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Plasma membrane density of $GABA_B$-R1a, $GABA_B$-R1b, GABA-R2 and trimeric G-proteins in the course of postnatal development of rat brain cortex.

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List of abbreviations

Baclofen, β-p-chlorophenyl-GABA; Ca_v, voltage-dependent calcium channels; GABA, γ-aminobutyric acid; $GABA_B$-R, metabotropic receptor for GABA; GIRKs, inwardly rectifying potassium channels; GPCR, G-protein-coupled receptor; G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $G_s\alpha$, G-protein stimulating adenylyl cyclase activity; $G_i/G_o\alpha$, G-proteins inhibiting adenylyl cyclase activity in pertussis-toxin sensitive manner; $G_q/G_{11}\alpha$, G- proteins stimulating phoshoplipase C in pertussis-toxin independent manner; [^{35}S]GTPγS, guanosine-5´-[γ-^{35}S] triphosphate; PD, postnatal day; P_i, inorganic phosphate, PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; SKF 97541, aminopropyl (methyl) phosphinic acid; w.w., wet weight.

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With the aim to understand the onset of expression and developmental profile of plasma membrane (PM) content /density of crucial components of $GABA_B$-R signaling cascade, $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2, $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ subunit proteins were determined by quantitative immunoblotting and compared in PM isolated from brain cortex of rats of different ages: between postnatal-day-1 (PD1) and 90 (PD90). PM density of $GABA_B$-R1a, $GABA_B$-R2, $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ was high already at birth and further development was reflected in parallel decrease of both $GABA_B$-R1a and $GABA_B$-R2 subunits. The major decrease of $GABA_B$-R1a and $GABA_B$-R2 occurred between the birth and PD15: to 55% (R1a, **) and 51% (R2, **), respectively. Contrarily, PM level of the cognate G-proteins $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ was unchanged in the course of the whole postnatal period of brain cortex development.

Maturation of $GABA_B$-R cascade was substantially different from ontogenetic profile of prototypical plasma membrane marker, Na,K-ATPase, which was low at birth and further development was reflected in continuous increase of PM density of this enzyme. Major change occurred between the birth and PD25. In adult rats, membrane content of Na, K-ATPase was 3-times higher than around the birth.

Key words: $GABA_B$-R, postnatal development, rat brain cortex, G-proteins, Na, K-ATPase.

Short title: Ontogenesis of $GABA_B$-R signaling in rat brain cortex

Introduction

$GABA_B$-receptors were defined as a class of bicuculline-insensitive GABA receptors for which (-)-baclofen is a specific agonist and phaclofen and 2-hydroxy-saclofen represent specific antagonists (Hill and Bowery 1981; Bowery *et al*., 1985; 1987; Kerr and Ong 1995). These receptors are not physically bound to ionic channels and belong to the family of G-protein coupled receptors, GPCR (Kerr and Ong 1995). Thus, the primary signal initiated by binding of GABA to $GABA_B$-R is transmitted further downstream by trimeric G proteins.

The central nervous system is known to contain high levels of all trimeric Gα subunits. The three species of inhibitory G-proteins, $G_i\alpha1$, $G_i\alpha2$ and $G_i\alpha3$ (Mumby *et al.* 1988), the long ($G_s\alpha L$) and short ($G_s\alpha S$) variants of the stimulatory $G_s\alpha$ protein (Bray *et al.* 1986), phosphoinositidase C-linked $G_q\alpha$ and $G_{11}\alpha$ proteins (Milligan 1993) as well as representatives of $G_{12}\alpha/G_{13}\alpha$ family of G-proteins (Harhammer *et al.* 1994, 1996) were identified in brain tissue in high amounts. The major G-proteins of brain, however, are members of $G_o\alpha$ family. The two isoforms of $G_o\alpha$ subunits, $G_o\alpha1$ and $G_o\alpha2$, represent up to 1% of the total membrane protein in the brain tissue (Giershick *et al.* 1986; Goldsmith *et al.* 1987, 1988 Milligan 1988, 1990). Accordingly, the content of Gβ subunits is very high in brain (Asano *et al.* 1988). It should be also mentioned that the complexity of biochemical composition of the brain tissue is not limited to G-proteins, but it is equally high for adenylylcyclase (AC) molecules because all the isoforms (ACI-X) of this key regulatory enzyme of GPCR-initiated cascades were identified in CNS and their PM content was found to respond readily to physiological state of experimental animals (Ujcikova *et al*. 2011).

Regulation of the specificity and efficiency of coupling between GPCRs and trimeric G-proteins in natural tissue such as brain is therefore highly complex. GPCRs usually exert their action through activation of preferential G-proteins (in a given cell type), however, a single type of receptor can be also coupled to several G-proteins (Boege *et al.* 1991; Gerhardt and

Neubig 1991; Raymond 1995; Dascal 1997; Gudermann *et al.* 1997; Hildebrandt 1997). Furthermore, the given type of G-protein may be activated by different receptors. Under such conditions, it is reasonable to assume that a complicated functional arrangement denominated as *cross talk* among individual members of G-protein-mediated cascades exists and provides an effective regulatory mechanism for the convergence or divergence of actions of a single neurotransmitter in nervous tissue.

Receptor-initiated activation of G-proteins results in the release of free Gα and Gβγ subunits from the non-active Gαβγ trimer; subsequently, both free Gα and Gβγ subunits mediate the signal transmission further downstream. Thus, besides the functional networks of Gi1α-, Gi2α-, Gi3α-, Go1α- and Go2α-mediated signaling, Gβγ-mediated cascades represent no less complicated regulatory circuits. The main Gβγ-regulated effectors of presynaptic $GABA_B$-receptors are P/Q-and N-type voltage-dependent Ca^{2+} channels (Chen and van den Pol 1998; Bussieres and El Manira 1999; Barral *et al.* 2000). $GABA_B$-receptors inhibit these Ca^{2+} channels at both excitatory and inhibitory terminals, thereby restricting neurotransmitter release. Depending on whether the terminal releases an inhibitory or excitatory neurotransmitter, the presynaptic $GABA_B$ receptors increase or decrease the excitability of the postsynaptic neuron (Pinard *et al.* 2010).

Presynaptic $GABA_B$ receptors restrict neurotransmitter release not only by inhibiting Ca^{2+} channels but also by retarding the recruitment of synaptic vesicles (Sakaba and Neher 2003). More recent evidence suggests that pre-synaptic $GABA_B$ -receptors may couple to inwardly rectifying Kir3-type K^+ channels (also designated GIRK channels) to inhibit glutamate release (Ladera *et al.* 2008; Fernandez-Alacid *et al.* 2009); however, Kir3 channels are generally considered as the main effectors of post-synaptic $GABA_B$-receptors (Pinard *et al.* 2010).

Binding of GABA to post-synaptic $GABA_B$-R results in activation of Kir3 channels, induction of K^+ efflux and hyperpolarization of postsynaptic membrane. This change of membrane potential shunts excitatory currents in a non-specific way. Finally, under such conditions, the so-called slow inhibitory post-synaptic potentials (IPSPs) are generated. Activation of post-synaptic $GABA_B$-receptors was also found to decrease the activity of Ca^{2+} channels, which inhibit dendritic Ca^{2+}-spike propagation (Perez-Garci *et al.* 2006).

The present state of knowledge about the plasma membrane part of $GABA_B$-receptor signaling cascade in the brain may thus be described as a mutually interrelated regulatory network of $GABA_B$-R, PTX-sensitive G-proteins of $G_i/G_o\alpha$ family, various AC isoforms and ionic channels such as $GABA_A$-R (Xu and Wojcik 1986; Simonds 1999; Sunahara and Taussig 2002; Padgett and Schlesinger 2010; Pinard *et al.* 2010). Functionally, in this network, primary inhibitory signals proceeding at receptor level are followed by both positive and negative feedback regulatory loops tuning the whole regulatory circuit to an optimum output (Padgett and Schlesinger 2010; Pinard *et al.* 2010). These circuits are therefore highly complex and important for brain function as GABA represents the main inhibitory neurotransmitter of mammalian brain.

Our previous results indicated that the plasma membrane density of $GABA_B$-R, determined by a saturation binding assay with antagonist [^{3}H]CGP54626, was highest in 1-day-old animals and then it was dramatically decreased in 15- and 90-day-old rats (Kagan *et al.* 2012). Intrinsic efficacy of $GABA_B$-receptors, measured as agonist-stimulated, high-affinity [^{35}S]GTPγS binding, was also high at birth (PD1, PD2), however, it *increased* further during the first two weeks of postnatal life and reached the maximum between PD9 and PD15. In older rats, both baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was decreased so that the level in adult rats (PD90) was not different from that in newborn animals.

The aim of our present work was to establish the structural correlate to these functional studies of $GABA_B$-R ontogenesis by determination of PM density of $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2, $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ subunit proteins by quantitative immunoblottig with specific antibodies. We have also determined PTX-insensitive $G_{12}\alpha$ protein as a test of maturation of intracellular "membrane traffic", as vesicular transport within the neuron is an important part of optimum functioning of CNS. The general trend of brain cortex maturation was screened by analysis of prototypical plasma membrane marker, ouabain-dependent Na, K-ATPase (EC 3.6.1.3).

2. Material and methods

The experiments were approved by Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic to be in agreement with Animal Protection Law of the Czech Republic as well as European Comunity Council directives 86/609/EEC.

Chemicals and radiochemicals

$GABA_B$-receptor agonists baclofen (β-p-chlorophenyl-GABA), SKF 97541 [3-aminopropyl (methyl) phosphinic acid] and antagonist [^{3}H]CGP 54626 (41.5 Ci/mmol, cat. no. R1088) were purchased from Tocris. [21, 22-^{3}H]ouabain (30 mCi/mmol; NET211001) was from Perkin Elmer. The complete protease inhibitor cocktail was from Roche Diagnostic (cat. no. 1697498). All other chemicals were of highest quality available.

Primary antisera and antibodies

$G_i1/G_i2\alpha$, $G_i3\alpha$ and $G_o1/G_o2\alpha$ subunit proteins were identified by antipeptide antibodies prepared as described originally by Gierschik *et al.* (1986), Goldsmith *et al.* (1987), Backlund *et al.* (1988) and Milligan (1988, 1990). These antisera were previously characterized in our laboratory (Ihnatovych *et al.* 2002a). Polyclonal antibodies oriented

against $GABA_B$-R1 (R-300, sc-14006), $GABA_B$-R2 (H-300, sc-28792), Gβ (T-20, sc-378) $G_z\alpha$ (I-20, sc-388), $G_{12}\alpha$(S-20, sc-409) and α subunit of Na, K-ATPase (H-300, sc-28800) were from Santa Cruz.

Isolation of plasma membrane-enriched fraction from rat brain cortex

Rat brain cortex was minced with a razor blade on a pre-cooled plate and diluted in STEM medium containing 250 mM sucrose, 20 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.6, fresh 1 mM PMSF plus protease inhibitor cocktail. It was then homogenized mildly in loosely-fitting Teflon-glass homogenizer for 5 min (2 g w. w. per 10 ml) and centrifuged for 5 min at 3500 rpm. Resulting post-nuclear supernatant (PNS) was filtered through Nylon nets of decreasing size (330, 110 and 75 mesh, Nitex) and applied on top of Percoll in Beckman Ti70 tubes (30 ml of 27.4 % Percoll in STE medium). Centrifugation for 60 min at 30000 rpm (65000xg) resulted in the separation of two clearly visible layers (Bourova *et al.* 2009). The upper layer represented plasma membrane fraction (PM); the lower layer contained mitochondria (MITO). The upper layer was removed, diluted 1:3 in STEM medium and centrifuged in Beckman Ti70 rotor for 90 min at 50000 rpm (175000xg). Membrane sediment was removed from the compact, gel-like sediment of Percoll, re-homogenized by hand in a small volume of 50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4 (TME medium), snap frozen in liquid nitrogen and stored at -80 ºC.

SDS-PAGE and immunoblotting

Aliquots of PM were solubilised in NuPAGE SDS Sample Buffer (4x) with an addition of NuPAGE Sample Reducing Agent (10x) according to manufacturer's instructions. Samples were heated at 70 °C for 10 minutes, loaded at 10 μg/well and resolved by NuPAGE 4-12% or 10% Bis-Tris polyacrylamide gels (10 wells,1 mm thick) using 3-(N-morpholino) propane sulfonic acid (MOPS), sodium dodecyl sulfate (SDS) running buffer with NuPAGE Antioxidant prior to blotting on nitrocellulose membranes (Protran, Schleicher & Schuell).

Molecular mass determinations were based on pre-stained molecular mass markers (Sigma, SDS 7B).

After SDS-PAGE, the proteins were transferred to nitrocellulose and blocked for 1 h at room temperature in 5% (w/v) low-fat milk in TBS-Tween buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20]. Antibodies were added in TBS-Tween containing 1% (w/v) low-fat milk and incubated for at least 2 h. The primary antibody was then removed and the blot washed extensively (3x10 min) in TBS-Tween. Secondary antibodies (donkey anti-rabbit IgG conjugated with horseradish peroxidase) were diluted in TBS-Tween containing 1% (w/v) low-fat milk applied for 1 h, and after three 10-min washes the blots were developed by ECL technique using Super Signal West Dura (Pierce) as substrate. The developed blots were scanned with an imaging densitometer ScanJett 5370C (HP) and quantified by Aida Image Analyzer v. 3.28 (Ray test).

[^{3}H]CGP54626 binding; *one-point assay*

Membranes (100 μg protein per assay) were incubated with 12 nM [^{3}H]CGP54626 in a final volume of 100 μl of binding mix containing (A) 50 mM Tris-HCl (pH 7.4) alone, (B) 50 mM Tris-HCl (pH 7.4) plus 2.5 mM $CaCl_2$ or (C) 50 mM Tris-HCl (pH 7.4) plus 5 mM $MgCl_2$ for 60 min at 30°C. The bound and free radioactivity was separated by filtration and determined by liquid scintillation as described. Non-specific binding was determined in the presence of 1 mM GABA.

Na, K-ATPase*;* *[^{3}H]ouabain binding*

Sodium plus potassium-activated, oubain-dependent Na, K-ATPase (E.C. 3.6.1.3) was determined by "one-point" [^{3}H]ouabain binding assay according to Svoboda *et al.* (1988). Membranes (50 μg of protein) were incubated with 20 nM [^{3}H]ouabain in a total volume of 0.45 ml of 5 mM $NaHPO_4$, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.6 (Mg-Pi buffer) for 90 min at 30 ºC. The bound and free radioactivity was separated by filtration through Whatman GF/B

filters in Brandel cell harvester. Filters were washed 3× with 3 ml of ice-cold incubation buffer and placed in 4 ml of scintillation cocktail (CytoScint, ICN). Radioactivity remaining on filters was determined after 10 h at room temperature by liquid scintillation. Non-specific binding was determined in the presence of 1 μM unlabelled ouabain.

Statistical analysis

The significance of difference between the immunoblot signal determined in fetuses 1-day before the birth (100%) and signals determined at different age intervals (PD1, PD2, PD5, PD9, PD10, PD15, PD25, PD35, PD42, PD47, PD90) was analyzed by one-way test of variance ANOVA followed by Bonferroni's post-hoc comparison test using GraphPad*Prism4*.

One-way ANOVA followed by Bonferroni's post-hoc comparison test was also used for statistical analysis of the difference of [^{3}H]CGP54626 and [^{3}H]ouabain binding to PM isolated from 1-, 15- and 90-day-old rats.

Protein determination

Lowry method was used for determination of membrane protein using bovine serum albumin (Sigma, Fraction V) as a standard. Data were calculated by fitting the calibration curve as a quadratic equation.

Results

Our previous results indicated an early functional maturation of $GABA_B$-R signaling cascade in rat brain cortex (Kagan *at al.* 2012). Agonists baclofen and SKF97541 exhibited significant efficiency (both potency and efficacy) already at PD2 and the highest number of $GABA_B$-R, determined as maximum binding capacity (B_{max}) for specific antagonist [^{3}H]CGP54626, was determined in 1-day-old animals (PD1). In older rats, the number of [^{3}H]CGP54626 binding sites was decreased, in contrast to agonist-stimulated G-protein activity, which was increased during the first two weeks of postnatal life. The maximum of

agonist-stimulated G-protein activity, measured as baclofen- or SKF97541-stimulated [^{35}S]GTPγS binding, was observed on PD14-15. Maximum of [^{35}S]GTPγS binding was followed by continuous decrease of G-protein activity till the adulthood (90-day-old rats).

Immunoblot analysis of plasma membrane density of $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2 and of the cognate, PTX-sensitive G-proteins performed in this work (**Fig. 1** and **Fig. 2**) indicated that expression level of $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2 and of all individual members of G_i/G_o family ($G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$[1] and Gβ subunit proteins) was high already around the birth, i.e. in fetuses 1-day before the birth (D-1) and in 1- and 2-day-old rats (PD1 and PD2). Subsequently, membrane density of $GABA_B$-R1a, $GABA_B$-R1b and $GABA_B$-R2 was decreased more or less in parallel till PD15 (**Fig. 1**). At this age interval, the $GABA_B$-R subunits represented 55 ± 15 % ($GABA_B$-R1a), 70 ± 17 % ($GABA_B$-R1b) and 51 ± 5 % ($GABA_B$-R2) of the level detected in newborn rats, 100%. In early postnatal period (up to PD15), PM expression level of $GABA_B$-R1b was lower than of $GABA_B$-R1a.

By contrast, the membrane density of all G-proteins ($G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$, $G_{12}\alpha$ [1] and Gβ) was unchanged in the course of the whole postnatal period, i.e. between PD2 and PD90 (**Fig. 2**). Expressed in more detail, the immunoblot signals of all G-proteins in PM samples containing the same amount of protein (10 μg) and prepared from fetuses 1 day before the birth and 1-, 2- , 5-, 9-, 10-, 15-, 25-, 35-, 42-, 47- and 90-day-old rats, were the same, i.e. not statistically different when compared with the control signal in fetuses 1-day before the birth, 100%. Thus, there was a clear disparity between development of receptor and G-proteins functionally participating in $GABA_B$-R signaling cascade: *membrane density* of *$GABA_B$-R subunit proteins was substantially decreased between the birth and "opening of eyes" period, while the cognate, trimeric G-proteins of Gi/Go family were unchanged.*

[1] Though being insensitive to PTX and thus unrelated to $GABA_B$-R, the ontogenetic profile of $G_{12}\alpha$ protein was also measured with the aim to obtain information about the important group of G-proteins regulating membrane traffic (Harhammer et al., 1994; 1996; Hildebrant et al., 1997). The developmental change of these proteins was similar to that of other G-proteins. The prenatal level was high and afterwards, it decreased slowly and continuously till the adulthood.

In the second part of our work, we have extended our recent results (Kagan *et al.* 2012) and compared antagonist [^{3}H]CGP54626 binding in ion-free, 2.5 mM $CaCl_2$ and 5mM $MgCl_2$ containing incubation media (**Fig. 3**) in PM isolated from 1-, 13- and 90-days old rats. The use of ion-free incubation medium was introduced by Ko *et al*. (2003) for determination of the number of μ-, δ- and κ-opioid receptors in monkey brain cortex and hypothalamus. Comparison of the level of [^{3}H]CGP54626 binding in these media was performed by a "one-point assay" at the constant concentration of 15 nM of this radioligand.

The decrease of [^{3}H]CGP54626 binding was noticed in all incubation media, however, due the low level of binding, this decrease was not significant in ion-free medium (**Fig. 3**). The highly significant decrease was measured in 2.5 mM $CaCl_2$ ($p<0.001$) and 5mM $MgCl_2$ ($p<0.01$) containing media. Please note that the level of binding in 2.5 mM $CaCl_2$ was much higher that in 5 mM $MgCl_2$. This result reflects and may be interpreted as a natural consequence of the presence of 2.5 mM calcium in extracellular medium surrounding $GABA_B$-R ligand binding site located on $GABA_B$-R1 (Padgett and Slesinger 2010; Pinard *et al.* 2010) and is in agreement with the previous agonist binding studies of $GABA_B$-R in rat brain cortex synaptosomes (Bowery *et al.* 1983). The decrease of $GABA_B$-R1a, $GABA_B$-R1b and $GABA_B$-R2 subunits (**Fig. 1**) proceeded in parallel with the decrease of antagonist binding (**Fig. 3)**. However, it was terminated at PD15, while antagonist binding was decreased further till the adulthood (PD90).

Postnatal development of $GABA_B$-R1, $GABA_B$-R2, G-proteins and ligand binding to $GABA_B$-R was substantially different from maturation of the prototypical plasma membrane marker, Na, K-ATPase (**Fig. 4A,B**). Membrane density of α-subunit of Na, K-ATPase was low at birth (PD1, PD2) and further development was reflected in a marked increase of this protein. The major increase occurred between the birth and PD25. Since this age interval, PM content of Na, K -ATPase was not significantly altered in PM isolated from 35-, 42- and 90-

day-old rats. The intensity of average immunoblot signal in adult rats (PD90) was 3.5-times higher than around the birth (day -1, PD1 and PD2).

Virtually the same results were obtained when selective inhibitor [^{3}H]ouabain was used for determination of Na, K-ATPase (**Fig. 4C**). The major increase of [^{3}H]ouabain binding in PM was noticed between the birth and PD25. Since PD25, the binding of this radioligand was not significantly different from the adult animals. [^{3}H]ouabain binding in 90-day-old rats (13.89 pmol.mg^{-1}) was 1.6x higher than in 15-day-old rats (8.64 pmol.mg^{-1}) and 2.6x higher than in fetuses 1 day before the birth (5.44 pmol.mg^{-1}). Thus, the overall maturation of brain cortex PM composition monitored by a developmental study of Na, K-ATPase molecules proceeds after the birth, while the level of $GABA_B$-signaling proteins is high at birth and further decreased ($GABA_B$-R) or unchanged (G-proteins).

Discussion

In the brain, $GABA_B$-R-initiated signal transfer to G-proteins and from G-proteins to adenylyl cyclase (AC) represents a rather intricate trans-membrane process (Bormann 1988; Boege *et al.* 1991; Padgett and Slesinger 2010) because all its pivotal components occur in multiple isoforms with distinct functional properties, and the cognate G proteins of G_i/G_o family exert both stimulatory and inhibitory effects on the overall AC activity, which represents the final outcome of the ten different isoenzymes, ACI-X (Backlund *et al.* 1988; Tang *et al.* 1992; Taussig *et al.* 1994; Simonds 1999; Sunahara and Taussig 2002). Our previous analysis of postnatal development of adenylyl cyclase in various brain areas indicated a marked activation of this enzyme in membranes prepared from 12-15-day-old rats (Ihnatovych *et al.* 2002b). The activity of the basal-, manganese-, fluoride-, GTP- and forskolin-stimulated AC was low at birth (PD1), increased sharply during the first two weeks of postnatal life, reached a maximum between P12 and PD15 and then decreased to the level in 18-day-old rats. The

maximum AC activities were roughly 4-times higher than those at birth. In older rats, AC activities were decreased further so that the level in adult animals (PD90) was about the same as that at birth (PD1). These results had also shown that there was a marked difference between the development of AC enzyme activity and protein content of individual AC isoforms. The immunoblot analysis indicated no significant change of ACI, but a continuous increase of ACII, IV and VI from PD1 to PD18. Since this age, membrane density of ACI, II, IV and VI was unchanged until the adulthood.

When considering other effectors of $GABA_B$-R but AC, pre-synaptic inhibition of voltage-gated Ca^{2+} channels (Ca_v) by $GABA_B$-R agonists has to be primarily considered (Dunlap and Fishbach 1981; Robertson and Taylor 1986; Dolphin 1990, 1991; Santos *et al.* 1995). Presynaptic inhibition of Ca_v by $GABA_B$-R agonists was demonstrated at early stages of postnatal development in rat somatosensory cortex at PD7 (Fukuda *et al.* 1993) and in hippocampus at PD6 (DiScenna *et al.* 1994). Postsynaptic $GABA_B$-receptors were found to be functioning in the cerebral cortex of rats only at postnatal day 17, i.e. 10 days later than pre-synaptic ones (Fukuda *et al.* 1993). It may be therefore suggested that the significant efficacy of baclofen and SKF97541 when activating G-proteins in newborn rat brain cortex (Kagan *et al.* 2012) is physiologically related to pre-synaptic inhibition of Ca_v channels mediated by G_o family of G-proteins. In accordance with this suggestion, PM density of $G_o\alpha$ and Gβ proteins was high already at PD1 and PD2 (**Fig. 2**).

G-protein regulated, inwardly rectifying potassium channels (GIRKs) represent another plausible candidate for interpretation of our data besides AC and voltage-gated Ca^{2+} channels (Ca_v). Activation of the $GABA_B$-R increases membrane conductance for potassium and reduces neuronal excitability by direct activation of the GIRK channels via free Gβγ subunits released from trimeric G-protein complex (Misgeld *et al.* 1995; Watts *et al.* 1996). The $GABA_B$-receptor was shown to be primarily K^+-linked in the hippocampus (Gahwiler and Brown 1985).

GIRK channel activation by $G_{i/o}$-coupled GPCR results in hyperpolarization of the neuron and inhibition of neuronal activity (Dascal 1997). In this way, similarly to the $GABA_B$-Ca_V currents, the $GABA_B$-GIRK currents are considered as inhibitory ones. In similarity with $GABA_B$-R expression patterns, the $GABA_B$-GIRK currents have been identified in many brain regions including hippocampus, thalamus and cerebellum (Gahwiler and Brown 1985; Watts *et al.* 1996).

Functional characterization of individual $GABA_B$-R subunits in heterologous expression systems revealed a remarkable property of $GABA_B$-receptors: $GABA_B$-R1/$GABA_B$-R2 subunits must be co-expressed to form a functional $GABA_B$-receptor dimmer; when expressed individually, the subunits failed to form physiologically normal receptors (Jones *et al.* 1998; Couve *et al.* 1998; Kaupmann *et al.* 1998; Kuner *et al.* 1999; Galvez *et al.* 2001; Padgett and Slesinger 2010). It has been also discovered that the $GABA_B$-R1 subunit contains an endoplasmic reticulum (ER) retention signal, which prevents forward trafficking of this receptor subunit (Margeta-Mitrovic *et al.* 2000). Dimerization of $GABA_B$-R1 with the $GABA_B$-R2 shields ER retention signal and permits surface expression of both $GABA_B$-R1 and $GABA_B$-R2. Yeast two-hybrid analysis revealed that the C-terminus of $GABA_B$-R1 and $GABA_B$-R2 was an important pre-requisite for heterodimerization of these subunits (White *et al.* 1998). CD spectroscopic analysis of a 30 amino acid sequence in C-termini of these proteins revealed a coiled-coil domain between the $GABA_B$-R1 and $GABA_B$-R2, which was required for the subunit-specific formation of the functional receptor dimmer (Kammerer *et al.* 1999). Interestingly, the C-terminus of $GABA_B$-R2 subunit also regulates lateral diffusion of the receptor in hippocampal neurons suggesting that it helps to control receptor expression levels at the plasma membrane (Pooler and McIlhinney 2007).

In this work, the postnatal development of $GABA_B$-R1a, $GABA_B$-R1b and $GABA_B$-R2 was studied in plasma membranes isolated from brain cortex of rats of different ages by

Western blotting. PM density of $GABA_B$-R1a, $GABA_B$-R1b and $GABA_B$-R2 was determined in parallel with trimeric Gα- and Gβ-subunits. Antagonist [^{3}H]CGP54626 binding was measured in the same membrane samples. Subsequently, data collected on $GABA_B$-R were compared with the ontogenetic profile of prototypical plasma membrane marker Na, K-ATPase, which was used as an indicator of general brain development.

The detailed immunoblot analysis clearly showed that PM density of all $GABA_B$-R subunits was high at the birth (in fetuses D-1, PD1 and PD2) and subsequently it was largely decreased till PD15 **(Fig. 1)**. The $GABA_B$-R subunits in PM isolated from 15-days-old rats represented 55 % ($GABA_B$-R1a), 70 % ($GABA_B$-R1b) and 51 % ($GABA_B$-R2) of the level in newborn rats (100%), respectively. The postnatal decrease of $GABA_B$-R1b subunit was relatively smaller when compared with $GABA_B$-R1a and $GABA_B$-R2. The decrease of $GABA_B$-R1a, $GABA_B$-R1b and $GABA_B$-R2 was accompanied by a decrease in the number of antagonist [^{3}H]CGP54626 binding sites, which was demonstrated in the presence of both calcium (2.5 mM $CaCl_2$) and magnesium (5 mM $MgCl_2$) ions. The ion-free buffer, which has been successfully used in μ-, δ- and κ-opioid receptor binding assays (Ko et al., 2003), was impropriate for radioligad binding assays of $GABA_B$-R.

In accordance with our data, the high levels of $GABA_B$-R1a in synaptic membranes isolated from brain cortex were also detected in the first postnatal days by Malitschek *et al.* (1998) and Fritschy *et al.* (1999) and these high levels of $GABA_B$-R1a were subsequently decreased till the adulthood. Both authors also described the different expression pattern for $GABA_B$-R1a and $GABA_B$-R1b isoforms. $GABA_B$-R1b was less abundant at birth than $GABA_B$-R1a, slightly increasing at postnatal days 10-14 and then decreasing till adulthood. Marked increase (3x) of PM density of Na,K-ATPase molecules was fully consistent with developmental study of Na,K-ATPase activity indicating manifold increase (5x) in membranes prepared by sucrose-density gradient centrifugation from the whole rat brain

(Samson and Quinn, 1967).

Our data thus indicated that the functional maturation of $GABA_B$-R signaling pathway is not finished at birth, in spite of the fact that these receptors are expressed in high amount (**Fig. 1**) and exhibit considerable ability to activate G-proteins with maximum of baclofen-stimulated [^{35}S]GTPγS binding at PD14-15 (Kagan *et al.* 2012). Increase of [^{35}S]GTPγS binding between the birth and PD14-15 was followed by a decrease in 18-day-old rats and further decrease till the adulthood (PD90). Accordingly, the peak value of [^{3}H]GABA binding was detected at PD14 in rat brain cortical slices by quantitative autoradiography and this high level of [^{3}H]GABA binding subsequently declined to the adult level (Turgeon and Albin 1994).

Contrarily, membrane density of all members of Gi/Go family of G-proteins was unchanged in the course of the whole postnatal development. The explanation why the "average" G-protein level in PM is unchanged in spite of the major change of G-protein function characterized by the peak level of baclofen- or SKF97541-stimulated [^{35}S]GTPγS binding at PD14-15 (Kagan at al., 2011), is unknown at present. It may be related to signaling via other effectors than AC (Ca_v and GIRKs); it may also reflect the fact that the data collected in mixture of all PM fragments do not reveal heterogeneities of protein composition in different PM compartments denominated as *membrane domains /rafts* (Moffett et al., 2000; Becher *et al.* 2001, 2004).

Conclusions

Our data indicate that the full complement of $GABA_B$-receptor protein molecules and cognate G-proteins exists in rat brain cortex already at birth. Functional maturation of $GABA_B$-R cascade in the course of the first two weeks of postnatal life was associated with a parallel decrease of plasma membrane density of $GABA_B$-R1a (55 ± 15 %) and $GABA_B$-R2 (51 ± 5%) subunits; $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$, $G_{12}\alpha$ and Gβ, $GABA_B$-R1b proteins were

unchanged. Decrease of $GABA_B$-R subunits proceeded together with the decrease of antagonist [^{3}H]CGP54626 binding measured in ion-free, 2.5 mM $CaCl_2$ or 5 mM $MgCl_2$. *The age interval between PD1 and PD14-15 represents the critical period for structural as well as functional maturation of $GABA_B$-R signaling cascade in rat brain cortex plasma membranes.*

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Figures

Fig. 1

Plasma membrane density of $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2 subunit proteins; *immunoblot analysis*

PM proteins (10 μg per well) were resolved by Invitrogen NuPAGE system in 4-12% gradient gel and identified by immunoblotting with specific antibodies as described in Methods. Data represent the average of five immunoblots ± S.E.M.

Significance of the difference between the immunoblot signal determined in fetuses 1 day before the birth (D-1, 100%) and signals determined at different ages (PD1, PD2, PD5, PD9, PD10, PD15, PD25, PD35, PD42, PD47, PD90) was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test using GraphPad*Prism4*.

$GABA_B$-R1a: D-1 vs 15 (**, $P < 0.01$), PD2 vs PD15 (**, $P < 0.01$), PD2 vs PD90 (**, $P < 0.01$), PD15 vs PD90 (NS, $P > 0.05$). **$GABA_B$-R1b:** D-1 vs PD15 (NS, $P > 0.05$), PD2 vs PD15 (NS, $P > 0.05$), PD15 vs PD90 (NS, $P > 0.05$), D-1 vs PD90 (**, $P < 0.01$), PD2 vs PD90 (*, $P < 0.05$). **$GABA_B$-R2:** D-1 vs PD15 (*, $P < 0.05$), PD2 vs PD15 (**, $P < 0.01$), PD2 vs PD90 (**, $P < 0.01$), PD15 vs PD90 (NS, $P > 0.05$).

Fig.2

Plasma membrane density of $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$, Gβ and $G_{12}\alpha$ subunit proteins; *immunoblot analysis*

The expression of G-proteins was analyzed in the same plasma membrane samples as those used for detection of $GABA_B$-R subunits. G-proteins were unchanged in the course of the whole postnatal period as there was no significant difference between immunoblot signals detected around the birth (D-1, PD1, PD2) and at all other ages: $G_i1/G_i2\alpha$ ($p > 0.05$); $G_i3\alpha$ ($p > 0.05$), $G_o\alpha$ ($p > 0.05$), $G_z\alpha$ ($p > 0.05$) and $G\beta$ ($p > 0.05$) at all ages. $G_{12}\alpha$ was decreased between PD1 and PD90 (*, $p < 0.05$).

Fig. 3

Decrease of [^{3}H]CGP54626A binding in the course of postnatal ontogenesis

PM (100 μg protein per assay) isolated from 1-(PD1), 13-(PD13) and 90-(PD90)-day-old rats were incubated with 12 nM [^{3}H]CGP54626A in 50 mM Tris-HCl with no additions (□, open columns), in 50 mM Tris plus 2.5 mM $CaCl_2$ (■, full columns) or in 50 mM Tris-HCl plus 5 mM $MgCl_2$ (≡, hatched columns). Non-specific binding was determined in the presence of 1 mM GABA. Data represent the average of 3 binding assays performed in quadruplicates ± S.E.M. Comparison of binding data in PM isolated from PD1, PD13 and PD90 was performed by one-way ANOVA followed by Bonferroni's post-hoc comparison test.

Open columns (□): PD1 versus PD13, NS, $p>0.05$; PD1 versus PD90, NS, $p>0.05$; PD13 versus PD90, NS, $p>0.05$. Full columns (■): PD1 versus PD13, **, $p<0.01$; PD1 versus PD90, ***, $p<0.001$; PD13 versus PD90, ***, $p<0.001$. Hatched columns (≡): PD1 versus PD13, NS, $p>0.05$; PD1 versus PD90, **, $p<0.01$; PD13 versus PD90, NS, $p>0.05$.

Fig. 4

Plasma membrane density of Na, K-ATPase; *immunoblot analysis and [^{3}H]ouabain binding*

Immunoblot detection of α-subunit of Na, K-ATPase was performed by polyclonal Ab (Santa Cruz, sc-28800). (A) Typical immunoblot. (B) Average of 5 immunoblots. The significance of the difference between the immunoblot signal determined in fetuses 1-day before the birth (100%) and signals determined at different ages (PD1, PD2, PD5, PD9, PD10, PD15, PD25, PD35, PD42, PD47, PD90) was analyzed by one-way ANOVA followed by Bonferroni's test using GraphPad Prism 4. Since PD5, the increase of Na,K-ATPase was highly significant (**, $p < 0.01$).

(C) [^{3}H]ouabain binding was measured as described in Methods. Data represent the average ± S.E.M. of three experiments performed in triplicates. Significance of the difference between the binding at different age intervals was analyzed by one-way ANOVA followed by Bonferroni's test: fetuses D-1 versus PD15 (*, $p<0.05$), D-1 versus PD25 (**, $p<0.01$), D-1 versus PD90 (**, $p<0.01$), PD15 versus PD25 (*, $p<0.05$), PD15 versus PD90 (**, $p<0.01$), PD25 versus PD90 (NS, $p>0.05$).

Fig. 1

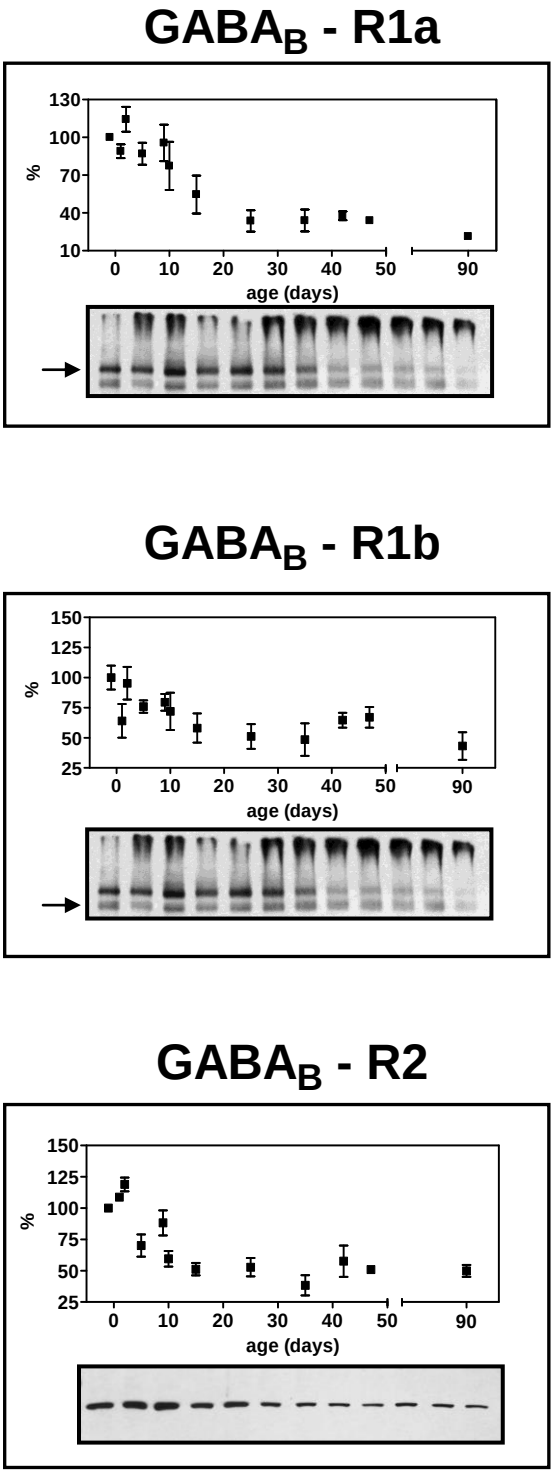

GABA$_B$ - R1a
130
100
70
40
10
%
0
10
20
30
40
50
90
age (days)
GABA$_B$ - R1b
150
125
100
75
50
25
%
0
10
20
30
40
50
90
age (days)
GABA$_B$ - R2
150
125
100
75
50
25
%
0
10
20
30
40
50
90
age (days)

Fig. 2

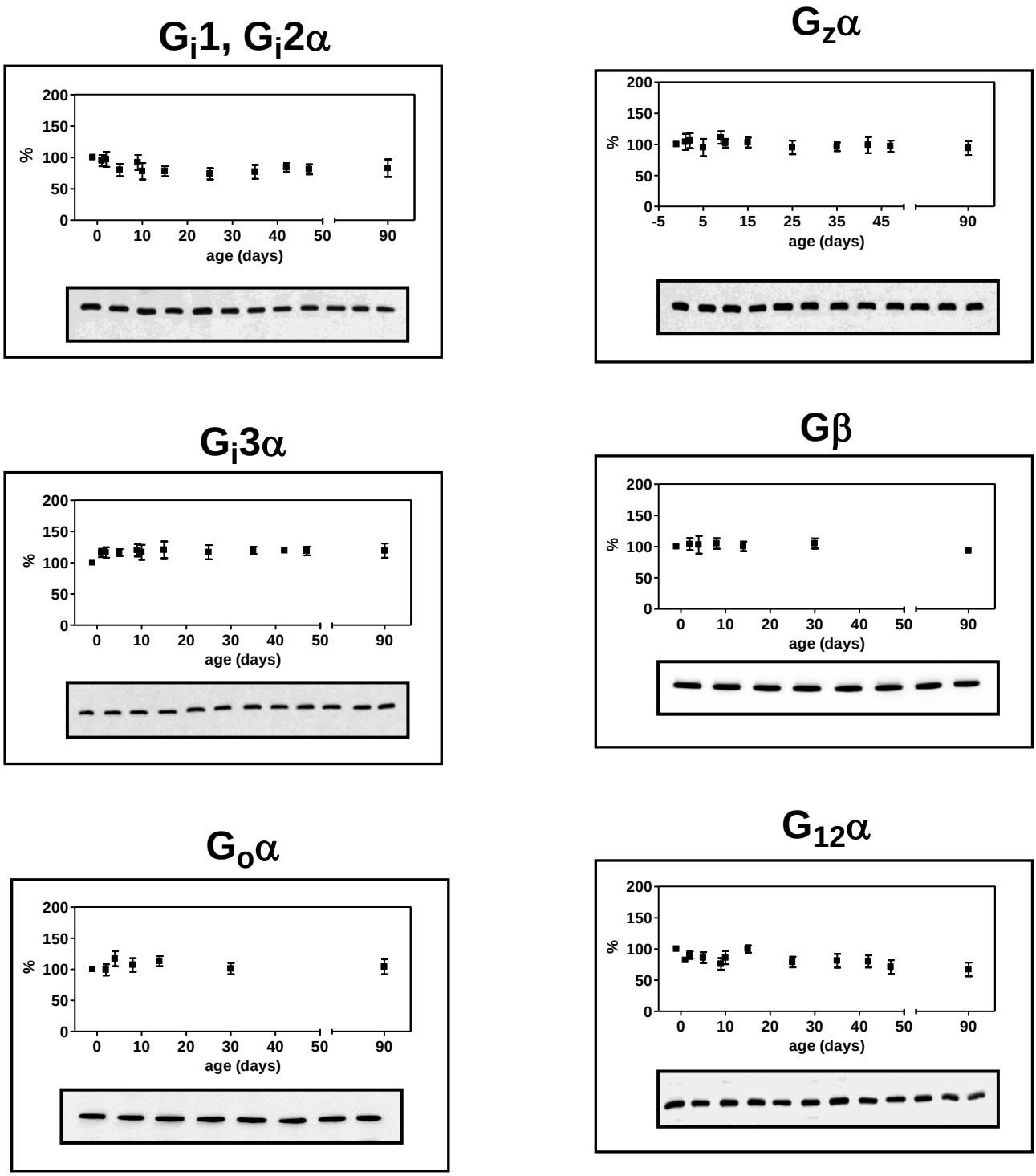

G_i1, $G_i2\alpha$
$G_z\alpha$
$G_i3\alpha$
$G\beta$
$G_o\alpha$
$G_{12}\alpha$
%
age (days)
0
10
20
30
40
50
90
-5
5
15
25
35
45
100
150
200

Fig. 3

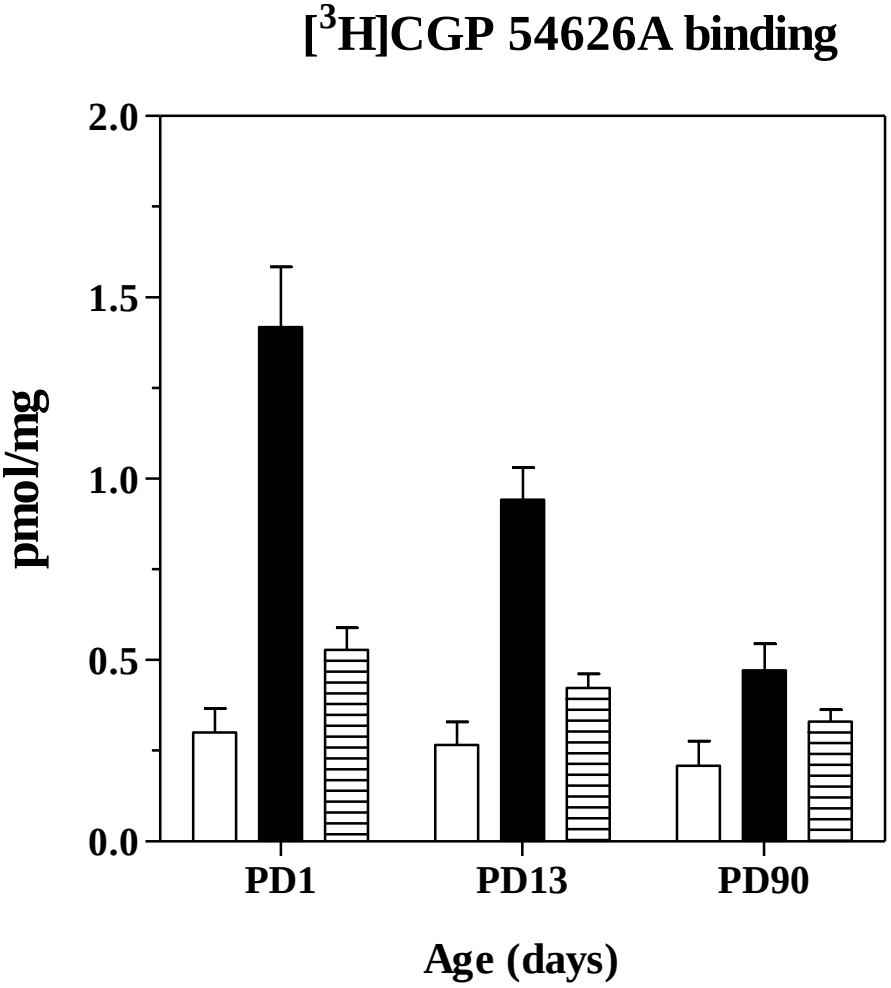
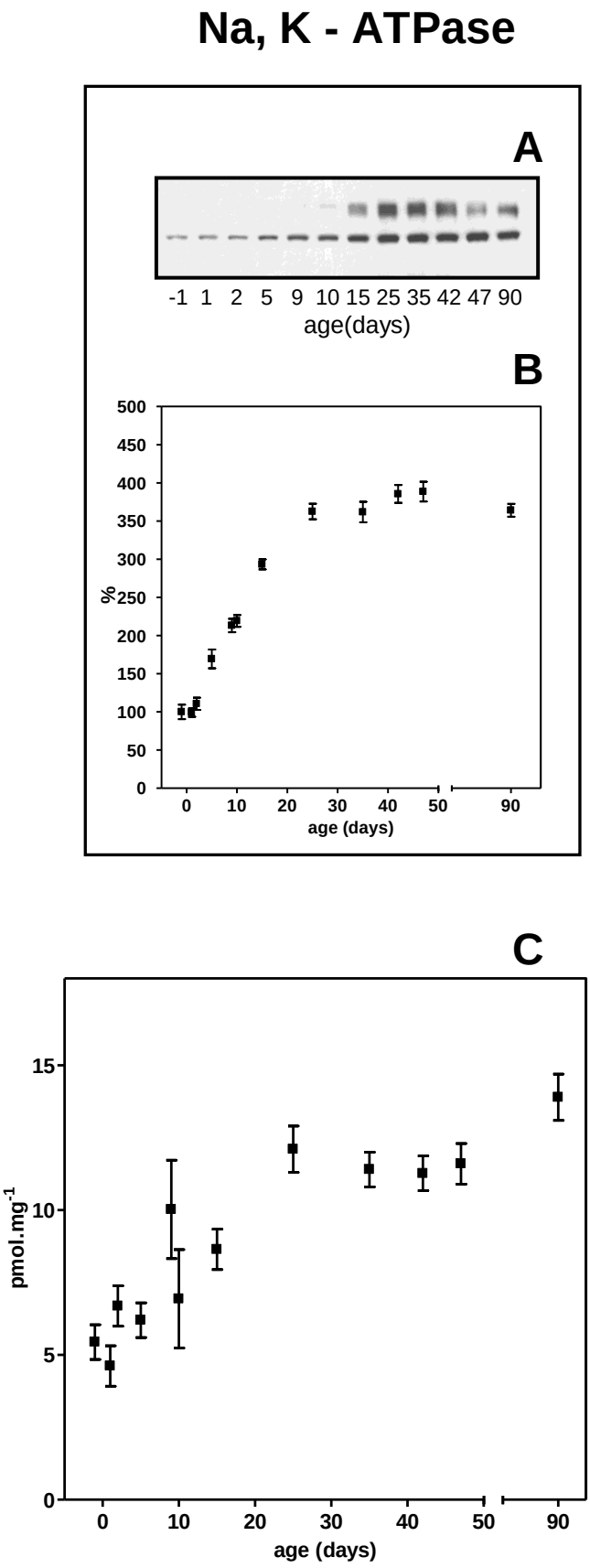

[3H]CGP 54626A binding
2.0
1.5
1.0
0.5
0.0
pmol/mg
PD1
PD13
PD90
Age (days)

Fig. 4


Na, K - ATPase
A
-1 1 2 5 9 10 15 25 35 42 47 90
age(days)
B
%
age (days)
C
pmol.mg-1
age (days)