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Age-related changes of the GABA-B receptor in the lumbar spinal cord of male rats and penile erection

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Abstract

Dorsal horn neurons of lumbosacral spinal cord innervate penile vasculature and regulate penile erection. GABAergic system is involved in the regulation of male sexual behavior. Because aging is frequently accompanied by a progressive decline in erectile function, the aim of this work was to examine age-related changes of the GABA-B receptor in the lumbar spinal cord. Sprague–Dawley rats of 10 and 21 days old, 3, 9 and 20 months old were used. GABA-B receptors were evaluated by quantitative autoradiography using [^{3}H]-Baclofen as ligand with or without GABA (10 μM) to determine the non-specific binding. Ten days after birth a homogeneous neuroanatomical distribution pattern was found in the gray matter, however at 20-day-old adult distribution emerged becoming heterogeneous with the highest binding values at layers II–III and X. In dorsal layers a significant decrease was observed in 9-month-old rats while layer X showed an earlier decrease (21-day-old). GABA-B receptor affinity showed significant age-dependent and regional increase. The GABA-B receptor decrease in aged rats seems not to be related to this receptor inhibitory function in penile erection. Moreover the changes found in GABA-B receptor binding anatomical distribution may indicate its role in the morphological development of the lumbar spinal cord rather than in the decline of the erectile function.

Keywords: GABA-B receptors; GABA; Baclofen; Spinal cord; Rat; Aging; Erectile dysfunction; Penile erection; Development; Quantitative autoradiography

Introduction

GABA-B receptor, mediating a slow and long-term metabotropic inhibition, has been identified at brain and spinal cord of rats by immunohistochemistry, autoradiography and in situ hybridization (Bowery et al., 1987; Charles et al., 2001; Chu et al., 1990; Kaupmann et al., 1997). GABA-B receptor function may depend on the receptor subunits composition and on their pharmacological properties (Bischoff et al., 1999; Hsueh, 1988; Mohler et al., 2001). These receptors are heterodimers of GABA-B R1 and GABA-B R2 proteins (Jones et al., 1998; White et al., 1998). Distinctive receptor isoforms are based on the presence of two GABA-B R1 splice variants (R1a and R1b) that were found to be associated with GABA-B R2. Both isoforms differ on their developmental regulation, regional distribution, cellular and subcellular expression, with the GABA-B R1b isoform predominating in adulthood (Fritschy et al., 1999; Mohler et al., 2001).

Developmental studies of GABA-B receptor in central nervous system have shown significant differences according to the age and to the evaluated region. High values of GABA-B receptor were described in brain during the first 3 weeks of life followed by a decline between postnatal day 28 and 23 months after birth. These receptor changes had been associated with a distinct pharmacological profile (Turgeon and Albin, 1994a,b). At spinal cord, Malitschek et al. (1998) described an increase of GABA-B receptor affinity up to 60 days old.

GABA-B receptors are involved in the modulation of different systems like pain, locomotion and plasticity. Penile erection seems to be also regulated by this receptor. The administration of GABA-transaminase inhibitors increases GABA concentration reducing the proportion of mounts that

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result in intromission (Paredes et al., 1993). Subcutaneous injection of baclofen (a GABA-B receptor agonist) decreases the number of males responding with glans erections in a dose-dependent manner (Leipheimer and Sachs, 1988) decreasing also the penile erection induced by subcutaneous administration of apomorphine (Zarridast and Farahvash, 1994). Moreover intrathecal injection of baclofen into the lumbosacral spinal cord but not into the thoracic segments produces a dose-related decrease in the number of erections, increasing the latency to the first glans erection without preventing rats from copula to ejaculation (Bitran et al., 1988).

Penile erection results from the interplay of endocrine, neurologic and vascular systems. Different neuropeptides and neurotransmitters like benzodiazepines (Martino et al., 1987), catecholamines (Bancila et al., 2002; Giuliano and Allard, 2001; Hancock and Peveto, 1979; Tang et al., 1998), neuropeptide Y (Clark et al., 1985), gamma aminobutiric acid (GABA) (Paredes et al., 1993), nitric oxide (NO) (Argiolas and Melis, 1995; Benelli et al., 1995; Nadelhaft and Booth, 1984), opioids (Wiesenfeld-Hallin and Sodersten, 1984) and oxytocin/vasopressin (Argiolas and Melis, 1995; Arletti et al., 1985; Melis et al., 1986, 2000) are also involved in this reaction.

The penile response is induced by the activation of parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) that projects towards the lumbosacral spinal cord (Mugnaini, 1985). One of the spinal pathways concerned in the induction and maintenance of penile erection in the rat involves pelvic nerves that emerge from the peripheral layers of dorsal horns and innervate penile vasculature (Hancock and Peveto, 1979; Lue et al., 1983; Nadelhaft and Booth, 1984).

The incidence of erectile dysfunction, defined as the persistent inability to achieve or maintain an erection sufficient for satisfactory sexual performance, is increased with age and with risk factors like neurovascular disease, atherosclerosis, diabetes mellitus and hypertension (Archer, 2002; Hsieh et al., 2004). However, other factors should be considered in the regulation of penile erection in aging.

Because of the frequently progressive decline on sexual activity observed with age and the relationship between this behavior and GABA-B receptor response, the aim of the present study was to examine if there are age-related changes in GABA-B receptor on the spinal cord region involved in penile erection.

Materials and methods

Experimental animals

Sprague–Dawley male rats, born and raised in the animal facilities of the IByME according to the international rules of FELASA (Federation of European Laboratory Animal Science Associations), were housed in a temperature-controlled room (22 °C), on a 12-h light/dark cycle (lights on at 07:00 h), with food and tap water available ad libitum. All procedures concerning animal care and use were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. Tissues from male rats of five different ages were used; age was selected according to sexual maturation: one before and one after masculinization (10 and 21 days old), two adult stages around the sexual maturation period (3 and 9 months old) and one aged stage (20 months) $n=5$ animals per group.

Tissue preparation

Animals were rendered unconscious by CO_2 inhalation and rapidly killed by decapitation. Spinal cords were removed by dorsal laminectomy, frozen with powdered dry ice and stored at −70 °C. Consecutive coronal sections (20 μm tick) of the lumbosacral region of the spinal cord were obtained using cryostat at −16 °C according to the Atlas of Paxinos and Watson, thaw-mounted onto gelatin 1% and chromium potassium sulfate 0.05% coated microscope slides (6 section / slide), air-dried at room temperature and stored at −70 °C until assayed. Adjacent sections were fixed and stained by Nissl technique for anatomical identification (Rexed, 1954).

Receptor autoradiography

GABA-B receptors were labeled using [^{3}H]-Baclofen ([^{3}H]-BCL, 42.9 Ci/mmol, Du Pont New Products, Boston, MA.) as ligand. The assay was carried out preincubating the spinal cord sections 40 min at room temperature in 50 mM Tris–HCl buffer (pH 7.4) followed by 20 min incubation at room temperature in Tris–HCl 50mM, $MgCl_2$ 2.5 mM, sucrose 190 mM buffer (pH 7.4) containing 20 nM [^{3}H]-BCL for single point assay and a 7.75 to 297.6 nM range for saturation assays. Non-specific binding was determined by adding 10 μM GABA to the incubation buffer. After incubation slides were rinsed 60 s in cold Tris–HCl 50 mM, $MgCl_2$ 2.5 mM buffer (pH 7.4), immediately dipped in deionized cold water and rapidly air dried at room temperature.

Slides containing incubated sections were exposed to tritium sensitive film ([^{3}H]-Hyperfilm-Amersham, Arlington Heights, IL), 60 days for single point assay and 75 days for saturation assay studies. Film exposure time was selected so that the optical densities in the regions to be measured lay in the linear response range of the film. Slides were exposed along with tritium standards ([^{3}H]microscales, Amersham). The generated autoradiograms were analyzed for quantitative densitometry using a video camera (CCD Sony-XC77), coupled to a Macintosh computer equipped with a video card (Data Translation) and a computerized image processing system NIH-Image software (developed by Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD). Approximately 12 spinal cord sections were evaluated per animal for each assay and optical density measurements were made bilaterally. Values were automatically converted to femtomol of [^{3}H]-ligand bound per milligram of wet weight tissue (fmol/mg wwt) using the curve generated with the co-exposed standards.

Statistical analysis

Results are presented as the mean ± standard deviation of at least two separate experiments, each one with 5 animals per group. Twelve sections per animal were used in single point assays and 6 sections in saturation assays. Results were analyzed using one-way analysis of variance (ANOVA) and comparisons among the five groups were made by the Fisher's PLSD test. Differences were considered significant when $p < 0.05$.

Results

Distribution of GABA-B receptors

Specific [^{3}H]-BCL binding was detected in the layers II–III, layer X and in the ventral horns. Changes on GABA-B receptor density were found between early stages of development and aging in all studied regions. 10-day-old animals showed a homogeneous distribution pattern of binding among the different regions of the gray matter (Fig. 1b). On the other hand the distribution observed from 21 days old and above resulted heterogeneous with a decreasing dorso-ventral density gradient (Fig. 1d). The binding observed in the ventral horns was just above non-specific binding (see next section) and homogeneous. No changes were observed from 21 days old and above in motoneuronal nuclei or the intermediolateral cell column region (data no shown).

Single point assay

Similar values of GABA-B receptor binding were found in layers II–III of 10 days, 21-day-old and 3-month-old animals. A significant decrease was observed in 9-month-old rats with respect to younger animals remaining similar in 20-month-old rats (Fig. 2a). On the other hand, in layer X, GABA-B receptor

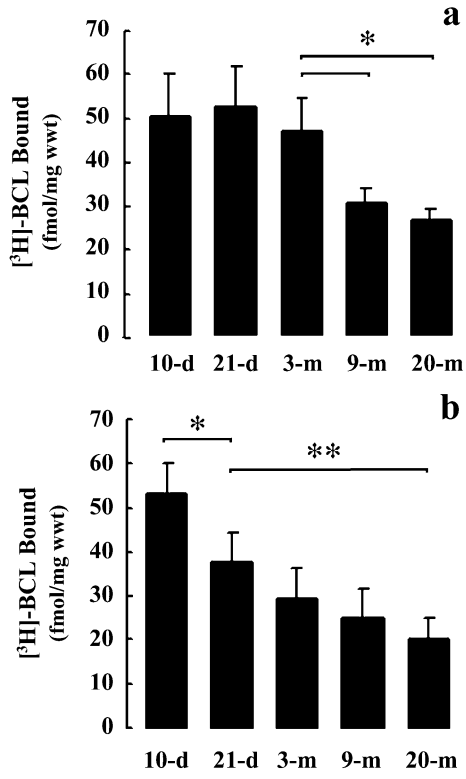

Fig. 2. Evolution of GABA-B receptor binding in the lumbosacral spinal cord by quantitative autoradiography. Autoradiograms were generated after the incubation of spinal cord section with 20 nM of [^{3}H]-BCL. Values are expressed in fmol/mg wwt, as the mean ± SD of 3 similar experiments (5 animals/group). (a) Layers II–III: 10 days old = 50.3 ± 13.3; 21 days old = 52.1 ± 9.5; 3 months old = 48.5 ± 12.0; 9 months old = 0.3 ± 3.1 and 20 months old = 26.3 ± 2.6. ANOVA: $F(4,20) = 5.55$, $p = 0.005$. *$p < 0.02$ vs. 3 months old. (b) Layer X: 10 days old = 53.3 ± 6.9; 21 days old = 37.6 ± 6.6; 3 months old = 29.4 ± 6.9; 9 months old = 24.9 ± 6.7 and 20 months old = 20.1 ± 4.1. ANOVA: $F(4,20) = 12.95$, $p < 0.001$, *$p < 0.01$ vs. 10 days old, **$p < 0.005$ vs. 21 days old.

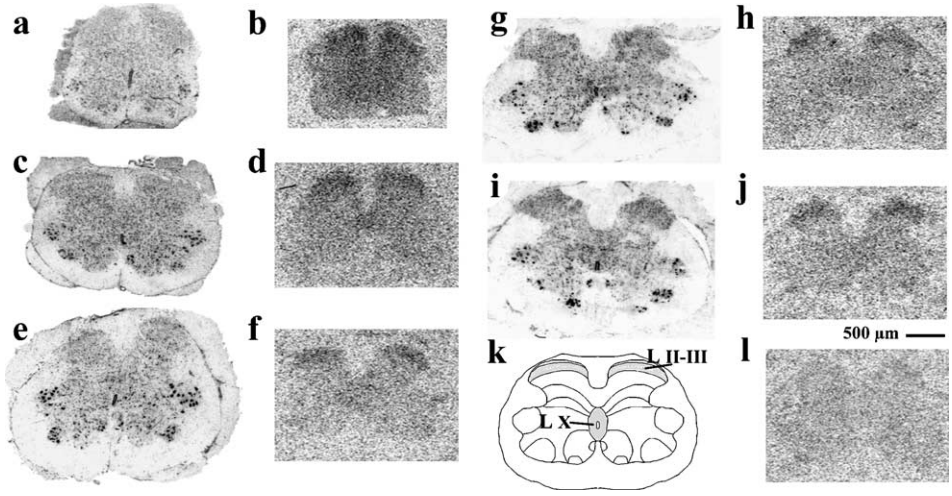

Fig. 1. Representative images of coronal sections from the lumbar six spinal cord of 10- (a, b) and 21-day-old (c, d) animals, 3- (e, f), 9- (g, h) and 20-month-old (i, j) animals. (a, c, e, g, i) sections stained using the Nissl technique; (b, d, f, h, j) autoradiograms generated using [^{3}H]-BCL (20 nM) as ligand. (l) Non-specific binding for 3-month-old animal, (k) diagram showing the regions analyzed (on the dorsal horn, Layers II–III and surrounding the central canal Layer X). Scale bar = 500 μm.

binding showed a marked decrease at 21-day-old rats with respect to younger animals and 3-, 9- and 20-month-old rats showed a further binding decrease with respect to 21-day-old rats that resulted significant only for the older group (Fig. 2b). In the ventral horns, specific binding was only detected in 10-day-old animals (Fig. 1d). Non-specific values were around 30% for the dorsal region, 55% for layer X and 90% for ventral horns except for 10-day-old animals where ventral horn non-specific binding was around 25%.

Saturation assay and linearization by Scatchard method

Saturation assays were performed in tissues from 10-day-old, 3- and 20-month-old animals using a 7.75 to 297.6 nM range of [^{3}H]-BCL. Maximum binding (Bm) and dissociation constant (Kd) were studied using the Scatchard method. Both, layers II–III and layer X showed significant decrease in Bm but beginning at different ages (Fig. 3). In layers II–III, Bm showed similar values in 10-day-old and 3-month-old rats, as it was expected from the single point assay, but a significant decrease of 48% was observed in 20-month-old animals. On the other hand layer X presented a significant 32% decrease in the Bm of 3-month-old rats with respect to young animals, with a further 35% decrease in oldest animals. Kd values showed significant differences in layer II–III at older age.

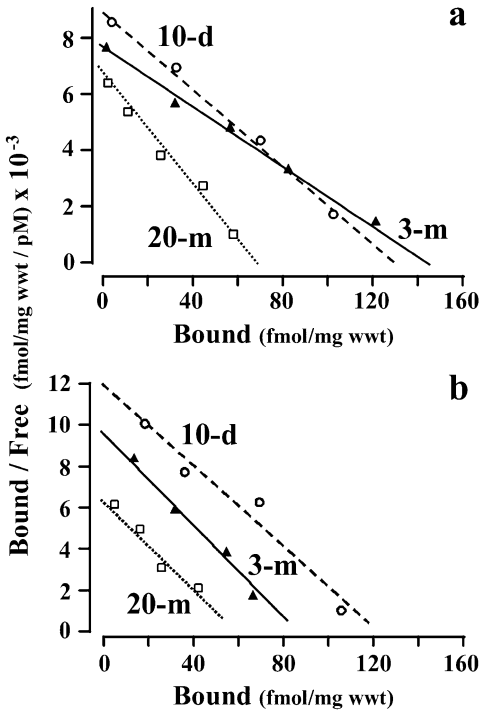

Fig. 3. Kinetic binding parameters of the GABA-B receptor in the lumbosacral spinal cord. GABA-B receptor binding was determined by quantitative autoradiography. Autoradiograms were generated after incubation with a 7.75 to 297.6 nM range of [^{3}H]-BCL. Values are expressed as nM^{-1} for the Kd and fmol/mg wwt for Bm, and were obtained by the linearization method described by Scatchard. (a) Layers II–III: 10 days old (○- -○) Kd=18.49±0.04, Bm=145.4±16.7; 3 months old (▲–▲) Kd=14.34±0.01 Bm=131.2±14.0; 20 months old (□. . .□) Kd*=10.10±0.05, Bm*=70.8±14.7. (b) Layer X: 10 days old (○- -○) Kd=10.10±0.02, Bm=120.7±6.2; 3 months old (▲–▲) Kd=9.10±0.05, Bm*=81.6±8.1; 20 months old (□. . .□) Kd=8.92±0.14, Bm*=53.4±10.3. Values represent the mean±S.D. of 2 similar experiments with 5 animals per group. *$p<0.05$ vs. previous age.

Discussion

GABA-B receptors of the lumbosacral spinal cord revealed a homogeneous distribution pattern among the different layers of the gray matter at 10-day-old male rat tissue. However this distribution became heterogeneous in older animals. By 21 days old, the adult pattern of GABA-B receptor distribution emerges. GABA-B receptor densities predominate in layers II–III and X and low binding was observed in the ventral horns from 21 days old and above. These data agree with the redistribution of these receptors in the spinal cord after the neonatal period, recently described by Sands et al. (2003). In contrast at brain, developmental studies have shown heterogeneous distribution of GABA-B receptors from the day of birth (Turgeon and Albin, 1994a,b).

GABA-B receptor affinity showed temporary changes during development. Malitschek et al. (1998) described an increase of GABA-B receptor affinity in brain cortex, cerebellum and spinal cord in rats up to 60 days old. Here we report a progressive increase of GABA-B receptor affinity in the spinal cord from neonatal period, and during maturation of the tissue up to aging (20 months old) with the overall GABA-B receptor dissociation constant decreased. Regional affinity differences, even within the same region of the spinal cord, were observed too. Significant affinity increase was observed in layers II–III of oldest evaluated animals, while earlier changes were observed in layer X. The expression of glutamic acid decarboxylase (GAD) in the rat cervical spinal cord showed a three-fold decline at the first two postnatal weeks (Somogyi et al., 1995). The progressive prenatal expression of GAD in both, the ventral and dorsal horns, and the postnatal disappearance of its expression in the ventral region weeks (Somogyi et al., 1995) seem to precede the pattern of GABA-B receptors density with the age observed in the present work.

GABA-B receptor function may depend on its subunit composition (Bischoff et al., 1999; Hsueh, 1988; Mohler et al., 2001; White et al., 1998). Sands et al. (2003) have shown a different developmental pattern to each of the three known GABA-B receptor subunit at spinal cord, from neonatal period up to adulthood (28 days old). They have described an increase on GABA-B-R1b over time, not changes at GABA-B-R1a and a decreased GABA-B-R2 expression. Because the heterodimerization of GABA-B subunits (R1a or R1b with R2) appears to be a prerequisite for the receptor function, the reduction of R2 at adulthood may be the responsible regulator of the declined binding seen in our work.

Our present work is consistent with an earlier report indicating that GABA-B receptor binding declines during development (Turgeon and Albin, 1994b, Moran et al., 2001) however we are reporting the first description of GABA-B receptor behavior at aging period, showing a progressive density decline and affinity increase. Consistently, aged rats have shown a decrease of GABA-B receptor binding in the

brain inferior colliculus with a concomitant decline of GABA concentration (Caspary et al., 1990; Milbrandt et al., 1994). The observed decrement in GABA-B receptors pattern may respond to the decline of GABA levels by aging.

GABA-B receptor stimulation is described to produce inhibition of penile erection (Bitran et al., 1988; Leipheimer and Sachs, 1988; Zarridast and Farahvash, 1994). Because we have found a decrease of GABA-B receptor density in the lumbosacral spinal cord of aged rats involved in penile erection and considering that this receptor mediates long-term inhibition, it seems unlikely that GABA-B receptor was entirely responsible for the erectile function decline at aging. However it should not be forgotten that the principal role of GABA-B receptor appears to be the regulation of primary afferent neurotransmitter release (Towers et al., 2000). So, other factors altered by aging may be involved in erectile dysfunction. The action of the NO on hypothalamic oxytocin neurons is widely accepted as the main pathway involved in the regulation of penile erection (Argiolas and Melis, 1995; Melis et al., 1997). Altered hypothalamic NO was found in aged rats (Sato and Tsukamoto, 2000; Serino et al., 1998). Different types of neurons of the lumbosacral spinal cord are involved in the regulation of penile erection. Recently, NADPH-diaphorase reactivity, that determines nitric oxide synthase function, was shown altered in the lumbar spinal cord of diabetic animals (Dorfman et al., 2004). NO changes in the spinal cord may also occur in aged animals. On one hand, inadequate NO levels result in arterial insufficiency and defective muscle relaxation producing erectile dysfunction (Andersson, 2003). On the other hand, the increased motoneuronal NO production in the aged spinal cord, may lead these cells to death (Kanda, 1996), generating a deficit in the innervations of penile musculature and inducing the decline of the erectile function. Moreover, oxytocin-like immunoreactivity decreases in the hypothalamus of old rats. These changes result from a reduction in the dendritic tree and in the number of varicosities along the neurons coming from the paraventricular nucleus of the hypothalamus, showing the existence of an involution process in the neurochemical organization of this nucleus (Calza et al., 1990). Besides, a decrease in oxytocin and its receptors was reported in the hypothalamus of aged rats (Arsenijevic et al., 1995; Lolova et al., 1996).

Conclusion

The present study shows a progressive maturation process of GABA-B receptor in the lumbar spinal cord remaining up to aging, with changes in distribution, anatomical localization, density and affinity that appear to be associated with a morphological role in the development of the central nervous system. Their effect on penile erection seems to be related to the alteration of other neurotransmitters modified with age.

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