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Developmental changes of calretinin immunoreactivity in the anterior thalamic nuclei of the guinea pig

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ABSTRACT

This study describes for the first time the distribution of the calcium-binding protein calretinin (CR) in the anterior thalamic nuclei (ATN) of the guinea pig during development. Brains from animals ranging from 40th embryonic day (E40) to 80th postnatal day (P80) were used in the study. No CR-immunoreactive (CR-ir) perikarya were present among the ATN at E40, but thick bundles of fibers containing CR were crossing the anteromedial nucleus (AM). The first CR-ir neurons appeared at E50 in the lateral part of the AM. At E60, the bundles of fibers disappeared and the whole area of AM displayed closely packed CR-ir perikarya. At this stage, CR also appeared in neurons of the anteroventral nucleus (AV), particularly in its lateral part and along its dorsal border. Moreover, from E50 short and thin bundles of fibers were observed in the medial part of the AV. The ATN of newborns (P0) already showed an adult-like CR distribution pattern – perikarya in the AM and AV were distributed more homogenously and their number was slightly decreased in comparison to E60. The anterodorsal nucleus (AD) was devoid of CR-ir neurons in all studied stages. In conclusion, our results demonstrate that calretinin appears for the first time in neurons of various anterior thalamic nuclei of the guinea pig between 40th and 60th day of prenatal development.

1. Introduction

Calcium ions play a crucial role in the regulation of multiple physiological processes and neuronal activities. In neurons, Ca^{2+} regulates synthesis of neurotransmitters (Spitzer et al., 2005), synaptic transmissions (Neveu and Zucker, 1996; Yang et al., 1999), gene expression (Fields et al., 2005; Hardingham and Bading, 1998), apoptosis (Bennett and Huxlin, 1996; Krebs, 1998) and many more processes throughout the ontogeny. On account of these various functions, the intracellular level of Ca^{2+} should be tightly controlled in a temporal and spatial dimension. This important role is played by a family of calcium-binding proteins (CaBPs), which comprises over 200 members in humans. Some CaBPs, including parvalbumin, calbindin and calretinin, are widely distributed in the brain. The last of these three proteins is examined in this study. Calretinin (CR) is a highly conserved 29 kDa protein, which is homologous to calbindin D_{28k} with a 58% sequence identity (Rogers, 1987). Although CR is considered a calcium buffer (Faas et al., 2007; Schwaller, 2009), there exists some evidence that CR may also act as a Ca^{2+} sensor (Billing-Marczak and Kuźnicki, 1999; Schwaller et al., 1997). The exact functions of calretinin have not yet been established. Nevertheless, CR takes part in such processes as neuroprotection against excitotoxic cell death (D'Orlando et al., 2001, 2002; Lukas and Jones, 1994) and modulation of neuronal excitability (Camp and Wijesinghe, 2009; Gall et al., 2003; Schurmans et al., 1997).

Anterior thalamic nuclei (ATN) complex is a key component of a very significant circuit of the limbic system processing, classically described as the Papez circuit (Papez, 1995). The main functions of the ATN are related to modulation of emotional and motivational states (Ghika-Schmid and Bogousslavsky, 2000; Papez, 1995; Xiao and Barbas, 2002a,b; Young et al., 2000), as well as to mnemonic processes, such as spatial and working memory (Aggleton et al., 2010; Byatt and Dalrymple-Alford, 1996; Méndez-López et al., 2009; Parker and Gaffan, 1997; Van Groen et al., 2002; Xiao and Barbas, 2002a). Recent studies have shown that each nucleus within the ATN complex, in regard to its connections and electrophysiological properties, may play a different role in the processes mentioned (Aggleton et al., 2010; Albo et al., 2003; Vann and Aggleton, 2003; Vertes et al., 2001; Xiao and Barbas, 2002a).

Abbreviations: AD, anterodorsal nucleus; AM, anteromedial nucleus; ATN, anterior thalamic nuclei; AV, anteroventral nucleus; CaBPs, calcium-binding proteins; CR, calretinin.

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Although the distribution of calretinin is well-known in the thalamus of various adult species (Fortin et al., 1996, 1998; Münkle et al., 2000; Winsky et al., 1992), very few studies have concerned this matter in the developing thalamus. Here, we assume that CR expression is different at various stages of the ontogeny and also among the individual nucleus of the anterior thalamus. If so, it may bring evidence of different functions of each nucleus within the complex during development. Therefore, the aim of this study is to reveal alterations in the distribution of calretinin within the ATN during the development of the guinea pig.

2. Materials and methods

2.1. Tissue preparation

All experimental protocols were approved by the Local Ethical Commission for Animal Experimentation (in accordance with EU Directive 2010/63/EU for animal experiments). The study was performed on the brains of the Dunkin-Hartley guinea pigs (*Cavia porcellus*), ranging from 40th embryonic day to 80th postnatal day, which correspond to the stage of sexual maturity. Animals were obtained from the Polish Mother's Health Centre in Łódź, Poland. The pregnant females and pups were kept in standard lighting (12 h light/12 h dark) and feeding (chow and tap water ad libitum) conditions.

Twelve postnatal animals (four animals for each stage: P0, P20, and P80 – newborn, 20th and 80th day after birth, respectively) were euthanized by an intraperitoneal injection of pentobarbital (Morbital, Biowet, Poland; 2 ml/kg body weight), immediately followed by the perfusion of a fixative solution – 4% buffered paraformaldehyde (pH 7.4; 4 °C). The brains were removed from the skulls and postfixed by immersion for 48 h in the fixative solution at 4 °C, then washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and passed through the graded solutions (19% and 30%) of sucrose in PBS until they sunk. After freezing, 10 μm-thick coronal sections were cut using cryostat and stored at −80 °C.

Twelve embryos (four embryos for each stage: E40, E50, E60 – 40th, 50th, 60th day of gestation, respectively) were obtained from the pentobarbital-euthanized pregnant guinea pigs. The day of the insemination was designated as gestational day 0 (E0). The embryos were quickly removed from the uterus and their brains underwent the same procedure as the brains of the postnatal animals.

2.2. Immunohistochemical procedures

Sections through the anterior thalamus from the postnatal and embryonic brains were processed for two immunohistochemical methods: a routine single-labeling immunofluorescence and DAB method.

2.2.1. Immunofluorescence

After triple wash in cold PBS, the sections were preincubated for 1 h with a solution of 10% normal donkey serum (diluted in PBS), then washed three times in cold PBS and incubated overnight with a solution of mouse monoclonal antibodies raised against calretinin (1:2000, Swant, Switzerland). The antibodies were diluted in PBS containing Triton X-100 (0.3–0.5%) and 1% normal donkey serum. In order to show the binding sites of the antibodies, the sections were incubated for 1 h with a solution of secondary antibodies conjugated with Cy 3 (1:10,000 Jackson ImmunoLabs, USA). Finally, the sections were washed three times in cold PBS and cover-slipped in buffered carboxyglycerol (pH 7.8). All staining procedures were carried out at room temperature.

2.2.2. DAB method

After triple wash in cold PBS, the sections were preincubated for 30 min in 0.3% H_2O_2 diluted in methanol and then for 20 min with a solution of 10% normal donkey serum (diluted in PBS). The sections were incubated overnight with a solution of mouse monoclonal antibodies raised against calretinin (1:1000, Swant, Switzerland). The antibodies were diluted in PBS containing Triton X-100 (0.3–0.5%) and 1% normal donkey serum. After triple wash in cold PBS, the sections were incubated for 30 min with ImmPRESS Reagent (Vector Laboratories, USA), washed in cold PBS and incubated with a 3,3-diaminobenzidine substrate–chromogen solution (DakoCytomytation, USA). The sections were rinsed in tap water, dehydrated through graded alcohol series, cleaned in xylen and mounted in DPS. All staining procedures were carried out at room temperature.

The sections were examined with an Olympus BX51 microscope equipped with a CCD camera connected to a PC. Images were acquired with Cell-F software (Olympus GmbH, Germany). To prove the specificity of the antibodies, in some sections the primary antibodies were replaced with normal serum. No specific staining was detected.

In order to show the boundaries of the ATN, coronal sections through the anterior thalamus of the guinea pig (from each of studied stages) were processed for a standard Nissl staining, as well as for immunostaining using mouse anti-NeuN (Millipore, USA).

3. Results

The ATN of the guinea pig are comprised of three nuclei: the anterodorsal (AD), anteroventral (AV) and anteromedial (AM) (Fig. 1). The localization and structure of the ATN of the guinea pig was maintained throughout the examined development stages (Figs. 2A, 3A, 4A and 5A). The pattern of distribution of calretinin-immunoreactive (CR-ir) structures was constant throughout the rostrocaudal extent in all studied stages.

3.1. Embryonic day 40

No CR-ir perikarya were present in any nucleus of the anterior thalamus (Fig. 1A). The AM displayed an intensely immunostained CR-ir neuropil consisting of numerous long, thick bundles of fibers traversing the AM in a dorsomedial direction, which were observed in the dorsomedial part of the nucleus (Fig. 2A and C). The AV and AD displayed a weakly stained neuropil, consisting of short single fibers and punctate structures reminiscent of terminal boutons (Fig. 2B).

3.2. Embryonic day 50

At this stage, neurons containing CR appeared for the first time in the AM (Fig. 1B). These CR-ir neurons of medium size were found in the dorsolateral part of the nucleus (Fig. 3A). The perikarya showed fairly weak immunoreactivity, but some with more intense immunoreactivity were also detected. Mostly oval and polygonal CR-ir perikarya gave rise to short processes (Fig. 3C). The surrounding neuropil was observed as intensely stained, thick and long bundles of fibers, that crossed the ventral and medial part of the nucleus (Fig. 3A). In the AV, CR-ir neurons could not be detected, but a moderately stained neuropil contained single CR-ir fibers as well as bundles of fibers, which were generally finer and shorter than these observed in the AM (Fig. 3B). The AD was devoid of CR-ir perikarya and displayed only a weakly stained neuropil.

3.3. Embryonic day 60

At E60, CR-ir neurons occurred for the first time in the AV (Fig. 1C). Moreover, the bundles of fibers from the AM diminished and the whole area of the nucleus contained numerous closely packed CR-ir neurons (Figs. 1C and 4A). Most of these neurons had large polygonal-shaped perikarya, but smaller oval ones were also present. Various-length processes emerged from all CR-ir perikarya (Fig. 4B and C). The immunoreactivity of the neuropil, which was composed of short fibers and punctate structures reminiscent of terminal boutons, was strong, especially in the ventral part of the AM. In the AV, neurons showed higher density and stronger CR immunoreactivity in the lateral part of the nucleus as well as along its dorsal border (Fig. 4A). The immunostained perikarya ranged in shape from fusiform to polygonal, and in size from medium to large (Fig. 4D and E). The AV displayed a moderate CR-ir neuropil, consisting of fine and short bundles of fibers, that were crossing the medial part of the nucleus (Fig. 4D and E). The CR-positive neuropil in the AD was similar to the ones observed at the previous stages and it remained so throughout the rest of the studied stages (Fig. 4F).

3.4. Postnatal day 0

The ATN of newborns already showed adult-like pattern of the calretinin distribution (Fig. 1D). Many scattered CR-ir neurons were localized in the AM, nevertheless their number was slightly decreased in comparison to E60. The size and morphology of the perikarya were very similar to those found at E60. In the AV, CR-ir