Published in final edited form as:

Neuroscience. 2009 April 21; 160(1): 85–96. doi:10.1016/j.neuroscience.2009.02.043.

EXPRESSION OF GABA_A RECEPTOR $\alpha 3$ -, θ -, AND ϵ -SUBUNIT mRNAs DURING RAT CNS DEVELOPMENT AND IMMUNOLocalIZATION OF THE ϵ SUBUNIT IN DEVELOPING POSTNATAL SPINAL CORD

J.-R. PAPE^a, S. S. BERTRAND^b, P. LAFON^a, M.-F. ODESSA^b, M. CHAIGNIAU^a, J. K. STILES^c, and M. GARRET^{b,*}

^a Université de Bordeaux, CNRS, UMR 5228, France

^b Université de Bordeaux, CNRS, UMR 5227, 146 Rue Léo Saignat, 33076 France

^c Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA, USA

Abstract

Ionotropic GABA_A receptors are heteromeric structures composed of a combination of five from at least 16 different subunits. Subunit genes are expressed in distinct cell types at specific times during development. The most abundant native GABA_A receptors consist of $\alpha 1$ -, $\beta 2$ -, and $\gamma 2$ -subunits that are co-expressed in numerous brain areas. $\alpha 3$ -, θ -, And ϵ -subunits are clustered on the X chromosome and show striking overlapping expression patterns throughout the adult rat brain. To establish whether these subunits are temporally and spatially co-expressed, we used *in situ* hybridization to analyze their expression throughout rat development from embryonic stage E14 to postnatal stage P12. Each transcript exhibited a unique or a shared regional and temporal developmental expression profile. The thalamic expression pattern evolved from a restricted expression of ϵ and θ transcripts before birth, to a θ and $\alpha 3$ expression at birth, and finally to a grouped ϵ , θ and $\alpha 3$ expression post-partum. However, strong similarities occurred, such as a grouped expression of the three subunits within the hypothalamus, tegmentum and pontine nuclei throughout the developmental process. At early stages of development (E17), ϵ and θ appeared to have a greater spatial distribution before the dominance of the $\alpha 3$ subunit transcript around birth. We also revealed expression of $\alpha 3$, θ , and ϵ in the developing spinal cord and identified neurons that express ϵ in the post-natal dorsal horn, intermediolateral column and motoneurons. Our findings suggest that various combinations of $\alpha 3$ -, θ - and ϵ -subunits may be assembled at a regional and developmental level in the brain.

Keywords

neuroepithelium; cortex; hindbrain; motoneurons; intermediolateral column

The physiological actions of GABA, the major inhibitory neurotransmitter in the adult CNS, are mediated by the activation of ionotropic and metabotropic receptors (Sieghart, 2000). Ionotropic GABA_A receptors are heteropentameric structures generated by coassembly of $\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, ϵ , θ and π subunits (Sieghart, 1995; Hevers and Luddens, 1998). Many studies have demonstrated that the subunit composition determines both functional

*Corresponding author. Tel: +33-557571686; fax: +33-556901421. Maurice.garret@u-bordeaux2.fr (M. Garret).

properties and subcellular localization of GABA_A receptors. The major native GABA_A receptor subtype is believed to be composed of $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits (Pirker et al., 2000; Olsen and Sieghart, 2008). The encoding genes of these three subunits are clustered on the same chromosome, suggesting a possible relationship with a genetic control of native GABA_A receptor composition.

The most recently identified subunits (ϵ and θ) of the GABA_A receptor gene family are clustered on the X chromosome along with the $\alpha 3$ subunit (Davies et al., 1997; Garret et al., 1997; Whiting et al., 1997; Bonnert et al., 1999). The $\alpha 3$ -, θ - and ϵ -subunits show striking overlapping expression patterns throughout the adult rat brain, including major neuronal groups with a broad range of influence, such as the cholinergic, dopaminergic, serotonergic, and noradrenergic systems. These data suggest that these subunits may assemble to form novel GABA_A receptors, although properties of native ϵ - and θ -containing receptors have not been fully characterized (Bonnert et al., 1999; Kasparov et al., 2001; Sergeeva et al., 2005; Wagner et al., 2005; Ranna et al., 2006; Taupignon et al., 2008). The co-expression of $\alpha 3$, θ and ϵ also suggests they share regulatory elements. However, it is not known whether these subunits have specific or common developmental regulations of their expression patterns.

It is well established that the subunit composition and the function of GABA_A receptors changes during CNS development (Maric et al., 2001; Ben-Ari et al., 2007). They are expressed early in the embryonic stages where they drive the excitatory action of GABA in immature neurons. GABA_A receptor signaling has been shown to be crucial for proliferation, migration, differentiation, and synaptogenesis (Ben-Ari, 2002; Meier, 2003; Meier et al., 2003; Lujan et al., 2005). Expression of different α , β , γ and δ subunits undergoes major temporal changes. For example, $\alpha 2$, $\alpha 3$, and $\beta 3$ subunits are expressed as early as embryonic stage E14; from birth to adulthood, their relative abundance is decreased, as compared to $\alpha 1$ and $\beta 2$ (Laurie et al., 1992; Yu et al., 2006). Conditional gene knockout has revealed the role of specific subunits in neuronal development (Ramadan et al., 2003) and several lines of evidence also suggest that the expression of some subunits is required for normal development (Ferguson et al., 2007). It is also now recognized that spinal cord development and physiology is regulated by GABA_A neurotransmission (Delpy et al., 2008; Knabl et al., 2008; Wang et al., 2008).

Taken together, this prompts us to analyze the expression of $\alpha 3$, ϵ and θ subunits in the developing rat CNS. We show here that each subunit has a unique temporal expression in the brain resulting in overlapping postnatal regional expression. We also detected expression of the three subunits in the embryonic spinal cord and have extended this study by immunolocalizing the expression of ϵ subunit in the developing spinal cord.

EXPERIMENTAL PROCEDURES

Tissues

Time-pregnant Wistar dams were purchased from Janvier Company (Le Genest-St. Louis, France). The experiments were carried out on whole embryos (E14, E17 and E19) and postnatal (P0, P06, P12) rat brains and spinal cords. All experiments complied with international guidelines on the ethical use of animals in the European Communities Council Directive dated 24 November 1986 (86/609/EEC) and with the guidelines of the Institutional Animal Care and Use Committee of Bordeaux University (agreement number AP2/5/2006). A significant effort was made to reduce the number of animals by limiting the number of dams used. Each developmental group consisted of for animals ($n=4$) coming from two dams. The gender of embryos/postnatal pups was not differentiated in our studies.

***In situ* hybridization**

The different nucleotidic probes used correspond to sequences with no significant sequence identity with other known GABA_A receptor subunit nucleotide sequences or other known nucleotide sequences available in databases. The rat GABA_A receptor ϵ probe sequence was obtained from the rat ϵ nucleotide sequence (nucleotides 1246–1728). The rat $\alpha 3$ and human θ cDNA probes derived from the $\alpha 3$ and θ sequences (nucleotides 1319–1742, accession number LO_8492 and 828–1884 accession number AF_144648, respectively). The specificity of these probes has previously been validated (Moragues et al., 2000). Radiolabeled antisense and sense cRNA probes were prepared by *in vitro* transcription of the pBluescript subclones with ³⁵S-UTP (>1000 Ci/mmol; Amersham, Saclay, France) using T7 or T3 RNA polymerase. The ³⁵S-labeled probes were purified on G50-sephadex and used at 20.10⁶ cpm/ml.

In situ hybridization was carried out on embedded (Tissue-Tek, USA) tissues processed into sagittal 14- μ m-thick cryostat serial sections. Embedded tissues corresponded to whole animals for embryonic stages, whole heads for newborns and brains for later stages. Sections were thaw mounted on gelatin-coated slides stored at –80 °C until used. Prior to hybridization, slices were fixed in a 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min. Hybridization was performed overnight at 55 °C (Le Moine et al., 1995). The hybridization solution was as follows: 20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1× Denhardt's, 250 mg/ml yeast tRNA, 100 mg/ml salmon sperm DNA, 0.1% sodium dodecyl sulfate (SDS), and 0.1% sodium thiosulfate. After 25 min of RNase A treatment (20 μ g/ml) at 37 °C, the slides were washed with 2× saline-sodium citrate (SSC) (twice for 5 min), 13 SSC (5 min), and 0.5× SSC (5 min), at room temperature, and rinsed in 0.1× SSC at 65 °C (twice for 30 min). The sections were dehydrated in graded alcohol series, dried and subsequently exposed to Kodak Bio Max Film for 6 weeks' exposure in the darkness of X-ray cassettes. For each subunit studied, hybridization, film exposure, digitization and quantification of sections were conducted in the same experiment. A slide carrying 14 sections of brain paste standards was exposed together with experimental sections. Films were then developed using standard photographic procedures.

Film autoradiographs were scanned at high resolution (1200 ppi) and analyzed with the aid of an image analysis software (Image Tools, UTHSCSA) that transforms the density of hybridization signal over a given tissue area into a mean grey level. Mean grey levels were arbitrarily ranked on a scale and graded relative to the highest signal. The complete pattern of ratings was arbitrarily distributed into four levels: none, weak, moderate and intense expression. The hybridization level of a given region is the compilation of all the punctuated measurements achieved on the region extent. Picture selection and digitalization of figures were done using Adobe Photoshop CS (ASI, USA) software.

Antibodies and immunohistochemistry

The rabbit antiserum to ϵ was directed at the N-terminal sequence of the protein (NH₂-WRVGPHIKLENKPPC-COOH) (Moragues et al., 2000). The anti-*Trypasonoma brucei* procyclin mouse monoclonal antibody was obtained from Cederlane (Hornby, Ontario, Canada).

Wistar rats were overdosed with pentobarbital until reflexes were lost and immediately perfused transcardially with 150 ml saline followed by 400 ml of a fixative solution containing 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains and spinal cords were dissected out and soaked overnight at 4 °C in phosphate buffer containing 20% sucrose. The tissues were then stored at –80 °C, and 50 μ m sections were prepared in a

cryostat and collected. Sections were incubated with the purified ϵ -antibody (1:500) or with antiprocyclin (1:1000) for 24 h at room temperature in a 0.1 M phosphate buffer, containing 0.2% Triton X-100 and 4% normal goat serum. For fluorescence labeling, sections were then incubated with goat antirabbit IgGs coupled with Alexa 568 (1:1000; Jackson ImmunoResearch, West Grove, USA) and/or goat antimouse IgGs coupled with fluorescein isothiocyanate (FITC) (1:200, Jackson ImmunoResearch). After washing, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined under a fluorescence microscope (Zeiss). DAB labeling was performed by incubation with peroxidase-labeled goat antimouse IgG (Vector) diluted 1:150 in PBS mixed with 1% normal goat serum at room temperature. The sections were visualized with the Vectastain ABC Kit (Vector). Fluorogold (1%; Fluorochrome, Denver, CO, USA) was injected intraperitoneally into rats 2 days before perfusion (Leong and Ling, 1990).

Western blot analysis on Wistar rat brain and spinal cord tissue was performed as described previously (Moragues et al., 2000).

RESULTS

Description of θ -, ϵ - and $\alpha 3$ -subunit expression patterns throughout rat brain development

In our study, we used *in situ* hybridization to analyze the mRNA expression patterns for three different GABA_A receptor subunits ($\alpha 3$, θ and ϵ) at six developmental stages (E14, E17, E19, P0, P06, P12) in the rat brain. No specific staining was seen in any of the developmental stages by the corresponding sense probes (data not shown). We arbitrarily divided the developing rat brain into nine areas of interest: cortex, striatum, thalamus, preoptic area (POA)/anterior hypothalamic area (AHA), hypothalamus, colliculus, tegmental area, pontine nuclei and medulla. Regions of positive hybridization were identified and mapped according to anatomical parcellation given by the atlas of the developing rat nervous system (Paxinos et al., 1994). Two strong tendencies emerged when the results were examined (Figs. 1, 2 and 3): from embryonic day 14 to postnatal day 12, specific brain regions were progressively recruited to express one or more GABA_A receptor subunits, and the level of expression of these subunits rose gradually. Patterns of subunit expression are illustrated in Fig. 1 for prenatal stages, Fig. 2 for postnatal stages, and are depicted in detail in Fig. 3 by a comprehensive table of histograms summarizing the expression levels of $\alpha 3$, ϵ , θ subunit transcripts for each developmental stage and brain region.

Regarding the expression of the θ subunit transcript, a noticeable early expression in the hindbrain exists in areas like the tegmentum, pontine nuclei and medulla. As development proceeds, by E17, the expression expands into the diencephalon while other important regions, like the cortex, colliculus and striatum, remain silent. Three interesting regional profiles showed residual or no detectable expression in the cortex, a weak but persistent expression in the thalamus and a permanent and temporally increasing expression in the tegmentum, pontine nuclei and medulla (Fig. 3).

Interestingly, the ϵ mRNA expression pattern is very wide at E14 and covers several scattered regions, including the cortex, thalamus, hypothalamus, colliculus and tegmentum (Figs. 1 and 3). The staining always matches with neuroepithelia, i.e. cortical or thalamic neuroepithelia, which give rise to dedicated mature regions. This early neuroepithelial induction highlights three remarkable ϵ expression profiles. A very early transcript expression is visible in the cortex, which peaks in intensity around E17, only to later disappear during the rest of the brain development. Such a transient temporal pattern is also partially found in the colliculus, where ϵ transcripts are no longer detected after E14 (Figs. 1 and 3). A variant to this latter profile is observable in the TH with a singular u-curve indicating expression recovery after birth. Although the postnatal expression of ϵ transcripts

is weak, this result observed in the thalamus clearly underlines the possibility for the organism to switch on/off ε subunit expression in a narrow temporal window (Fig. 3). The third type of pattern shows a weak, followed by a moderate, and then a high level of transcripts within several regions throughout brain development. This temporally increasing expression is clearly visible in a broad range of structures, including the POA/AHA, hypothalamus, tegmentum, pontine nuclei and medulla (Figs. 2 and 3). By the end of gestation (E19 to P0), a strong ε signal is clearly detected in the subfornical organ and the vascular organ of the lamina terminalis, which amplifies until P12 (Fig. 2).

A spatiotemporal comparison of θ , ε and $\alpha 3$ subunit expression patterns

Originally restricted to cortical and hypothalamic regions, $\alpha 3$ subunit mRNA staining expands at birth to all regions examined thus far, except for the striatum (Figs. 2 and 3). By E17, $\alpha 3$ exhibits a weak to moderate transcript expression in the mid- and hindbrain (i.e. the colliculus, tegmentum, pontine nuclei and medulla) and later follows a pattern similar to the one described for ε and θ . It is important to note that this similarity is observed at the scale of the selected regions and may not exist systematically when an examination was carried out at the level of each nucleus. For example, although the hypothalamus displays a signal for the three subunits, only ε and θ transcripts were detected in the lateral hypothalamic area (Fig. 2). The cortex and colliculus show little or no detectable staining for ε and θ transcripts regardless of the considered developmental stage, whereas the $\alpha 3$ signal is visible all throughout the chosen developmental window. Once again, remarkable $\alpha 3$ profiles emerged with a delayed expression in the hypothalamus starting at birth and an interesting transient expression in POA/AHA, starting prenatally around E19, ending postnatally around P06 and peaking in intensity around birth (Fig. 3). Our results are in line with previous results describing the presence of $\alpha 3$ transcripts in the cortex, hypothalamus and thalamus throughout rat brain development (Laurie et al., 1992).

Apart from the earliest stages, i.e. E14 and E17, we found a recurrent overlapping expression for ε and θ in the majority of the studied regions. Indeed, a thorough investigation provided a detailed panel of structures showing both an ε and θ signal: lateral and medial POAs; hypothalamic nuclei (Arc, DM, Mnu, VMH; see abbreviations above); brainstem nuclei (LC, DR, RPa; Fig. 2). On the other hand, $\alpha 3$ displays a spatiotemporal pattern of expression both intrinsically specific (i.e. cortex and colliculus) and similar to ε and θ . Fig. 3 clearly shows that the birth event is not linked to drastic changes in the number of expressed subunits in the regions but rather to subtle changes in expression levels.

Expression of the ε subunit in the developing rat spinal cord

In the spinal cord, we observed $\alpha 3$, θ and ε mRNAs expression all throughout the E14–E19 period. Because a Northern blot analysis revealed ε -subunit mRNA expression in humans (Whiting et al., 1997), we first tested expression of this subunit in postnatal rat spinal cords using immunohistochemical methods.

As expected, a Western blot analysis with ε -antiserum revealed a band at 70 kDa in membrane extracts from the hypothalamus (Moragues et al., 2000), as well as from the spinal cord (Fig. 4A). A band of approximately 120 kDa was also detected in spinal cord protein extracts, suggesting expression of the long ε -subunit splice variant (Sinkkonen et al., 2000; Moragues et al., 2000). The insert found in the long form of the rodent ε -subunit has been proposed to cross-react with a monoclonal antibody directed against procyclin, a surface glycoprotein coat of *Trypanosoma brucei* (Armah et al., 2006). In order to establish this cross-reactivity, we expressed the long and short forms of ε in COS-7 cells. Labeling showed that the long splice variant is revealed by both antiprocyclin and anti- ε (Fig. 4B), whereas the short form is only revealed by anti- ε antibodies (not shown). All neurons

labeled with anti-epsilon were also labeled with antiprocyclin (Fig. 4C, D) in brain nuclei, where expression of the ϵ -subunit was demonstrated (Moragues et al., 2000, 2002; Sinkkonen et al., 2000). Because antiprocyclin appeared to be a more efficient probe than anti- ϵ , we decided to use this antibody to study ϵ expression in the spinal cord.

Fig. 5 illustrates the gradual development of ϵ receptor expression from P0 to P12 at the different levels of the spinal cord. At P0, in all spinal segments (i.e. the cervical, thoracic and lumbar segments), immunoreactivity was detected in neurons located at the border of the superficial dorsal horn (SDH; arrows in Fig. 5) and in lamina VII. As development proceeds, ϵ expression expands into laminae X and IX and invades all of lamina VII (Fig. 5). In the thoracic spinal cord, a specific population of cells was labeled in the intermediolateral column region (CML) in lamina VII. As the CML contains the majority of the sympathetic preganglionic neurons (SPNs), we performed immunohistochemistry on sections from animals that had SPNs pre-labeled by i.p. injections of fluorogold, which is taken up by peripheral axons to retrogradely label motoneuronal and intermediolateral neurons (Leong and Ling, 1990). I.p. injections of fluorogold located SPNs and motoneurons, as described previously (Deuchars et al., 2001; Wilson et al., 2005), with labeled neurons evident in lamina IX at the three different levels of the cord (Fig. 6A, B, C) and SPNs in the thoracic segments (Fig. 6B, D). It was apparent that some of the neurons immunolabeled for ϵ were fluorogold positive (arrows in Fig. 6) in both lamina IX and the CML, indicating that at least a fraction of motoneurons and SPNs express the ϵ subunit.

DISCUSSION

In this study, we focused on the expression profile of ϵ , θ and $\alpha 3$ GABA_A receptor subunits whose genes are clustered on chromosome X. Except for $\alpha 3$ (Laurie et al., 1992; Pirker et al., 2000), previous studies have provided little information regarding the developmental expression of ϵ and θ within rodent brain (Sinkkonen et al., 2000), although GABA neurotransmission plays a major role during CNS development. In addition, expression of ϵ subunit in the spinal cord has not yet been considered. Here, we show that each subunit has both a specific temporal and local expression profile in some brain areas, but they are also co-expressed in many domains. In addition, we identify expression of the ϵ subunit in different spinal cord neurons.

Expression profile of ϵ subunit transcripts in the developing rat brain

A very transient cortical expression of the ϵ transcript at E14 and ending between E17 and E19 is visible. The distribution of the signal remains strictly confined to cortical neuroepithelium. The subsequent break in expression after E17 is certainly due to changes of gene expression within neurons, and not to the death of a population of neurons which, in the rat cortex, occurs massively in the third and fourth postnatal weeks (Aghajanian and Bloom, 1967; Oppenheim, 1991). By E14, radial and tangential migrations for native pyramidal neurons and GABAergic interneurons, respectively, start from germinal zones and are expected to end around E17 in the rat (Parnavelas, 2000; Marin and Rubenstein, 2001). In the cortex, migrating GABAergic interneurons must be synchronized with putative pyramidal cells before invading the cortical plate and then settling down at the proper layer (Lopez-Bendito et al., 2008). Our results suggest the existence of a GABAergic message mediated by the ϵ subunit during a short developmental interval (E14/E17). Because $\alpha 3$ is clearly expressed in the neocortex at this developmental stage as well (see Fig. 3), we can envisage the presence of a functional $\alpha 3\beta \epsilon$ assembly. In regard to this, it has been recently demonstrated that the human $\alpha 3\beta 1\epsilon$ receptor subtype expressed in *Xenopus* oocytes was 100-fold more sensitive to GABA than $\mu 3\beta 1\gamma 2$ (Ranna et al., 2006). The transient expression of $\alpha 3\beta \epsilon$ with high affinity for GABA would fit in with the sequential arrival of migrating neurons.

Between E14 and E17, diencephalic (e.g. the POA/AHA and hypothalamus), mesencephalic (e.g. the colliculus) and myeloencephalic regions (e.g. the medulla) display an early expression of ϵ mRNA subunit, which gradually amplifies until P12. The ϵ transcript level reaches its maximum level of detection only in the tegmental and hypothalamic regions. For example, in the POA and hypothalamus, a strong signal is seen in anatomically well defined nuclei such as Arc, DM, Mnu or VMH, suggesting a probable link with the maturation of both the modulatory and neuroendocrine systems of the brain. This could involve the maturation of GnRH neurons which express ϵ (Moragues et al., 2003) and achieve their migration from the olfactory placode around E17. These neurons need an appropriate GABAergic environment to settle down properly at the right place in medial septum and POA (Bless et al., 2000; Heger et al., 2003).

Expression profile of $\alpha 3$ subunit transcripts in the developing rat brain

Specific regional and temporal changes in the expression of $\alpha 3$ have been previously demonstrated during differentiation, maturation and aging of the rat neocortex. Briefly, $\alpha 3$ is a predominant GABA_A subunit in differentiating neurons migrating to the cortical plate, and a high level of $\alpha 3$ protein expression is maintained postnatally in deep cortical layers until the first month (adolescence onset), before slowly decreasing for the rest of the lifespan (Maric et al., 2001; Yu et al., 2006). Our results confirm and complete this $\alpha 3$ expression profile observable within the inner layers of the developing rat neocortex. At E14, the cortex consists of a primordial plexiform layer that is split before E17 by the cortical plate of postmitotic neuroblasts (Jacobson et al., 1978; Miller, 1988). Although weakly detected, $\alpha 3$ transcript expression clearly exists by E14, which then reaches a moderate intensity level by E17 and finally stabilizes until P12. This kinetic of expression happens to be independent from the stratification of cortical neurons (layers I–VI), which is normally established by P06 (Jacobson et al., 1978; Miller, 1988). This is in agreement with previous data showing a differential expression of GABA_A receptor subtypes in the developing neocortex dependent on afferent innervating features (Paysan et al., 1997).

Beyond the cortex, we also detected expression of the $\alpha 3$ subunit at P12 in the glomerular and external plexiform layers of the olfactory bulb, thalamus, colliculus, amygdala and cranial nerve nuclei, as observed in the adult rat (Wisden et al., 1992; Pirker et al., 2000). In the developing rat brain, the $\alpha 3$ transcript was already observed in the cortex, the thalamus and, to a minor degree, the colliculus and the cerebellum (Laurie et al., 1992). Our present data provide additional information showing that the $\alpha 3$ transcript is also expressed in cerebral regions such as the hypothalamus, tegmentum, pontine nuclei and medulla. We noted that $\alpha 3$ expression endowed many profiles, as it is present very early in the hypothalamus (E14), relatively later (P0) in the thalamus and transiently between E19 and P06 within the POA/AHA. Interestingly, GABA_A receptors containing the $\alpha 3$ subunit were shown to predominate in the reticular thalamic nucleus and the loss of the $\alpha 3$ subunit in knockout mice is critical for both the maturation of the GABA_A receptor and the formation of GABAergic synapses. These findings have suggested a role for $\alpha 3$ in the regulation of the thalamocortical function (Fritschy and Mohler, 1995; Huntsman et al., 1999; Pirker et al., 2000; Studer et al., 2006). Our study points out that $\alpha 3$ GABA_A-mediated transmission in the thalamus and its corollary, a functional thalamocortical loop, cannot start before birth.

Comparison of ϵ , θ and $\alpha 3$ expression profiles in the developing rat brain

It is widely accepted that the various GABA_A receptor subunits arose as a consequence of gene duplication events that occurred during chordate evolution, thereby modifying both gene promoters and primary sequences (Russek, 1999; Darlison et al., 2005). In addition, nucleotide sequence analysis has shown a mammalian-specific evolution of ϵ and θ subunits (Martyniuk et al., 2007). Thus, a simultaneous study of ϵ , θ and $\alpha 3$, based on their common

chromosomal origin, was appropriate in order to indicate a temporary association and dissociation of their gene expressions during rat CNS development.

Expression of the human θ -subunit was reported in various brain regions, including the hypothalamus, amygdala, hippocampus, substantia nigra, dorsal raphe and locus coeruleus. Surprisingly, θ produces a functional receptor when co-expressed (and probably coassembled) with an α , a β and a γ -subunit (Bonnert et al., 1999). More recently, *in situ* hybridization revealed a particularly strong signal for the θ and ε subunit mRNA in rat locus coeruleus, in which co-expression was detectable from the first post-natal day (Moragues et al., 2000, 2002; Sinkkonen et al., 2000). Such perfect overlapping between ε and θ subunit profiles extended to other brainstem and hypothalamic nuclei.

The present study confirms a moderate to strong expression of these subunits in the same regions, namely the hypothalamus, tegmentum, pontine nuclei and medulla, visible from E17/E19 onwards. We also noticed that P12 ε and θ expression profiles do not differ from those described in adults (Moragues et al., 2000); this is consistent with findings for other subunits (Laurie et al., 1992). In particular, we found a ε and θ co-distribution in the dorsal raphe and the locus coeruleus leading us to make the assumption of a native receptor recruiting ε and/or θ subunits, in accordance with the distinctive demands of the developing midbrain. This is of interest since GABA_A receptors are strongly involved in trophic effects of GABA on embryonic brainstem monoamine neurons (Liu et al., 1997).

Taken together our data suggest that $\alpha 3$ -, θ - and ε -subunits shared some common regulatory elements as well as specific regulation. It is particularly obvious for $\alpha 3$ that have substantial expression in areas where ε and θ are not expressed. It is also the case for θ and ε that are not co-expressed in some restricted areas in developing (this work) and adult animals (Moragues et al., 2002).

Expression of the ε subunit in the developing rat spinal cord

The developing spinal cord expresses several GABA_A receptor subunits, including $\alpha 3$ (Serafini et al., 1998a,b). In addition, the present data showed the expression of θ and ε from the 14th gestational day onward. Expression of the ε subunit mRNA in spinal cord was demonstrated by a Northern blot analysis of human tissues (Whiting et al., 1997). Our analyses, using Western blot analysis, clearly corroborate these data. We also identified neurons expressing this subunit, particularly within the SDH and CML. It must be noted that the antibody used on spinal cord sections is directed to the long form of ε subunit (see Fig. 4); we cannot completely rule out the fact that, in the spinal cord, some neurons labeled by this antibody do not express the short form of ε . It is likely that this long form of ε does not form functional receptors, contrary to that of the short form (Davies et al., 2002).

RT-PCR experiments have identified ε mRNA expression in substantia gelatinosa tissue. This expression was related to specific pharmacological properties of inhibitory post-synaptic currents. Our detection of ε protein expression in some neurons within SDH supports this finding (Takahashi et al., 2006). The output neurons for the sympathetic nervous system, SPNs, are considered as critical links in particular reflex responses to maintain homeostasis. They are located mainly in the CML where they form the final common pathway from the CNS in the regulation of sympathetic function. SPNs are a functionally heterogeneous group of neurons involved in regulating the heart, adrenal medulla, vascular smooth muscle, liver, kidney and many other organs. They are subjected to inputs descending from higher brain regions, as well as strong influences from local interneurons, e.g. GABAergic interneurons located in lamina X (Deuchars et al., 2005). It is interesting to note that a tonic GABAergic inhibition was identified very recently in CML neurons. These GABAergic currents were sensitive to diazepam but insensitive to zolpidem

(Wang et al., 2008). This particular pharmacology could result from the expression of ϵ since we found a similar pharmacology in locus coeruleus neurons that express this subunit (Taupignon et al., 2008).

Our data identify the expression of the ϵ -subunit containing GABA_A receptors in ventral horn laminae (e.g. the ventromedial area and lamina IX) that contain the locomotor pacemakers and the motoneurons, respectively. The GABAergic system is a major neuromodulator of spinal motor networks that control posture and locomotion (Cazalets et al., 1994; Bertrand and Cazalets, 1999). During maturation, the GABAergic synaptic transmission switches from excitatory to inhibitory in the ventral part of the cord (Wu et al., 1992). In later stages of development, the level of GABA declines and glycine becomes the dominant inhibitory neurotransmitter between the two sides of the spinal cord (Allain et al., 2004, 2006). However, a recent study shows that part of the commissural interneurons, responsible for the coordination of locomotor activities located in the ventromedial area of spinal cord, are GABAergic interneurons (Weber et al., 2007).

Taken together, our data suggest a role for the ϵ subunit in GABAergic neurotransmission that control pain pathway processing, autonomic nervous system and locomotor pattern generation and modulation.

CONCLUSION

The fact that GABA_A receptor subunit genes are found in clusters suggests that such an arrangement facilitates their coordinate, developmental, and/or tissue-specific expression (Steiger and Russek, 2004). Evidence from the expression analysis indicates that GABA_A receptor subunit genes can be regulated by shared binding sites but also by unique regulatory elements (Steiger and Russek, 2004). Our data showed that these properties are shared by the θ - $\alpha 3$ - ϵ cluster. Thus, we provide evidence that during rat brain development $\alpha 3/\epsilon$, $\alpha 3/\theta$, θ/ϵ or $\alpha 3/\theta/\epsilon$ is co-expressed in regions suggesting that these subunit combinations may be included into native GABA_A receptor complexes. These subunits are expressed early in the CNS suggesting a possible involvement in development and in many aspects of the physiology as well.

Acknowledgments

This work has been supported by the CNRS, the University of Bordeaux 2 and the "Conseil Régional, Aquitaine." We would like to thank Laura Cardoit for technical assistance and Dr. Philippe Ciofi for helpful comments and discussions.

Abbreviations

AHA	anterior hypothalamic area
CML	intermediolateral column
FITC	fluorescein isothiocyanate
POA	preoptic area
SPN	sympathetic preganglionic neuron
SSC	saline-sodium citrate

Abbreviations used in the figures

Arc	arcuate nucleus
-----	-----------------

BTP	basal telencephalic plate
C	colliculus
CML	intermediolateral column
CPu	caudate putamen
CVD	caudoventrodorsal reticular nucleus
Cx	cortex
DLL	lateral lemniscus dorsal nucleus
DM	dorsomedial hypothalamic nucleus
DR	dorsal raphe nucleus
HIP	hippocampus
HT	hypothalamus
LH	lateral hypothalamic area
LC	locus coeruleus
LPO	lateral preoptic area
Md	medulla
Mnu	mamillary nuclei
MPO	medial preoptic area
Ob	olfactory bulb
Pn	pontine nuclei
RPa	raphe pallidus nucleus
SDH	superficial dorsal horn
SFO	subfornical organ
SC	spinal cord
Tg	tegmentum
TH	thalamus
VDB	vertical diagonal band
Vent	cardiac ventricle
VLL	lateral lemniscus ventral nucleus
Vm	ventromedian thalamic nucleus
VMH	ventromedian hypothalamic nucleus

References

- Aghajanian GK, Bloom FE. The formation of synaptic junctions in developing rat brain: a quantitative electron microscopic study. *Brain Res* 1967;6(4):716–727. [PubMed: 4169903]
- Allain AE, Bairi A, Meyrand P, Branchereau P. Ontogenic changes of the GABAergic system in the embryonic mouse spinal cord. *Brain Res* 2004;1000(1–2):134–147. [PubMed: 15053961]

- Allain AE, Bairi A, Meyrand P, Branchereau P. Expression of the glycinergic system during the course of embryonic development in the mouse spinal cord and its co-localization with GABA immunoreactivity. *J Comp Neurol* 2006;496(6):832–846. [PubMed: 16628621]
- Armah, HB.; Switzer, RC., III; Osmand, AP.; Stiles, JK. 2006 Abstract viewer/itinerary planner, Program No. 527.15/D40, online. Society for Neuroscience; Atlanta, GA: 2006. Procyclin-like immunoreactive populations of neurons in mouse brain.
<http://www.abstractsonline.com/viewer/SearchResults.asp>
- Ben-Ari Y. Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci* 2002;3(9):728–739. [PubMed: 12209121]
- Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev* 2007;87(4):1215–1284. [PubMed: 17928584]
- Bertrand S, Cazalets JR. Presynaptic GABAergic control of the locomotor drive in the isolated spinal cord of neonatal rats. *Eur J Neurosci* 1999;11(2):583–592. [PubMed: 10051758]
- Bless EP, Westaway WA, Schwarting GA, Tobet SA. Effects of gamma-aminobutyric acid(A) receptor manipulation on migrating gonadotropin-releasing hormone neurons through the entire migratory route in vivo and in vitro. *Endocrinology* 2000;141(3):1254–1262. [PubMed: 10698203]
- Bonnert TP, McKernan RM, Farrar S, le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Brown N, Wafford KA, Whiting PJ. Theta, a novel gamma-aminobutyric acid type A receptor subunit. *Proc Natl Acad Sci U S A* 1999;96(17):9891–9896. [PubMed: 10449790]
- Cazalets JR, Sqalli-Houssaini Y, Clarac F. GABAergic inactivation of the central pattern generators for locomotion in isolated neonatal rat spinal cord. *J Physiol* 1994;474(1):173–181. [PubMed: 8014893]
- Darlison MG, Pahal I, Thode C. Consequences of the evolution of the GABA_A receptor gene family. *Cell Mol Neurobiol* 2005;25(3–4):607–624. [PubMed: 16075381]
- Davies PA, Kirkness EF, Hales TG. Modulation by general anaesthetics of rat GABA_A receptors comprised of alpha 1 beta 3 and beta 3 subunits expressed in human embryonic kidney 293 cells. *Br J Pharmacol* 1997;120(5):899–909. [PubMed: 9138697]
- Davies PA, McCartney MR, Wang W, Hales TG, Kirkness EF. Alternative transcripts of the GABA_A receptor epsilon subunit in human and rat. *Neuropharmacology* 2002;43(4):467–475. [PubMed: 12367593]
- Delpy A, Allain AE, Meyrand P, Branchereau P. NKCC1 co-transporter inactivation underlies embryonic development of chloride-mediated inhibition in mouse spinal motoneuron. *J Physiol* 2008;586(4):1059–1075. [PubMed: 18096599]
- Deuchars SA, Brooke RE, Deuchars J. Adenosine A1 receptors reduce release from excitatory but not inhibitory synaptic inputs onto lateral horn neurons. *J Neurosci* 2001;21(16):6308–6320. [PubMed: 11487654]
- Deuchars SA, Milligan CJ, Stornetta RL, Deuchars J. GABAergic neurons in the central region of the spinal cord: a novel substrate for sympathetic inhibition. *J Neurosci* 2005;25(5):1063–1070. [PubMed: 15689541]
- Ferguson C, Hardy SL, Werner DF, Hileman SM, Delorey TM, Homanics GE. New insight into the role of the beta3 subunit of the GABA_A-R in development, behavior, body weight regulation, and anesthesia revealed by conditional gene knockout. *BMC Neurosci* 2007;8:85. [PubMed: 17927825]
- Fritschy JM, Mohler H. GABA_A-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* 1995;359(1):154–194. [PubMed: 8557845]
- Garret M, Bascles L, Boue-Grabot E, Sartor P, Charron G, Bloch B, Margolskee RF. An mRNA encoding a putative GABA-gated chloride channel is expressed in the human cardiac conduction system. *J Neurochem* 1997;68(4):1382–1389. [PubMed: 9084408]
- Heger S, Seney M, Bless E, Schwarting GA, Bilger M, Mungenast A, Ojeda SR, Tobet SA. Overexpression of glutamic acid de-carboxylase-67 (GAD-67) in gonadotropin-releasing hormone

- neurons disrupts migratory fate and female reproductive function in mice. *Endocrinology* 2003;144(6):2566–2579. [PubMed: 12746320]
- Hevers W, Luddens H. The diversity of GABA_A receptors. Pharmacological and electrophysiological properties of GABA_A channel subtypes. *Mol Neurobiol* 1998;18(1):35–86. [PubMed: 9824848]
- Huntsman MM, Munoz A, Jones EG. Temporal modulation of GABA_A receptor subunit gene expression in developing monkey cerebral cortex. *Neuroscience* 1999;91(4):1223–1245. [PubMed: 10391431]
- Jacobson S, Butters N, Tovskey NJ. Afferent and efferent subcortical projections of behaviorally defined sectors of prefrontal granular cortex. *Brain Res* 1978;159(2):279–296. [PubMed: 103596]
- Kasparov S, Davies KA, Patel UA, Boscan P, Garret M, Paton JF. GABA_A receptor epsilon-subunit may confer benzodiazepine insensitivity to the caudal aspect of the nucleus tractus solitarii of the rat. *J Physiol* 2001;536 (Pt 3):785–796. [PubMed: 11691872]
- Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU. Reversal of pathological pain through specific spinal GABA_A receptor subtypes. *Nature* 2008;451 (7176):330–334. [PubMed: 18202657]
- Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABA_A receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 1992;12(11):4151–4172. [PubMed: 1331359]
- Le Moine C, Normand E, Bloch B. Use of non-radioactive probes for mRNA detection by in situ hybridization: interests and applications in the central nervous system. *Cell Mol Biol (Noisy-le-grand)* 1995;41(7):917–923.
- Leong SK, Ling EA. Labelling neurons with fluorescent dyes administered via intravenous, subcutaneous or intraperitoneal route. *J Neurosci Methods* 1990;32(1):15–23. [PubMed: 2186224]
- Liu J, Morrow AL, Devaud L, Grayson DR, Lauder JM. GABA_A receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *J Neurosci* 1997;17(7):2420–2428. [PubMed: 9065503]
- Lopez-Bendito G, Sanchez-Alcaniz JA, Pla R, Borrell V, Pico E, Valdeolmillos M, Marin O. Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. *J Neurosci* 2008;28(7):1613–1624. [PubMed: 18272682]
- Lujan R, Shigemoto R, Lopez-Bendito G. Glutamate and GABA receptor signalling in the developing brain. *Neuroscience* 2005;130 (3):567–580. [PubMed: 15590141]
- Maric D, Liu QY, Maric I, Chaudry S, Chang YH, Smith SV, Sieghart W, Fritschy JM, Barker JL. GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and facilitates neurite outgrowth via GABA_A autoreceptor/Cl[−] channels. *J Neurosci* 2001;21(7):2343–2360. [PubMed: 11264309]
- Marin O, Rubenstein JL. A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci* 2001;2(11):780–790. [PubMed: 11715055]
- Martyniuk CJ, Aris-Brosou S, Drouin G, Cahn J, Trudeau VL. Early evolution of ionotropic GABA receptors and selective regimes acting on the mammalian-specific theta and epsilon subunits. *PLoS ONE* 2007;2(9):e894. [PubMed: 17878929]
- Meier J. The enigma of transmitter-selective receptor accumulation at developing inhibitory synapses. *Cell Tissue Res* 2003;311 (3):271–276. [PubMed: 12658435]
- Meier J, Akyeli J, Kirischuk S, Grantyn R. GABA_A receptor activity and PKC control inhibitory synaptogenesis in CNS tissue slices. *Mol Cell Neurosci* 2003;23(4):600–613. [PubMed: 12932440]
- Miller. Development of projection and local circuit neurons in neocortex. In: Peters, AJE., editor. *Cerebral cortex*. Vol. 7. London: Plenum; 1988. p. 133-176.
- Moragues N, Ciofi P, Lafon P, Odessa MF, Tramu G, Garret M. cDNA cloning and expression of a gamma-aminobutyric acid A receptor epsilon-subunit in rat brain. *Eur J Neurosci* 2000;12(12):4318–4330. [PubMed: 11122342]
- Moragues N, Ciofi P, Lafon P, Tramu G, Garret M. GABA_A receptor epsilon subunit expression in identified peptidergic neurons of the rat hypothalamus. *Brain Res* 2003;967(1–2):285–289. [PubMed: 12650990]

- Moragues N, Ciofi P, Tramu G, Garret M. Localisation of GABA_A receptor epsilon-subunit in cholinergic and aminergic neurones and evidence for co-distribution with the theta-subunit in rat brain. *Neuroscience* 2002;111(3):657–669. [PubMed: 12031352]
- Olsen RW, Sieghart W. GABA_A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 2008;60:243–260.
- Oppenheim RW. Cell death during development of the nervous system. *Annu Rev Neurosci* 1991;14:453–501. [PubMed: 2031577]
- Parnavelas JG. The origin and migration of cortical neurones: new vistas. *Trends Neurosci* 2000;23(3):126–131. [PubMed: 10675917]
- Paxinos, G.; Ashwell, KWS.; Törk, I. Atlas of the developing rat nervous system. 2. San Diego: Academic Press; 1994.
- Paysan J, Kossel A, Bolz J, Fritschy JM. Area-specific regulation of gamma-aminobutyric acid type A receptor subtypes by thalamic afferents in developing rat neocortex. *Proc Natl Acad Sci U S A* 1997;94(13):6995–7000. [PubMed: 9192680]
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA_A receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 2000;101(4):815–850. [PubMed: 11113332]
- Ramadan E, Fu Z, Losi G, Homanics GE, Neale JH, Vicini S. GABA_A receptor beta3 subunit deletion decreases alpha2/3 subunits and IPSC duration. *J Neurophysiol* 2003;89(1):128–134. [PubMed: 12522165]
- Ranna M, Sinkkonen ST, Moykkynen T, Uusi-Oukari M, Korpi ER. Impact of epsilon and theta subunits on pharmacological properties of alpha3beta1 GABA_A receptors expressed in *Xenopus* oocytes. *BMC Pharmacol* 2006;6:1. [PubMed: 16412217]
- Russek SJ. Evolution of GABA_A receptor diversity in the human genome. *Gene* 1999;227(2):213–222. [PubMed: 10023064]
- Serafini R, Ma W, Maric D, Maric I, Lahjouji F, Sieghart W, Barker JL. Initially expressed early rat embryonic GABA_A receptor Cl[−] ion channels exhibit heterogeneous channel properties. *Eur J Neurosci* 1998a;10(5):1771–1783. [PubMed: 9751149]
- Serafini R, Maric D, Maric I, Ma W, Fritschy JM, Zhang L, Barker JL. Dominant GABA_A receptor/Cl[−] channel kinetics correlate with the relative expressions of alpha2, alpha3, alpha5 and beta3 subunits in embryonic rat neurones. *Eur J Neurosci* 1998b;10(1):334–349. [PubMed: 9753142]
- Sergeeva OA, Andreeva N, Garret M, Scherer A, Haas HL. Pharmacological properties of GABA_A receptors in rat hypothalamic neurons expressing the epsilon-subunit. *J Neurosci* 2005;25 (1):88–95. [PubMed: 15634770]
- Sieghart W. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* 1995;47(2):181–234. [PubMed: 7568326]
- Sieghart W. Unraveling the function of GABA_A receptor subtypes. *Trends Pharmacol Sci* 2000;21(11):411–413. [PubMed: 11121567]
- Sinkkonen ST, Hanna MC, Kirkness EF, Korpi ER. GABA_A receptor epsilon and theta subunits display unusual structural variation between species and are enriched in the rat locus ceruleus. *J Neurosci* 2000;20(10):3588–3595. [PubMed: 10804200]
- Steiger JL, Russek SJ. GABA_A receptors: building the bridge between subunit mRNAs, their promoters, and cognate transcription factors. *Pharmacol Ther* 2004;101(3):259–281. [PubMed: 15031002]
- Studer R, von Boehmer L, Haenggi T, Schweizer C, Benke D, Rudolph U, Fritschy JM. Alteration of GABAergic synapses and gephyrin clusters in the thalamic reticular nucleus of GABA_A receptor alpha3 subunit-null mice. *Eur J Neurosci* 2006;24(5):1307–1315. [PubMed: 16987218]
- Takahashi A, Tokunaga A, Yamanaka H, Mashimo T, Noguchi K, Uchida I. Two types of GABAergic miniature inhibitory postsynaptic currents in mouse substantia gelatinosa neurons. *Eur J Pharmacol* 2006;553:120–128. [PubMed: 17064685]
- Taupignon AI, Belujon P, Baufreton J, Grandoso L, Boué-Grabot E, Batten TFC, Ugedo L, Garret M. Inhibitory transmission in locus coeruleus neurons expressing GABA_A receptor epsilon subunit has a number of unique properties. *FENS Abstr* 2008;4:011–25.

- Wagner DA, Goldschen-Ohm MP, Hales TG, Jones MV. Kinetics and spontaneous open probability conferred by the epsilon subunit of the GABA_A receptor. *J Neurosci* 2005;25(45):10462–10468. [PubMed: 16280584]
- Wang L, Spary E, Deuchars J, Deuchars SA. Tonic GABAergic inhibition of sympathetic preganglionic neurons: a novel substrate for sympathetic control. *J Neurosci* 2008;28(47):12445–12452. [PubMed: 19020037]
- Weber I, Veress G, Szucs P, Antal M, Birinyi A. Neurotransmitter systems of commissural interneurons in the lumbar spinal cord of neonatal rats. *Brain Res* 2007;1178:65–72. [PubMed: 17920568]
- Whiting PJ, McAllister G, Vassilatis D, Bonnert TP, Heavens RP, Smith DW, Hewson L, O'Donnell R, Rigby MR, Sirinathsinghji DJ, Marshall G, Thompson SA, Wafford KA, Vasilatis D. Neuronally restricted RNA splicing regulates the expression of a novel GABA_A receptor subunit conferring atypical functional properties. *J Neurosci* 1997;17(13):5027–5037. [PubMed: 9185540]
- Wilson JM, Hartley R, Maxwell DJ, Todd AJ, Lieberam I, Kaltschmidt JA, Yoshida Y, Jessell TM, Brownstone RM. Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. *J Neurosci* 2005;25(24):5710–5719. [PubMed: 15958737]
- Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* 1992;12(3):1040–1062. [PubMed: 1312131]
- Wu WL, Ziskind-Conhaim L, Sweet MA. Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J Neurosci* 1992;12(10):3935–3945. [PubMed: 1403091]
- Yu ZY, Wang W, Fritschy JM, Witte OW, Redecker C. Changes in neocortical and hippocampal GABA_A receptor subunit distribution during brain maturation and aging. *Brain Res* 2006;1099(1):73–81. [PubMed: 16781682]

Fig. 1.

In situ hybridization histochemistry for $\alpha 3$ -, ϵ - and θ -mRNAs in sagittal sections of rat embryos. Low-power bright-field photomicrographs of E14-, E17-, E19-whole fetal bodies, as indicated at the top. Representative adjacent sagittal sections were hybridized with $\alpha 3$ -, ϵ - and θ - ^{35}S -labeled antisense probes, as indicated on the left side. Scale bars=5 mm.

Fig. 2.
In situ hybridization histochemistry for $\alpha 3$ -, ϵ - and θ -mRNAs in sagittal sections of rat brains. Low-power bright-field photomicrographs of whole heads (P0) or brains (P06, P12), as indicated at the top. Representative adjacent sagittal sections were hybridized with $\alpha 3$ -, ϵ - and θ - ^{35}S -labeled antisense probes, as indicated on the left side. Scale bars=5 mm.

Fig. 3.

Schematic representation of the expression of $\alpha 3$ (grey bars), ϵ (dark grey bars) and θ (black bars) mRNA in selected entire regions of the developing rat brain from E14 to P12. The POA/AHA region covers both the POA and the AHA. Four different arbitrary ratings symbolizing none or undetectable, weak, moderate and strong signal were systematically reported in the table to achieve a complete dynamic overview of $\alpha 3$, ϵ and θ expression throughout development.

Fig. 4.

Analysis of ϵ -subunit expression. (A) Western blot analysis of spinal cord (SC) and hypothalamus (HT) protein extracts from 12-day-old rats using ϵ -specific antiserum. The molecular masses (kDa) of marker proteins (Bio-Rad) are indicated on the left side. (B) COS-7 cells were transiently transfected with the long splice variant of rat ϵ -subunit. Fluorescent double-labeling was obtained after labeling cells with a monoclonal antibody directed at the insert found in the long rodent ϵ -variant, followed by FITC-conjugated secondary antibodies (B1) and a polyclonal antibody directed at the N-terminal peptide followed by Alexa Fluor 588-conjugated antibodies (B2). (C, D) Fluorescent double-labeling in frontal sections through the locus coeruleus (C) and B9 nucleus (D) performed as in B. Scale bars=10 μ m (B); 100 μ m (C); 20 μ m (D).

Fig. 5. ϵ -Immunoreactivity in cervical (top lane), thoracic (middle lane) and lumbar (bottom lane) frontal sections in P0, P06 and P12 rat spinal cords using an antiprocylin monoclonal antibody that detects the long form of ϵ subunit. At all developmental stages, immunolabeling of the ϵ -subunit could be observed within lamina IX containing the somatic motoneurons (Mn), the superficial dorsal horn (SDH, detailed in the inset in the P06 lumbar section) and in lamina VII (the area boxed in the P06 cervical section is shown at higher magnification in the inset). In thoracic segments, a specific labeling was observed in the intermediolateral column (CML, shown at higher magnification in the inset in the P0 thoracic section). Scale bars=300 μ m.

Fig. 6.

Photomicrographs showing fluorogold fluorescence (A1–C1) and labeling for the ϵ -subunit (A2–C2) in the frontal section of the spinal cord in the P12 cervical (A), P12 thoracic (B) and P06 lumbar segments (C). Sections were analyzed 2 days after the i.p. injection of fluorogold, immunolabeling of ϵ -subunit was revealed with Alexa Fluor 568–conjugated secondary antibodies. Neurons in lamina IX were double labeled (A, C, white arrows), suggesting they are motoneurons. In the thoracic segments (B), neurons in the intermediolateral column (CML, white frames in B1 and B2, detailed in b1 and b2) exhibit dual immunostaining (white arrows) for fluorogold (b1) and the ϵ -subunit (b2), suggesting they are SPNs. Black arrows point to single-labeled neurons. Scale bars=300 μ m (A, B); 100 μ m (C). The lower left panel displays a schematic drawing of the location of A to C in frontal spinal cord sections.