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Substance P receptor in the rat indusium griseum during postnatal development

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ABSTRACT

The presence of substance P (SP) receptor (Neurokinin-1 receptor, NK1R) in the indusium griseum (IG) and anterior hippocampal continuation (AHC) during postnatal development was studied by immunocytochemistry (ICC). NK1R-immunopositive neurons ($NK1R_{IP-n}$) first appeared in both areas on postnatal day (P) 5. From P5 onward, their distribution pattern was adult-like. In sagittal sections $NK1R_{IP-n}$ formed a narrow strip of neurons and dendrites that were located over the corpus callosum (cc); in coronal sections they were found in a roughly triangular area at the base of the cingulate cortex (Cg) on the dorsal surface of the cc. $NK1R_{IP-n}$ were also found in the AHC, which is considered as a subcallosal extension of the IG, located ventral to the genu of the cc. At all ages studied, IG $NK1R_{IP-n}$ sent dendrites to the contralateral IG, the underlying cc, and the Cg. Moreover, $NK1R_{IP-n}$ located in the Cg and the cc sent dendrites to the IG. The present findings are in line with previous ICC studies describing dopaminergic and serotoninergic afferents to the IG. Together these data suggest that, through NK1R, SP could play an important role in regulating the release mechanisms of these afferents and that it could be an important developmental factor. Notably, IG neurons could be activated by cortical and intracallosal afferents.

1. Introduction

The corpus callosum (cc), the largest fiber tract in the brain, connects the two cerebral hemispheres (Innocenti, 1986; Raybaud, 2010) and is overlain by a longitudinal, symmetric structure consisting of two thin layers of gray matter denominated indusium griseum (IG; Sturrock, 1978a,b; Wyss and Sripanidkulchai, 1983; Künzle, 2004; Laplante et al., 2013; Di Ieva et al., 2015). The IG continues rostrally toward the ventral portion of the genu of the cc and dorsal to the taenia tecta. This subcallosal extension is denominated anterior hippocampal continuation (AHC; Wyss and Sripanidkulchai, 1983; Laplante et al., 2013). Studies exploring their connections and neuroanatomical characteristics suggest that the two structures are the continuation of the hippocampal formation (Wyss and Sripanidkulchai, 1983; Laplante et al., 2013). The physiological role of the IG in the adult is still unknown (Raybaud, 2010), but during early cc formation the IG, together with the glial wedge, guides callosal fibers to the midline and to their targets in the contralateral hemisphere by secreting guidance molecules such as the chemorepellent Slit2 and RyK receptor (Lindwall et al., 2007; Shu and Richards, 2001; Shu et al., 2003; Richards et al., 2004; Raybaud, 2010; Keeble et al., 2006).

Immunocytochemical and autoradiographic rat and cat studies have suggested the presence of a variety of afferent projections to the IG and AHC. Other immunocytochemical studies have identified several cell populations expressing parvalbumin (Parv), neuronal nuclear antigen, and various reuptake transporter systems (for a review see Laplante et al., 2013).

Moreover, in two recent studies assessing the presence of intracallosal neurons expressing neurokinin-1 receptor (NK1R), the receptor showing the highest affinity for substance P (SP; Harrison and Geppetti, 2001; Onaga, 2014), Barbaresi et al. described NK1R-immunopositive neurons ($NK1R_{IP-n}$) in the IG (Barbaresi et al., 2015, 2017). It has been extensively demonstrated that Substance P may have a growth-stimulatory effect and plays an important role as a trophic factor and in many regions of the central nervous system (Quirion and Dam, 1986).

The present study, therefore, explored the possible involvement of SP in early postnatal development using an antibody against NK1R (Shigemoto et al., 1993) to demonstrate the presence of $NK1R_{IP-n}$ neurons in the IG/AHC of rats of different ages, from post-

Abbreviations: AHC, Anterior hippocampal continuation; cc, corpus callosum; Cg2, Cingulate cortex, Area 2; CPu, Caudate-putamen nucleus; gcc, Genu of the corpus callosum; GCL, Granular cell layer; IG, Indusium griseum; LV, Lateral ventricle; ML, Medial molecular layer; SFi, Septofimbrial nu; vhc, Vent hip commissure; 3V, Third ventricle.

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natal day (P) 0 to P30, and to assess their size, morphology and distribution.

2. Material and methods

2.1. Animals

The study involved 43 Sprague-Dawley albino rats of different ages whose care and handling were approved by the Animal Research Committee of Marche Polytechnic University in accordance with National Institutes of Health guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used; indeed, several rats came from a previous study (see Barbaresi et al., 2017).

2.2. Immunocytochemistry (ICC)

Animals were from three litters. The day of birth (the first 24 h after birth) was considered as P0. Animals were examined at six different ages: P0, P5, P10, P15, P20, and P30.

Perfusion, cutting and immunocytochemistry were as described in a previous paper (Barbaresi et al., 2017). In brief, animals, anaesthetized with chloral hydrate (12% in Phosphate Buffer, PB; 0.1 M; pH 7.4), were perfused with saline followed by 4% paraformaldehyde and 20% saturated picric acid in PB (0.1 M; pH 7.4). Brains were removed, postfixed for 12 h, and cut into 60-μm-thick sections (3 consecutive; coronal and sagittal) using a freezing microtome. Two sections were reacted with NK1R antibody, whose specificity has been tested by Shigemoto et al. (1993). The third section was stained with neutral red (NR; Fluka Chemie GmbH, Buchs, Switzerland) or toluidine blue (TB; Merck KGaA, Darmstadt, Germany; both in 1% in aqueous solution). In some cases sections reacted for ICC were also stained with NR or TB.

2.3. Characterization of the NK1R antibody

The NK1R antibody was made in rabbit against a peptide corresponding to amino acid residues 349–407 of rat SP receptor. Its specificity has been tested through preabsorption with *trp E*-SPR fusion protein, which abolished all staining (see Fig. 2c in Shigemoto et al., 1993), and has successfully been employed in previous studies of $NK1R_{IP\text{-}n}$ distribution in the rat striatum (Shigemoto et al., 1993), CNS (Nakaya et al., 1994), cerebral cortex (Kaneko et al., 1994), periaqueductal gray matter (Barbaresi, 1998) and cc (Barbaresi et al., 2015, 2017).

2.4. Distribution

$NK1R_{IP\text{-}n}$ distribution in the IG was drawn using a camera lucida attached to a Leitz Orthoplan microscope equipped with a 10 × or a 40 × objective (Leica, Wetzlar, Germany). Its borders were defined by comparing sections stained with NR or TB with data from previous cytoarchitectonic studies (Wyss and Sripanidkulchai, 1983; Laplante et al., 2013) and atlases (Paxinos and Watson, 1982; Zilles, 1985).

2.5. Soma size

IG/AHC $NK1R_{IP\text{-}n}$ for soma size analysis were randomly selected according to the following criteria: (i) neurons must be intensely labeled and show a clearly distinguishable morphology; (ii) cell bodies must be located centrally in the 60-μm section depth, to minimize cutting of dendrite branches near the section surface; (iii) dendrites must not be excessively obscured by other heavily stained processes from neighboring cells; and (iv) dendritic trees must not show discontinuity with the cell body. The soma size of IG/AHC neurons was obtained by pooling data from 2 rats per age group.

The outlines of all somata were drawn with a camera lucida attached to a Leitz Orthoplan microscope equipped with a 100 × objective (Leica). Soma profiles were digitized using an Epson Perfection 3170 scanner (resolution, 500 dpi) connected to a Power Macintosh G5. $NK1_{IP\text{-}n}$ size, measured as μm^2, was calculated with the NIH Image program (Rasband and Bright, 1995). The histograms in Fig. 12 were constructed by assembling the areas of IG and AHC $NK1_{IP\text{-}n}$ from two rats per time point. Student's *t*-test was used for statistical comparisons. A p value ≤ 0.05 was considered statistically significant.

2.6. Photomicrographs

Photomicrographs of $NK1R_{IP\text{-}n}$ were acquired using an Eclipse E 600 microscope (Nikon-Italia, Firenze, Italy) provided with a DS-Vi1 color camera (Nikon Instruments, Europe BV, Kingston, Surrey, UK). Photographic montage of neurons was created in Adobe Photoshop CS4 Extended (v. 11.0; Adobe System Inc., CA, USA); images were cropped to the appropriate size and adjusted only for brightness and contrast.

3. Results

Sections from P0, P5 and P10 were reacted together with those from P30 animals; the overlying cerebral cortex, caudate putamen, globus pallidus and mesencephalon were the positive control. The immunocytochemical experiments provided excellent Golgi-like staining of neurons and processes in sections from all postnatal age groups.

3.1. P0. Distribution of IG $NK1R_{IP\text{-}n}$. Sagittal sections

IG $NK1R_{IP\text{-}n}$ were not detected at P0; however, a dense plexus of intensely labeled fibers (likely glial processes; Horie et al., 2000) extended from the base of the medulla to the floor of the fourth ventricle in these sections. Labeled neurons and dendrites were also detected in the caudate putamen, globus pallidum, and the subcortical plate of the cerebral cortex.

3.2. P5-P30. Distribution of IG/AHC $NK1R_{IP\text{-}n}$. Sagittal and coronal sections

This stage was characterized by the appearance of IG $NK1R_{IP\text{-}n}$ and by a progressive increase in cell body size and dendritic arborization. At P5, $NK1R_{IP\text{-}n}$ distribution in the IG was already adult-like (Figs. 1, 2A, 3A and 4A). At P5 and all subsequent ages the labeled neurons in the sagittal sections formed a narrow strip of cells and dendrites over the anterior two thirds of the cc (Figs. 4, 5D and E); IG $NK1R_{IP\text{-}n}$ formed clusters of two or four cells, although individual neurons were also observed (Fig. 4). In the sagittal sections the dendritic trees of these neurons were oriented longitudinally and the primary dendrites emerged from opposite poles and clustered into dense bundles (Figs. 4, 5D and E). In some cases secondary dendritic branches were oriented vertically toward the cerebral cortex or ventrally toward the cc (Figs. 4 and 5, 10C). The $NK1R_{IP\text{-}n}$ found in front of the genu of the cc had their soma and dendrites oriented vertically, with secondary branches directed toward the cc. Several IG areas contained only dense bands of dendrites and were interspersed with smaller areas containing both neurons and dendrites (Figs. 4, 5D and E).

In accordance with this organization, the coronal sections exhibited one to four-six neurons per level (Figs. 1, 3A–D and 5), whereas only dendrites were detected in the adjacent sections and/or the contralateral IG (Figs. 3, 5A and C). Several IG $NK1R_{IP\text{-}n}$, including