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Postnatal development of GABA and calbindin cells and fibers in the prefrontal cortex and basolateral amygdala of gerbils (*Meriones unguiculatus*)

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Abstract

The postnatal maturation of immunohistochemically stained gamma-amino-butyric acid (GABA) and calbindin (CB) cells and fibers were quantitatively examined in the prefrontal cortex (PFC) and the basolateral amygdala (BLA) of the Mongolian gerbil (*Meriones unguiculatus*). Animals of different ages, ranging from juvenile (postnatal day (PD)14, PD20, PD30), to adolescent (PD70), adult (PD180, PD540) and aged (PD720) were analyzed. Results reveal an increase in GABAergic fiber densities between PD14-20 in the PFC and the BLA with a concomitant decrease in cell density. After PD70 GABA fiber density slightly decreases again in the BLA, while there is a further slow but significant increase in the PFC between PD70 and PD540. Fibers immunoreactive for the calcium binding-protein CB, which is predominantly localized in particular GABAergic subpopulations, also accumulate between PD14 and PD20 in the PFC and BLA, while a concomitant decrease in cell density is only seen in the BLA. Both areas reveal a decrease of CB cells between PD30 and PD70, which parallels with a decrease of CB fibers in the PFC. However, there is no particular 'aging-effect' in the fiber or cell densities of GABA or CB in any of the investigated areas in old animals.

In conclusion, we here demonstrate long-term dynamics in cell and fiber densities of the GABAergic system until late in development which might correspond to the prolonged maturation of other neuroanatomical and functional systems.

Keywords: Gamma-amino-butyric acid; Calbindin; Calcium-binding proteins; Immunohistochemistry; Development; Limbic system

1. Introductory statement

Gamma-amino-butyric acid (GABA) is probably the most important inhibitory neurotransmitter in the mammalian nervous system. It is usually expressed in local interneurons, which can modulate and even control the neuronal activity of cortical and subcortical output neurons. Further, GABA has been shown to exert important morphogenetic influences during development (Chronwall and Wolff, 1980; Nguyen et al., 2001) and to play an essential role in reactive plasticity and reorganization processes during development and adulthood (Dawirs et al., 1997; Hensch, 2005; Merzenich et al., 1983; Zito and Svoboda, 2002). Thus, GABA has a central part in shaping and maintaining of neuronal networks.

Within the GABAergic population several classes of subpopulation can be distinguished according to their content of calcium-binding proteins (Baimbridge et al., 1992) and corresponding different maturation patterns. One of these proteins is calbindin (CB) which is, e.g. found in Marinotti, Neuroglia and Double Bouquet cells within the cortex, i.e. in cells, that primarily innervate distal parts and spines of pyramidal dendrites (Conde et al., 1994; DeFelipe et al., 1989; Gabbott and Bacon, 1996; Lund and Lewis, 1993) and appear and mature relatively early (Alcantara et al., 1993). In the amygdala, CB cells are distributed differently in the various nuclei (Kemppainen and Pitkanen, 2000), but in contrast to the prefrontal cortex (PFC), CB varicosities are found in the basolateral amygdala (BLA) to form basket-like structures around unlabelled projection neuron somata (Berdel and Morys, 2000; Kemppainen and Pitkanen, 2000; Legaz et al., 2005; Muller et al., 2003). This points to the

Abbreviations: GABA, gamma-amino-butyric acid; CB, calbindin; PFC, prefrontal cortex; mPFC, medial prefrontal cortex; BLA, basolateral amygdala; PD, postnatal day; PV, parvalbumin

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particularly interesting role of CB in this subcortical area, as it is widely known, that axo-somatic synapses have an exceptionally powerful control over target neurons compared to distal dendritic or spine contacts. In the cortex, these baskets are usually built by GABAergic cells containing parvalbumin, another calcium-binding protein, or other substances such as cholecystokinin (Conde et al., 1994; Kawaguchi and Kubota, 1998).

Despite this difference, the GABAergic innervation patterns of the PFC and the BLA bear marked resemblances (Carlsen, 1988; Muller et al., 2006), although the origin and function of the PFC and the BLA are quite diverse, which is a reason for choosing these particular two structures for investigation in the current study. Further reasons are the high interconnection and thus potential interrelation of the PFC and BLA during development and their distant positions in the brain, which imply divergent developmental patterns. In addition, both areas belong to one main circuit, characterized by the mesolimbic prefrontal dopamine projections, which originate in the ventral tegmental area and the substantia nigra (Björklund and Lindvall, 1984; Fallon and Ciofi, 1992). This dopamine fiber innervation is of particular interest as it shows a prolonged maturation until adulthood in the rodent medial PFC (mPFC) (Dawirs et al., 1993; Kalsbeek et al., 1988), while it stays relatively stable after PD20 in the gerbil amygdala or entorhinal cortex (Brummelte and Teuchert-Noodt, 2006). This is in line with the general developing pattern, with the PFC being one of the last areas to reach adult stages (Mrzljak et al., 1990; Van Eden et al., 1990), while the amygdala maturates relatively early after birth (Joseph, 1999; Morys et al., 1999).

The prenatal and early postnatal maturation of the GABAergic population in the cortex, with particular emphasis on the visual cortex, has been intensively investigated in the last two decades (Chronwall and Wolff, 1981; Del Rio et al., 1992; Parnavelas, 1992; Van Eden et al., 1989; Wolff et al., 1984). However, less research has been done concerning the late postnatal development and aging effects of GABAergic and CB fibers and concerning different cortical or subcortical areas. It is assumed that GABA exhibits a high synaptic plasticity and can help to reorganize, shape and modulate neuronal circuits not only during development (Chen et al., 2002; Teuchert-Noodt, 2000). This compensatory effect in plastic processes might be reflected in changes of the GABAergic or CB fiber densities even during adulthood and aging. As it is further supposed, that the cortex might continuously adapt to new situations and experiences by (re)arranging neuronal networks (Bagorda et al., 2006; Holtmaat et al., 2006; Trachtenberg et al., 2002), the current study was conducted to examine the life long progression of GABAergic and CB structures in two areas of the mesolimbocortical circuit, the mPFC and BLA.

2. Experimental procedures

A total of 60 male Mongolian gerbils (*Meriones unguiculatus*) were used for this study. Breeding gerbils were obtained from Harlan Winkelmann (Borchen, Germany). The animals were bred in standard cages (Macrolon type 4) and, after weaning on postnatal day (PD) 30, were reared individually in standard cages (Macrolon type 3). All gerbils were kept under natural day/night cycles with food and water being provided *ad libitum*. Seven experimental animal groups of different ages were investigated to cover convincing periods of the life span of gerbils: PD14 ($n = 11$), PD20 ($n = 6$) (juvenile), PD30 ($n = 12$) (weaning), PD70 ($n = 11$) (young adult), PD180 ($n = 8$), PD540 ($n = 8$) (adult) and PD720 ($n = 4$) (aging). Gerbils were chosen due to their very small genetic variability (Thiessen and Yahr, 1977), and their rich wild-type like behavioral repertoire (Rosenzweig and Bennett, 1969). All experimental procedures were approved by the appropriate committee for animal care in accordance with the European Communities Council Directive.

2.1. Immunohistochemistry

Animals were transcardially perfused under deep chloralhydrate anesthesia (1.7 g/kg, i.p.). The perfusion was performed with 200 ml 0.05 M phosphate buffer (pH 6.2), containing 1% sodium metabisulfite, followed by 750 ml 5% glutaraldehyde with 1% sodium metabisulfite in 0.1M phosphate buffer (pH 7.5), with appropriate amounts of solutions for younger animals. Immediately after perfusion, the brains were removed and postfixed for 30 min. Coronar sections of 50 μm were cut with a vibratome (Vibratome Series 1000, Technical Products International Inc.) of which every 3rd was used for GABA and CB immunostaining, respectively. For GABA staining sections were collected in wash buffer at 4 °C and rinsed 3 × 10 min followed by a preincubation in 10% normal goat serum and 0.4% Triton X-100 (Sigma) for 30 min. Subsequently, the sections were incubated with rabbit anti-GABA (ImmunoStar, Hudson, WI), diluted 1:5000 with 1% normal goat serum and 0.4% Triton X-100 for 48 h. Sections used for CB staining were treated in almost the same manner, but collected and rinsed in 0.05 M Tris–HCl buffered saline (pH 7.5, TBS), and were additionally incubated in 1% H_2O_2 for 10 min. The primary antibody was mouse anti-calbindin (Sigma, diluted 1:3000, for 18 h). The following rinses, all three times for 10 min, and dilutions were all done in TBS. The sections were rinsed and incubated for 30 min in biotinylated goat anti-rabbit antibody (Sigma) for GABA and biotinylated goat-anti-mouse antibody (Sigma) for CB staining, respectively, diluted 1:20 with 1% normal goat serum, rinsed again and incubated with ExtraAvidin-Peroxidase (Sigma) diluted 1:20 for 30 min. After another rinse the sections were stained in 0.05% 3.3-diaminobenzidine (Sigma) with 0.01% H_2O_2 for 4 min. Then the sections were washed, mounted on glass slides, dried overnight, dehydrated with ethanol, cleared with xylene and cover slipped with DePeX (Serva, Heidelberg, Germany). To avoid deviations due to possibly lateralized innervation densities of GABA and CB only right hemispheres were used for analyses.

For quantification of fiber densities, brain sections were chosen in areas of interest by means of anatomical characteristics according to brain atlases of the rat (Paxinos and Watson, 1986) and the mouse (Valverde, 1998). The BLA and mPFC subregions Cg1 and Cg3, with the latter being further divided into layer III and layer V, were chosen for investigation due to the clear presence of GABAergic and CB fibers and cells. The average number of analyzed sections was 5 per animal and region. In the defined region of each section all detectable fiber fragments were visualized in standard test fields using a bright field microscope (BX61, Olympus, Hamburg, Germany) and a digital camera for microscopy (ColorView II, SIS, Münster, Germany). Calbindin sections were investigated using 200-fold magnification, GABA sections at 600-fold magnification.

To account for a possible interaction of fiber density and cell density or size of the investigated area, these parameters were measured additionally for the PFC and BLA at 200-fold and 20-fold magnification, respectively. Digital images were adjusted in contrast and intensity before fibers, cells or the size of the area were quantified by software for image analysis (KS300, Jenoptik, Jena, Germany). For further details of the quantification process see (Brummelte et al., 2006; Brummelte and Teuchert-Noodt, 2006). The fiber density was calculated as a percentage of the evaluated test area, the cell density as number of cells per test area. Lightly stained cells (cf. qualitative results) were excluded in the counting by a minimum threshold of gray values for cell recognizing. All analyses were done by a person blind to the age of individual animals.

2.2. Data analysis

Measurements were computed as arithmetic means by-case and by-group ± S.E.M. The overall size of the particular area in which fiber densities were measured as well as the number of GABA or CB cells were integrated as