped coronal sections through cortical layers indicated by roman numbers where GIRK1 (**A1**), GIRK2 (**B1**) and GIRK3 (**C1**) mRNA-containing cells (white silver grains) appear localized in the different layers. Detail of GIRK1-IR cells in layer V (**A2**), GIRK2- IR cells in layer V (**B2**) and GIRK3-IR cells in layer III (**C3**). Scale bars = 400 μm in A1-C1; 20 μm in A2-C2.

Figure 5.- Expression of GIRK subunit and 5-HT$_{1A}$ receptor mRNAs and detail of positively immunostained neurons in rat hippocampus. Dark-field photomicrographs from emulsion-dipped coronal sections through the hippocampal region showing the presence of mRNA coding for GIRK1 (**A1**), GIRK2 (**B1**), GIRK3 (**C1**) and 5-HT$_{1A}$ (**D1**). Detail of GIRK1-IR cells in the hilus of the dentate gyrus (**A2**), GIRK2-IR cells in the CA3 field (**B2**), GIRK3-IR cells in the CA1 field (**C2**) and 5-HT$_{1A}$ receptor-IR cells in the CA3 field of the hippocampus (**D2**). Scale bars = 400 μm in A1-D1; 20 μm in A2-D2.

Figure 6.- Expression of GIRK subunit mRNAs in emulsion-dipped sections and detail of positively immunostained neurons in rat habenular nuclei. Dark-field photomicrographs from coronal sections through the habenular region showing the presence of mRNA coding for GIRK1 (**A1, A2**), GIRK2 (**B1, B2**) and GIRK3 (**C1, C2**). **A2, B2** and **C2** are higher magnification photomicrographs of the medial habenular nucleus. Detail of GIRK1-IR cells in the medial habenular nucleus (**A3**), GIRK2-IR cells in the lateral habenular nucleus (**B3**) and GIRK3-IR cells in the medial habenular nucleus (**C3**). Scale bars = 400 μm in A1-C1; 50 μm in A2-C2; 20 μm in A3-C3.

Figure 7.- Distribution of GIRK subunit mRNAs in the rat thalamus as observed on emulsion-dipped sections under dark-field illumination. Photographs show GIRK1 (**A1-A3**), GIRK2 (**B1-B3**) and GIRK3 (**C1-C3**) mRNAs. Scale bars = 400 μm in A1-C1; 100 μm in A2-C2; 50 μm in A3-C3.

Figure 8.- Bright-field photomicrographs showing GIRK1 subunit positively immunostained neurons in the supraoptic nucleus (**A1, A2**) and the paraventricular nucleus of the hypothalamus (**B1, B2**). Scale bar = 100 μm in A1-B1; 20 μm in A2-B2.

Figure 9.- Distribution of GIRK subunit mRNAs in the rat cerebellum. Emulsion-dipped sections show GIRK1 (**A1-A3**), GIRK2 (**B1-B3**) and GIRK3 (**C1-C3**) mRNAs. **A3-C3** illustrate cresyl violet staining in the same labeled sections showed in A2-C2, respectively. Arrowheads in A2-C2 and A3-C3 point to Purkinje cells. Note the absence of GIRK1 mRNA in Punkinje cells in A2 and A3. Scale bars = 400 μm in A1-C1; 50 μm in A2-C2 and A3-C3.

Figure 10.- Detail of GIRK2 subunit-immunostained neurons for the in the rat red nucleus (**A1, A2**). Scale bars = 100 μm in A1; 20 μm in A2.

Figure 11.- GIRK2 immunoreactive neural cells in the rat substantia nigra (**A**) and a dark-field photomicrograph of this area showing the presence of mRNA coding for GIRK2 (**B**). Detail of neural cell bodies in the substantia nigra compact part (**C**) and reticular part (**D**). Scale bars = 100 μm in A, B; 20 μm in C and D.

Figure 12.- High-magnification photomicrographs showing the simultaneous detection of two GIRK mRNAs in different brain areas. One of the subunits was detected by using ^{33}P-labeled oligonucleotides (^{33}P) and the other with digoxigenin-labeled probes (Dig) as indicated. Photomicrographs were taken from the dentate gyrus (**A-C**), the pyramidal layer of the hippocampus (CA1 field) (**D-F**), the parietal cortex (**G-I**) and the thalamus (**J-L**). White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 10 μm.

Figure 13.- High-magnification photomicrographs showing the simultaneous detection of two species of mRNA using Dig- labeled probes for vGluT mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of GIRK subunits GIRK1 (**A1-D1**), GIRK2 (**A2-D2**) and GIRK3 (**A3-D3**) in the dentate gyrus of the hippocampus **(A1-A3),** pyramidal layer of the hippocampus (CA1 field) (**B1-B3**), parietal cortex **(C1-C3)** and thalamus (**D1-D3**). White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 20 μm.

Figure 14.- High-magnification photomicrographs showing the simultaneous detection of two species of mRNA using Dig- labeled probes for GAD mRNA and ^{33}P-labeled oligonucleotide probes for the mRNA of GIRK subunits GIRK1 (**A1-D1**), GIRK2 (**A2-D2**) and GIRK3 (**A3-D3**) mRNA in the dentate gyrus of the hippocampus (**A1-A3**), pyramidal layer of the hippocampus (CA1 field) (**B1-B3**), parietal cortex (**C1-C3**), and reticular thalamic nucleus (**D1-D3**). White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 20 μm.

Figure 15.- High-magnification photomicrographs showing the simultaneous detection of two species of mRNA using ^{33}P-labeled oligonucleotide probes for GIRK1 (**A-D**) and GIRK2 (**E-H**) subunits (silver grains) and Dig- labeled probes for ChAT mRNA in the nucleus of the vertical limb of the diagonal band. White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 20 μm.

Figure 16.- High-magnification photomicrographs showing the simultaneous detection of two species of mRNA using ^{33}P-labeled oligonucleotide probes for the serotonin receptor 5-HT$_{1A}$ (silver grains) and Dig- labeled probes for the mRNAs of GIRK subunits: GIRK1 (**A1-E1**), GIRK2 (**A2-E2**) and GIRK3 (**A3-E3**) in the dentate gyrus of the hippocampus (**A1-A3**), pyramidal layer of the hippocampus (CA1 field) (**B1-B3**), parietal cortex (**C1-C3**), septum (**D1-D3**), dorsal raphe nucleus (**E1-E3**). White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 20 μm.

Figure 17.- High-magnification photomicrographs showing the simultaneous detection of two species of mRNA using ^{33}P-labeled oligonucleotide probes for GIRK1 (**A**), GIRK2 (**B**) and GIRK3 (**C**) subunits (silver grains) and Dig-labeled probes for the serotonin transporter mRNA in the dorsal raphe nucleus. White arrowheads point to single Dig-labeled cells. White arrowheads point to single Dig-labeled cells. Black arrowheads point to single ^{33}P-labeled cells. Black-and-white arrowheads point to double-labeled cells. Scale bar = 20 µm.

TABLE OF ABBREVIATIONS

AD	anterodorsal thalamic nucleus
APTS	3-aminopropyltriethoxysilane
Arc	arcuate nucleus
CA1	field CA1 of hippocampus
CA2	field CA2 of hippocampus
CA3	field CA3 of hippocampus
ChAT	choline acetyl transferase
CM	central medial thalamic nucleus
CPu	caudate-putamen
DG	dentate gyrus of the hippocampus
Dig	digoxigenin
DMH	dorsomedial hypothalamic area
DR	dorsal raphe nucleus
DS	dorsal septal nucleus
ECL	enhanced chemiluminiscence system
ER	endoplasmic reticulum
GAD	glutamic acid descarboxylase
GIRK	G-protein inwardly rectifying potassium channel (Kir3)
GL	granular cell layer of cerebellum
5-HT	serotonin
IC	inferior colliculus
ICM	major island of Calleja
IG	indusium griseum
IP	interpeduncular nucleus
IPSP	inhibitory postsynaptic potential
IR	immunoreactive or immunoreactivity
Kir3	G-protein inwardly rectifying potassium channel (GIRK)
LC	locus coeruleus
LDDM	laterodorsal thalamic nucleus, dorsomedial part
LDVL	laterodorsal thalamic nucleus, ventrolateral part
LHb	lateral habenular nucleus
MG	medial geniculate nucleus

MHb	medial habenular nucleus
MnR	median raphe nucleus
MS	medial septal nucleus
NGS	normal goat serum
Pa	paraventricular nucleus
PFCx	prefrontal cortex
Pir	piriform cortex
PK	Purkinje cell layer
Pn	pontine nuclei
Rt	reticular thalamic nucleus
SDS	sodium dodecyl sulfate
SNC	substantia nigra, compact part
SNR	substantia nigra, reticular part
SO	supraoptic nucleus
ST	serotonin transporter
Ts65Dn	segmental tryisomy 16, Down syndrome model
Tu	olfactory tubercle
VDB	nucleus of the vertical limb of the diagonal band
vGluT	vesicular glutamate transporter
VMH	ventromedial hypothalamic nucleus
VS	ventral septal nucleus
wv	weaver mouse
ZI	zona incerta

TABLES

Table 1.- Estimated densities of immunoreactive cells and of mRNAs encoding GIRK subunits and 5-HT$_{1A}$ receptor in different regions of the rat brain.

The relative abundance of immunostained cell bodies is indicated as: "**++++**" very abundant (more than 2500 labeled elements/mm^2); "**+++**" abundant (1500-2500 labeled elements/mm^2); "**++**" moderate expression (1000-1500 labeled elements/mm^2); "**+**" low expression (less than 1000 labeled elements/mm^2); "-" consistent absence of detectable staining; "n.d" not determined.

The relative densities of labeled mRNAs obtained by semiquantification of film autoradiograms are expressed as: "**+++**" very high (relative optical density higher than 0.5); "**++**" moderate (relative optical density from 0.35 to 0.5); "**+**" low (relative optical density lower than 0.35); "-" very low or undetectable; "n.d" not determined.

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Table 1. Estimated densities of immunoreactive cells and of mRNAs encoding GIRK subunits and 5-HT$_{1A}$ receptor in different regions of the rat brain.

Brain area	GIRK1		GIRK2		GIRK3		5HT$_{1A}$	
	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA
Primary cortical areas (Motor, Somatosensory, Auditory, Visual cortices)								
Layer I	-	-	intense neuropil	-	-	-	-	-
Layer II-III	+++	++	abundant fibres	++	+++	++	++	-
Layer IV	+++	+++	+	++	++	+++	++	-
Layer V	+++	++	+++	++	++	++	+++	+
Layer VI	+++	++	+++	++	++	++	+++	+
Prelimbic, Infralimbic, anterior Cingulate, Insular and Orbital Cortices, and Parietal and Temporal association cortical areas								
Layer I	-	-	intense neuropil	-	-	-	-	-
Layers II-VI	++	++	abundant fibres	++	++	++	++	-
Piriform cortex	++++	+++	++++	+++	+++	++	++	+
Entorhinal and Retrosplenial Cortices								
Layer II	++	+++	abundant fibres	++	++	++/+++	++	++
Inner layers	++	+/++	abundant fibres	++	++	+/++	++	+
Hippocampus and related areas			intense neuropil					
Dentate gyrus	++++	+++	++++	+++	++++	++	++++	+++
Ammon´s horn								
CA1	+++	+++	+++	+++	+++	++	++	++
CA2	+++	+++	+++	+++	+++	++	++	++
CA3	+++	+++	+++	+++	+++	++	+++	++
Subiculum and presubiculum	+++	+++	++	++	++	++		
Taenia tecta	+++	n.d	+++	n.d	++	n.d	+	n.d
Septum and Diagonal Band								
Ventral septal nucleus	+++	+	abundant fibres	++	abundant fibres.	+	n.d	++
Medial septal nucleus	+++	+	abundant fibres	++	abundant fibres	+	+	++
Dorsal septal nucleus	++	+	abundant fibres	++	abundant fibres	+	n.d	++
Ventral limb of the diagonal band	++	+	+++	++	+++	+	+	-
Basal Ganglia								
Caudate-putamen	+++	+	-	+	++	+	+	-
Globus pallidus	+	-	+	-	-	-	-	-
Amygdala	++	+	-	++	+++	+	++	-
Epithalamus			abundant fibres					
Lateral habenula	++++	+	+	++	++	+	+	-
Medial habenula	++++	++	+++	+++	+++	+	++++	-

Thalamus								
Anterodorsal nucleus	+++	++	+++	+++	++	++	+	-
Reticular nucleus	+++	++	++	++	++	++	+	-
Centromedial nucleus	+++	++	++	++	++	++	+	-
Laterodorsal nucleus, dorsomedial part	+++	++	+++	+++	++	++	+	-
Laterodorsal nucleus, ventrolateral part	+++	++	++	++	++	++	+	-
Hypothalamus								
Dorsomedial nucleus	+++	+	+	++	+++	+	+	+
Ventromedial nucleus	+++	+	+	+++	+++	+	+	++
Arcuate nucleus	++++	-	+++	+++	n.d	+	+++	-
Paraventricular nucleus	++++	+	+++	++	+++	+	++++	-
Supraoptic nucleus	++++	-	+++	+++	+++	-	++++	-
Brainstem								
Red nucleus	+	n.d	+	n.d	+	n.d	+	-
Ventral tegmental area	++	-	+ abundant fibres	+++	abundant fibres	-	n.d	-
Substantia nigra			abundant fibres		abundant fibres			
Compact part	++	-	+++	+++	+	-	+	-
Reticular part	+	-	-	+++	-	-	-	-
Dorsal raphe nucleus	+++	+	+	++	++	+	+++	+++
Pontine nuclei	+++	++	+++	++	++	+	++	+
Locus coeruleus	n.d	+	n.d	++	n.d	+	n.d	++
Cerebellum								
Granule cell layer	+++	+++	+++	+++	+++	+++	-	-

The relative abundance of immunostained cell bodies is indicated as: "++++" very abundant (more than 2500 labeled elements/mm^2); "+++" abundant (1500-2500 labeled elements/mm^2); "++" moderate expression (1000-1500 labeled elements/mm^2); "+" low expression (less than 1000 labeled elements/mm^2); "-" consistent absence of detectable staining; "n.d" not determined.

The relative densities of labeled mRNAs obtained by semiquantification of film autoradiograms are expressed as: "+++" very high (relative optical density higher than 0.5); "++" moderate (relative optical density from 0.35 to 0.5); "+" low (relative optical density lower than 0.35); "-" very low or undetectable; "n.d" not determined.