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Ontogenetic Development of $GABA_B$-Receptor Signaling Cascade in Plasma Membranes Isolated From Rat Brain Cortex; the Number of $GABA_B$-Receptors Is High Already Shortly After the Birth

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Summary

Our data indicate the significant intrinsic efficacy of $GABA_B$-receptors in rat brain cortex already at birth (PD1, PD2). Subsequently, baclofen- and SKF97541-stimulated G-protein activity, measured by agonist-stimulated, high-affinity [^{35}S]GTPγS binding assay, was increased; the highest level of both baclofen and SKF97541-stimulated [^{35}S]GTPγS binding was detected between PD10 and PD15. In older rats, baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was continuously decreased so, that the level in adult, 90-days old animals, was not different from that in newborn animals. The potency of G-protein response to baclofen (characterized by EC_{50} values) was also high at birth but unchanged by further postnatal development. An individual variance among different agonists was observed in this respect as the potency of SKF97541 response was decreased between the birth and adulthood. Accordingly, the highest plasma membrane density of $GABA_B$-R, determined by saturation binding assay with antagonist [^{3}H]CGP54626, was measured in 1-day old animals (2.27±0.08 pmol · mg^{-1}). The further development was reflected in a decrease of [^{3}H]CGP54626 binding as the B_{max} values of 1.38±0.05 and 0.93±0.04 pmol · mg^{-1} were determined in PM isolated from 13- and 90-days old rats, respectively.

Key words

Postnatal development • $GABA_B$-receptor • G-protein coupling/ activation • Baclofen • SKF97541

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Introduction

Historically, $GABA_B$ receptors were pharmacologically distinguished from $GABA_A$ receptors as bicuculline-insensitive GABA binding sites for which agonist is (-)-baclofen (Hill and Bowery 1981, Bowery *et al.* 1983, 1985, 1989, 1993, Hill *et al.* 1984, Hill 1985). After discovery of specific antagonists, $GABA_B$ receptors were defined as a class of bicuculline-insensitive GABA receptors for which (-)-baclofen is a specific agonist and phaclofen and 2-hydroxy-saclofen are specific antagonists (Kerr and Ong 1995). Later, more potent agonist SKF97541 was introduced and electrophysiologically characterized at pre- and postsynaptic binding sites on neurons in rat brain slices (Seabrook *et al.* 1990). $GABA_B$-receptors are not physically bound to an ionic channel and belong to the family of G-protein-coupled receptors, GPCRs (Bowery *et al.* 1983, 1985, 1989, 1993, Kerr and Ong 1995). Thus, the signal initiated by binding of GABA to these receptors is transmitted further downstream by trimeric G-proteins.

$GABA_B$-R agonist stimulation of G-protein activity (measured as high-affinity [^{35}S]GTPγS binding or [^{32}P-γ]GTPase assays) was important experimental evidence indicating that the effect of $GABA_B$-R agonists is mediated *via* trimeric G-proteins (Bowery *et al.* 1983, 1985, 1989, 1993). Close correlation between distribution of baclofen-stimulated GTPase activity and regional distribution of $GABA_B$-receptors in rat brain supported this idea. Furthermore, baclofen-stimulated GTPase *in vitro* was significantly inhibited by pertussis toxin (PTX) and specific antipeptide antisera oriented against $G_i\alpha$

subunit proteins (Sweeney and Dolphin 1992). Electrophysiological studies using specific antisera indicated that both PTX-sensitive $G_i\alpha$ and $G_o\alpha$ subunit proteins were activated by $GABA_B$-R agonists (Dolphin 1990, 1991).

With the aim to understand the maturation of $GABA_B$-R signaling cascade more fully, the early postnatal development of functional coupling between $GABA_B$-R and the cognate G-proteins was studied in plasma membranes isolated from rat brain cortex. The dose-response curves of the two potent agonists baclofen and SKF97541 were determined by high-affinity [^{35}S]GTPγS binding assay and compared in rats of different ages; the number of $GABA_B$-R was determined by saturation binding assay with specific antagonist [^{3}H]CGP54626.

Methods

Materials

$GABA_B$-receptor agonists baclofen (β-p-chlorophenyl-GABA), SKF97541 [3-aminopropyl (methyl) phosphinic acid] and antagonist [^{3}H]CGP54626 (41.5 Ci/mmol, cat. no. R1088) were purchased from Tocris. [^{35}S]GTPγS (1250 Ci/mmol) was from Perkin-Elmer (NEG030H). Complete protease inhibitor cocktail was from Roche Diagnostic (cat. no. 1697498). All other chemicals were of highest quality available.

Isolation of plasma membrane-enriched fraction from rat brain cortex

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 Community Council directives 86/609/EEC.

Rat brain cortex was minced with razor blade on 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 (65000 x g) 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 (175000 x g). 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 °C.

Agonist-stimulated [^{35}S]GTPγS binding
Dose-response curves

Membranes prepared from 2-, 14- and 90-day-old rats of selected ages were incubated with (total binding, B_{total}) or without (basal binding, B_{basal}) increasing concentrations of $GABA_B$-R agonists baclofen and SKF97541 (10^{-10}-10^{-3} M) in final volume of 100 µl of reaction mix containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 20 µM GDP, 0.2 mM ascorbate and [^{35}S]GTPγS (about 100-200,000 dpm per assay) for 30 min at 30 °C. The binding reaction was terminated by dilution with 3 ml of ice-cold 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$ and filtration through Whatman GF/C filters on Brandel cell harvester. Radioactivity remaining on the filters was determined by liquid scintillation using Rotiszcint Eco Plus cocktail. Non-specific binding was determined in parallel assays containing 10 µM unlabelled GTPγS. Data were analyzed by GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and B_{basal}, B_{max} and EC_{50}, values calculated according to the method of least-squares by fitting the data with sigmoidal dose-response curve.

"One-point assay"

With the aim to screen PM prepared from all age intervals under the same assay conditions, membranes (20 µg protein per assay) were incubated with ($B_{agonist}$) or without (B_{basal}) 1 mM baclofen or 100 µM SKF97541 in final volume of 100 µl of reaction mix containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 20 µM GDP, 0.2 mM ascorbate and [^{35}S]GTPγS (1-2 nM) for 30 min at 30 °C. The binding reaction was discontinued by dilution with 3 ml of ice-cold 2 mM HEPES, pH 7.4, 0.15 mM $MgCl_2$ and immediate filtration through Whatman GF/C filters on Brandel cell harvester. Radioactivity remaining on the filters was determined by liquid scintillation using Rotiszcint Eco Plus cocktail. Non-specific GTPγS binding was determined in parallel assays containing

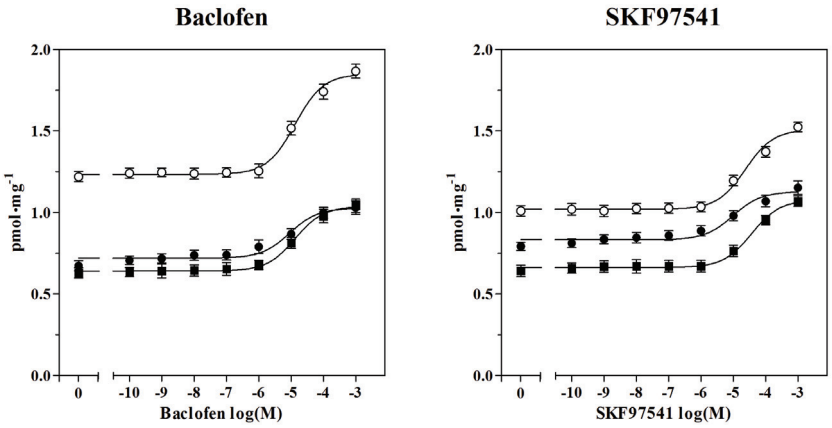

Fig. 1. Dose-response curves of baclofen and SKF97541-stimulated [^{35}S]GTPγS binding in PM isolated from 2-, 14- and 90-day-old rats. PM were isolated in parallel from brain cortex of 2 (●)-, 14 (○)- and 90 (■)-day-old rats and the high-affinity [^{35}S]GTPγS binding was measured in the presence of increasing concentrations of GABA$_B$-R agonists (-)-baclofen (left) or (-)-SKF97541 (right panel) in different age groups as described in Methods. The binding data were fitted by sigmoidal dose-response curves using GraphPad Prism 4 and represent the average of three experiments ± S.E.M. Differences between the averaged dose-response curves corresponding to PM prepared from 2-(PD2), 14-(PD14) and 90-days (PD90) old rats were statistically analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* comparison test. **Left** (baclofen): PD2 (●) versus PD14 (○), p<0.0001, ****; PD14 (○) versus PD90 (■), p<0.0001, ****; PD2 (○) versus PD90 (■), NS, p>0.05. **Right** (SKF97541): PD2 (●) versus PD14 (○), p<0.05, *; PD14 (○) versus PD90 (■), p>0.05, NS; PD2 (○) versus PD90 (■), p<0.0001, ****.

10 μM GTPγS. The binding data were analyzed by GraphPad Prism 4 and represent an average ± S.E.M. of 3 experiments.

[^{3}H]CGP54626 binding; saturation binding study

Membranes (100 μg protein per assay) were incubated with increasing concentrations of GABA$_B$-antagonist [^{3}H]CGP54626 (0.06-36.8 nM) in final volume of 100 μl of binding mix containing 50 mM Tris-HCl (pH 7.4) plus 2.5 mM $CaCl_2$ for 60 min at 30 °C. The bound and free radioactivity was separated by filtration through Whatman GF/B filters in Brandel cell harvestor. Filters were washed 3x with 3 ml of ice-cold incubation buffer and radioactivity remaining and placed in 5 ml of scintillation cocktail (Rotiszint Eco Plus). The non-specific binding was determined in the presence of 1 mM GABA in binding mix. Data were analyzed by GraphPad Prism 4 and K_d and B_{max} values calculated according to the method of the least-squares by fitting the data with rectangular hyperbola.

Protein determination

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

Results

The efficacy and potency of GABA$_B$-receptors in plasma membranes isolated from brain cortex of 2-, 14- and 90-days old rats was determined as baclofen- and SKF97541-stimulated, high-affinity [^{35}S]GTPγS binding in the presence of 20 μM GDP in reaction mix to suppress the low-affinity binding of this non-hydrolysable analog of GTP (Bourova *et al.* 2009). Dose-response curves were measured in 0.1 nM-1 mM range of baclofen or SKF97541 concentrations and the significance of differences among PM prepared from 2- (PD2), 14- (PD14) and 90-days (PD90) old rats was analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* comparison test using GraphPad Prism 4 software (Fig. 1).

Both agonists exhibited the significant ability to increase the basal level of binding measured in the absence of agonist (B_{basal}) already in 2-day-old animals (PD2). This ability was further increased in the course of the first two weeks of postnatal life (compare PD2 and PD14, Fig. 1), but virtually unchanged when viewed over the whole period of brain development as the averaged dose-response curve corresponding to PD2 was not significantly different from that measured in adult rats (PD90). The same applied to the net-increment of agonist stimulation (Δ) and % stimulation of the basal level of [^{35}S]GTPγS binding (Table 1). The highest baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was measured between postnatal day 10 and 15 and than it steeply and continuously decreased towards the adult level (Fig. 2).

The potency (EC_{50} estimates) of G-protein response to baclofen was not significantly different in membranes prepared from 2-, 14- and 90-day-old rats, but decreased from the birth to adulthood in the case of SKF97541 (Table 1). This finding is compatible with electrophysiological studies of brain maturation indicating an altered sensitivity to different GABA$_B$-R agonists or antagonists and similar trends of postnatal changes of GABA$_B$-R efficacy (Bernasconi *et al.* 1992, Hosford *et al.* 1992, Marescaux *et al*. 1992, Lin *et al.* 1993, Kubová *et al*. 1996, Mareš 2008).

Table 1. Maximum response (B_{max}) and affinity (EC_{50}) of baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding in PM isolated from 2-, 14- and 90-days old rats**.**

A(-)-baclofen	**2-days**	**14-days**	**90-days**
B_{basal}	0.72 ± 0.01	1.23 ± 0.02	0.64 ± 0.01
B_{max}	1.03 ± 0.02	1.85 ± 0.03	1.04 ± 0.01
$\Delta = B_{max} - B_{basal}$	0.31	0.62	0.40
$100 \times B_{max} / B_{basal}$	152 %	152 %	166 %
EC_{50} (μM)	9.00 (4.46-18.15)	13.34 (7.81-22.88)	13.26 (9.96-17.65)
B(-)-SKF97541			
B_{basal}	0.83 ± 0.01	1.02 ± 0.01	0.66 ± 0.01
B_{max}	1.13 ± 0.02	1.51 ± 0.02	1.08 ± 0.02
$\Delta = B_{max} - B_{basal}$	0.30	0.49	0.42
$100 \times B_{max} / B_{basal}$	142 %	152 %	168 %
EC_{50} (μM)	9.79 (5.30-18.10)	23.45 (14.34-38.35)	36.51 (21.87-60.95)

B_{basal} (pmol · mg^{-1}), binding in the absence of agonist; B_{max} (pmol · mg^{-1}), binding at saturating agonist concentration; $\Delta = B_{max} - B_{basal}$, net-increment of agonist stimulation; $100 \times B_{max} / B_{basal}$, % stimulation of the basal level by agonist. EC_{50} (μM), agonist concentration inducing half-maximum stimulation (95 % confidence limit). B_{max}, B_{basal} and EC_{50} values were determined by analysis of the sigmoidal dose-response curves of baclofen- (**A**) and SKF97541- (**B**) stimulated [^{35}S]GTPγS binding presented in Figure 1 by GraphPad Prism 4 and represent the average of three experiments ± S.E.M. The significance of difference between B_{basal}, B_{max} and EC_{50} values in PM prepared from 2 (PD2)-, 14 (PD14)- and 90 (PD90)-days-old rats was determined by one-way ANOVA followed by Bonferroni's *post-hoc* comparison test. **A (baclofen)**. B_{basal} (PD2 versus PD14, p<0.0001, ****; PD14 versus PD90, p<0.0001, ****; PD2 versus PD90, p>0.05, not significant. B_{max} (PD2 versus PD14, p<0.0001, ****; PD14 versus PD90, p<0.0001, ****; PD2 versus PD90, p>0.05, not significant. EC_{50} (PD2 versus PD14, p>0.05, NS; PD14 versus PD90, p>0.05, NS; PD2 versus PD90, p>0.05, NS. **B (SKF97541)**. B_{basal} (PD2 versus PD14, p<0.001, ***; PD14 versus PD90, p<0.0001, ****; PD2 versus PD90, p<0.001, ***. B_{max} (PD2 versus PD14, p<0.001, ***; PD14 versus PD90, p<0.0001, ****; PD2 versus PD90, p>0.05, NS. EC_{50} (PD2 versus PD14, p>0.05, NS; PD14 versus PD90, p>0.05, NS; PD2 versus PD90, p<0.01, **).

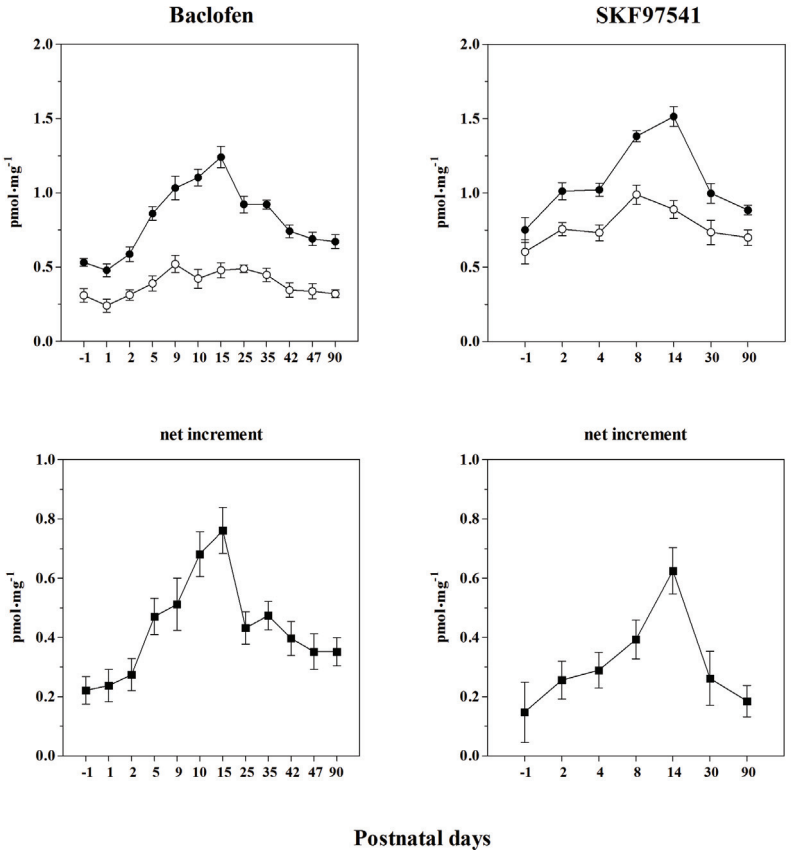

Fig. 2. Baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding; one-point assay. **Upper panels.** PM were isolated from fetuses (-1) and from 1-, 2-, 4-, 5-, 9-, 10-, 14-, 15-, 25-, 30-, 35-, 42-, 47- and 90-days old rats, frozen in liquid nitrogen and used only once. Baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was determined in different age groups as described in Methods in the presence (●, $B_{agonist}$) or absence (○, B_{basal}) of 1 mM baclofen (**left panel**) or 100 μM SKF97541 (**right panel**). The significance of difference between the two sets of data ($B_{agonist}$ versus B_{basal}) at all age intervals was analyzed by Student s t-test using GraphPad Prism 4: baclofen, p<0.0001, ****; SKF97541, p<0.0022, **. The same type of comparison ($B_{agonist}$ versus B_{basal}) was also performed at individual age intervals: **baclofen** [day -1 (*), PD2 (**), PD5(****), PD9(***), PD10(****), PD15(**), PD25(***), PD35(****), PD42(***), PD47(**), PD90(***)]. **SKF97541** [day -1 (NS), PD2 (NS), PD4(*), PD8(*), PD14(**), PD30(NS), PD90(NS)]. **Lower panels.** Difference between agonist-stimulated ($B_{agonist}$) and basal (B_{basal}) level of binding was expressed as the net-increment of agonist stimulation $\Delta = B_{agonist} - B_{basal}$. Data represent the average ± S.E.M. of three experiments.

The existence of the maximum of GABA$_B$-R agonist-stimulated [^{35}S]GTPγS binding between PD10 and PD15 (Fig. 2) has to be considered together with our previous data indicating the striking maximum of basal, manganese-, fluoride- and forskoline-stimulated AC activity in 12-day-old rats (Ihnatovych *et al.* 2002; see discussion for further details). Thus, the increase of baclofen- and SKF97541-stimulated G-protein activity during the first two weeks of postnatal life, its maximum in 10-15-day-old rats and the subsequent decrease is related in time to the maximum and subsequent decrease of AC activity.

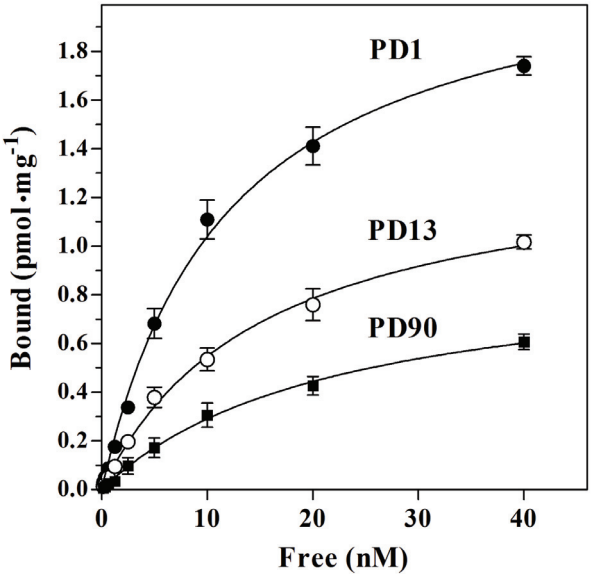

Fig. 3. Saturation of [^{3}H]CGP54626 binding sites in PM isolated from 1-, 13- and 90-day-old rats. Maximum number (B_{max}) and affinity (K_d) of specific [^{3}H]CGP54626 binding sites was determined in PM isolated in parallel from brain cortex of 1 (●)-, 13 (○)- and 90 (■)-days old rats by direct saturation binding assay as described in Methods. B_{max} (maximum binding capacity) and K_d (dissociation constant) of specific [^{3}H]CGP54626 binding sites were calculated by fitting the data by 1-site hyperbola by GraphPad Prism 4 and represent the average ± S.E.M. of 3 experiments. One-way ANOVA followed by Bonferroni's *post-hoc* comparison test was used for statistical analysis of the difference between B_{max} or K_d values in PM prepared from rats of different ages. B_{max} : PD1 versus PD13, $p<0.01$, **; PD13 versus PD90, $p<0.001$, ***; PD13 versus PD90, $p<0.05$, *. K_d: PD1 versus PD13, $p>0.05$, NS; PD13 versus PD90, $p<0.01$, **; PD13 versus PD90, $p<0.05$, *.

Plasma membrane density of GABA$_B$-R at different age intervals was measured by saturation binding study with specific antagonist [^{3}H]CGP54626. Data presented in Figure 3 indicated clearly that the highest PM density of GABA$_B$-R, estimated as the maximum binding capacity (B_{max}) of [^{3}H]CGP54626 binding sites, was detected in PM samples prepared from 1-day-old rats (2.27±0.08 pmol · mg^{-1}). The further development was reflected in a marked decrease of [^{3}H]CGP54626 binding as the B_{max} values of 1.38±0.05 and 0.93±0.04 pmol · mg^{-1} were determined in PM isolated from 13- and 90-days old rats, respectively. The dissociation constant (K_d) was increased from 11.8 nM (PD1) to 15.3 nM (PD13) and 22.1 nM (PD90), indicating the decreased affinity and qualitative change of GABA$_B$-R binding sites towards this antagonist in the course of rat brain cortex maturation.

Discussion

Data presented in this work (Figs 1 and 2) indicate a noticeable extent of compatibility of our present results with experimental data obtained by functional assays of adenylyl cyclase (AC) activity in the presence or absence of GABA$_B$-R agonists, which were previously reported by us (Ihnatovych *et al*. 2002). Maximum activation of baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding coincided with the developmental profile of AC activity. The maximum of agonist-stimulated G-protein activity (Fig. 2) as well as basal, fluoride-, GTP- and forskoline-stimulated AC (Ihnatovych *et al*. 2002) was found in the same period of brain development, between PD10 and PD15. However, marked difference between the two sets of data was noticed as well. Maturation of functional coupling of GABA$_B$-R with G-proteins preceded maturation of AC system because AC activity was low at birth while both baclofen and SKF97541 exhibited significant efficacy already at PD2 (Fig. 1).

Plasma membrane density of GABA$_B$-R determined by saturation binding study with specific antagonist [^{3}H]CGP54626 was also high, virtually the highest, when compared with 13- and 90-day-old rats (Fig. 3). It may be therefore suggested that the physiological significance of the high receptor number and significant efficacy of coupling of GABA$_B$-R with G-proteins shortly after the birth (at PD1 and PD2) is related to some other effectors but AC-cAMP system. Ionic channels and electrophysiological effects of GABA$_B$-R stimulation mediated by $G_o\alpha$ and Gβ subunits represent the obvious choice (Newberry *et al.* 1984a,b, Gähwiler *et al.* 1985, Bormann 1988, Bowery *et al.* 1989).

Comparison of EC_{50} values of agonist-stimulated [^{35}S]GTPγS binding indicated no significant difference in PM isolated from 2-, 14- and 90-day-old rats for

baclofen, but EC_{50} values of SKF97541 were clearly increased from the birth to adulthood (Table 1). This result suggests a developmental decrease in affinity of $GABA_B$-R response for the latter agonist and it is compatible with electrophysiological studies of brain function indicating the differences in sensitivity of $GABA_B$-R to individual agonists (Bernasconi *et al.* 1992, Hosford *et al.* 1992, Lin *et al*. 1992, Marescaux *et al.* 1992). Furthermore, epileptological studies of brain function indicated that anticonvulsant action of baclofen was unchanged during postnatal period (Kubová *et al.* 1996) but the detailed ontogenetic profile of anticonvulsant action of SKF97541 was not identical with that of baclofen (Mareš 2008). The time-span between PD12 and PD18 represented the most critical period in this respect.

Conclusions

Our data indicate significant intrinsic efficacy of $GABA_B$-receptors in rat brain cortex already at the birth (PD1, PD2). Subsequently, baclofen and SKF97541-stimulated G-protein activity, measured by high-affinity [^{35}S]GTPγS binding assay, was increased; the highest level of agonist-stimulated [^{35}S]GTPγS binding was detected between PD10 and PD15. In older rats, both baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was continuously decreased so, that level in adult, 90-days old animals was not different from that in newborn animals. This profile of ontogenetic development of $GABA_B$-R was similar to the maturation of AC activity (Ihnatovych *et al.* 2002).

The potency of G-protein response to baclofen (characterized by EC_{50} values) was high at birth and unchanged by further development. An individual variance among different agonists was observed in this respect as the potency of SKF97541 response was decreased when compared in 2- and 90-days old rats. Surprisingly, the plasma membrane density of $GABA_B$-R, determined by saturation binding assay as maximum binding capacity (B_{max}) for specific antagonist [^{3}H]CGP54626, was highest in 1-day old and then decreased in 13- and 90-days old animals.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

AC, adenylyl cyclase; cAMP, cyclic 3´,5´-[α-^{3}H] adenosine monophosphate, baclofen, β-p-chlorophenyl-GABA; GABA, γ-aminobutyric acid, $GABA_B$-R, metabotropic receptor for GABA, GPCR, G-protein-coupled receptor; G-proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; NS, not significant; PD, postnatal day; PBS, phosphate-buffered saline; PM, plasma membrane, PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; SKF97541, aminopropyl (methyl) phosphinic acid; w.w., wet weight, TCA, trichloroacetic acid.

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