Neuroscience 138 (2006) 487–499

EXPRESSION OF SUBSTANCE P, NEUROKININ 1 RECEPTORS (NK1) AND NEUROKININ 3 RECEPTORS IN THE DEVELOPING MOUSE RETINA AND IN THE RETINA OF NK1 KNOCKOUT MICE

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Abstract—To complete a series of studies on the expression of substance P and neurokinin receptors in mammalian retinas, we investigated the occurrence of these molecules in developing mouse retinas and in retinas of mice with genetic deletion of the neurokinin 1 receptor, the preferred substance P receptor. Using semi-quantitative reverse transcription–polymerase chain reaction, we measured detectable levels of the γ isoform of preprotachykinin A (a substance P precursor) mRNA at postnatal day 4. Neurokinin 1 receptor and neurokinin 3 receptor mRNAs were also detected at postnatal day 4. While γ preprotachykinin A and neurokinin 1 receptor mRNA levels significantly increased up to eye opening (postnatal day 11), neurokinin 3 receptor mRNA levels remained constant throughout development. Substance P, neurokinin 1 receptor and neurokinin 3 receptor immunoreactivities were present at postnatal day 5. Substance P was in amacrine cells, neurokinin 1 receptor in developing amacrine and bipolar cells and neurokinin 3 receptor in OFF-type cone bipolar cells. Interestingly, a transient increase in the density of neurokinin 1 receptor immunoreactive processes was observed at eye opening in lamina 3 of the inner plexiform layer, suggesting a role of substance P and neurokinin 1 receptor in this developmental phase. However, in neurokinin 1 receptor knockout retinas, besides a significant increase of the γ preprotachykinin A mRNA levels, no major changes were detected: neurokinin 3 receptor mRNA levels as well as substance P and neurokinin 3 receptor immunostainings were similar to wild types. Together with previous studies, these observations indicate that there are major differences in neurokinin 1 receptor expression patterns among developing mammalian retinas. The observations in neurokinin 1 receptor knockout mice may not be applicable to rats or rabbits, and substance P and neurokinin 1 receptor may play different developmental roles in different species. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: inner plexiform layer, bipolar cells, amacrine cells, vesicular acetylcholine transporter, neuropeptide receptors.

We have recently investigated the expression patterns of the substance P (SP) receptor, the neurokinin 1 receptor (NK1), in different mammalian retinas (Casini et al., 1997a, 2002; Catalani et al., 2004). In addition, the developmental patterns of this receptor have been investigated in rat and in rabbit retinas (Oyamada et al., 1999; Casini et al., 2000, 2004a). Overall, these studies have revealed remarkable differences between species in the distribution of NK1 in adult retinas, suggesting the involvement of SP and NK1 in retinal functions that are possibly related to species-specific behavioral habits (see Casini et al., 2002, for discussion). Similarly, differences have been found between the developmental profiles of NK1 expression in rat and in rabbit retinas, however the presence of transient changes in developing retinas of both species strongly suggests an involvement of NK1 in developmental events. In particular, in rabbit retinas, not only the expression pattern of NK1 undergoes dramatic rearrangements during development, with NK1 expressed by cholinergic amacrine cells in the immature retina and by dopaminergic amacrine cells after eye opening, but also the physiological actions of SP have been observed to influence different cell populations in developing and in adult retinas (Casini et al., 2004a). To complete this series of investigations of NK1 in different adult and developing mammalian retinas, in the present study we examined the developmental expression of NK1 in the mouse retina. In addition, the localization of SP and of another neurokinin receptor, the neurokinin 3 receptor (NK3), was also investigated. Finally, to determine possible effects caused by the genetic deletion of NK1, we examined the retinas of adult NK1 knockout (KO) mice.

SP is a member of the family of tachykinin peptides, which also includes neurokinin A (NKA), two NKA-related peptides (neuropeptide K and neuropeptide γ) and neurokinin B (NKB). Two distinct, structurally related genes encode for these peptides: SP, NKA and NKA-related peptides are the products of the preprotachykinin A (PPT-A) gene, while NKB is encoded by the preprotachykinin B

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Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; KO, knockout; NBL, neuroblastic layer; NKA, neurokinin A; NKB, neurokinin B; NK1, neurokinin 1 receptor; NK2, neurokinin 2 receptor; NK3, neurokinin 3 receptor; OPL, outer plexiform layer; PB, phosphate buffer; PND, postnatal day; PPT-A, preprotachykinin A; RT-PCR, reverse transcription–polymerase chain reaction; SP, substance P; VAChT, vesicular acetylcholine transporter.

0306-4522/06$30.00+0.00 © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2005.11.020

gene (see Otsuka and Yoshioka, 1993; Harrison and Geppetti, 2001, for review). In particular, four splice variants (α, β, γ and δ) derive from the transcription of the PPT-A gene (Harmar et al., 1990; Helke et al., 1990), and all of them encode SP (Springer et al., 2003).

In mammalian retinas, both tachykinin immunoreactivity and mRNA have been localized to mainly amacrine, displaced amacrine and ganglion cells (see Bagnoli et al., 2003; Brecha, 2004, for reviews). In the mouse retina, SP immunoreactivity is in a small population of mostly normally placed amacrine cells with their processes stratified at three distinct levels of the inner plexiform layer (IPL; Haverkamp and Wässle, 2000). Functionally, SP has been reported to be excitatory on ganglion cells of different vertebrate retinas (Dick and Miller, 1981; Glickman et al., 1982; Zalutsky and Miller, 1990) and on amacrine cells of rabbit retinas (Zalutsky and Miller, 1990). In addition, it stimulates retinal dopamine release in rat and rabbit (Tsang, 1986; Casini et al., 2004a). Finally, SP is likely to modulate transmitter release from isolated bipolar cells of the goldfish retina (Ayoub and Matthews, 1992).

The cellular actions of tachykinin peptides are mediated by neurokinin receptors, belonging to the superfamily of G protein-coupled receptors, and named NK1, neurokinin 2 receptor (NK2) and NK3, whose preferred ligands are SP, NKA and NKB, respectively (Otsuka and Yoshioka, 1993; Harrison and Geppetti, 2001; Hökfelt et al., 2001). In mammalian retinas, NK2 are not expressed (Oyamada et al., 1999), while different studies have reported the presence of NK1 in variable cell populations of rat, mouse and rabbit retina (Casini et al., 1997a, 2002; Catalani et al., 2004; Kim et al., 2005), and of NK3 primarily in OFF-type cone bipolar cells of rat, mouse and cat retina (Oyamada et al., 1999; Casini et al., 2000; Strettoi et al., 2002; Haverkamp et al., 2003; Feigenspan et al., 2004; Ghosh et al., 2004; Kao et al., 2004; Pignatelli and Strettoi, 2004). Concerning the localization of NK1 in adult mouse retinas, they are in a population of ON-type cone bipolar cells and in different subsets of amacrine cells, including GABA, glycine, tyrosine hydroxylase or SP containing amacrine cells (Catalani et al., 2004).

Available evidence concerning the expression of NK1 in developing rat and rabbit retinas strongly suggests that SP and NK1 receptors may mediate processes in the developing retina that are different from those in the retina capable of visual information processing (Casini et al., 2000, 2004a). In contrast, studies in the developing rat retina showed that NK3 appear only near the time of eye opening (Oyamada et al., 1999; Casini et al., 2000), when the main morphologic and functional characteristics of the retinal pathways have reached their maturity, and developmental functions of NKB and NK3 seem unlikely. The aim of the present study was to expand and complete this knowledge with observations in the developing mouse retina and in mice with genetic deletion of the NK1 gene.

EXPERIMENTAL PROCEDURES

Experiments were performed in compliance with the Italian law on animal care No. 116/1992, in accordance with the European Community Council Directive (EEC/609/86) and with guidelines established by the National Institutes of Health. All efforts were made to reduce both animal suffering and the number of animals used.

Animals

Both Swiss and Balb/c wild type mice at different postnatal ages were used in the developmental studies. Eye opening in our colonies was observed between postnatal day (PND) 11 and PND 13. In addition, NK1 KO mice kindly provided by Dr. Norma Davis (Harvard University, Boston, MA, USA) were also used. They were developed as previously described in detail (Bozic et al., 1996) and backcrossed to Balb/c mice. Animals were kept in a regulated environment (23±1 °C, 50±5% humidity) with a 12-h light/dark cycle (lights on at 8 a.m.) with food and water *ad libitum*. No appreciable effects of NK1 deletion were found on gross visual ability as evaluated by feeding and exploratory behavior. Eye blink and pupillary reflex were also normal.

Antibodies

A monoclonal antibody raised in rat and directed to SP (Cuello et al., 1979) was purchased from Chemicon (Temecula, CA, USA) and used at 1:500 dilution. A polyclonal antibody raised in rabbit and directed to the C-terminus of rat NK1 (Vigna et al., 1994) was also purchased from Chemicon and used at 1:200–1:400 dilution. An affinity-purified antiserum directed against a peptide sequence (410–417) of the intracellular tail of rat NK3 (kind gift of Dr. N. W. Bunnett, UCSF, San Francisco, CA, USA; Grady et al., 1996) was used at 1:500 dilution. Experiments for double-labeling immunofluorescence were performed using the antibody directed to NK1 in conjunction with a polyclonal antibody raised in guinea-pig and directed against the vesicular acetylcholine transporter (VAChT, Chemicon), used at 1:100 dilution.

Immunohistochemical procedures

Wild type mice at different postnatal ages as well as adult NK1 KO and corresponding Balb/c, wild type mice were anesthetized and killed with an overdose of chloral hydrate. The eyes were removed and immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 h. The fixed eyes were transferred to 25% sucrose in 0.1 M PB and stored at 4 °C. The retinas were cut with the whole eye in a plane perpendicular to the vitreal surface at 10 μm with a cryostat. The sections were mounted onto gelatin-coated slides and stored at −20 °C. For immunohistochemical processing, the sections were washed in 0.1 M PB and incubated overnight at 4 °C in SP, NK1 or NK3 antibodies diluted in 0.1 M PB containing 0.5–1% Triton X-100. Following washes in 0.1 M PB, the sections were incubated in secondary antibodies conjugated with Alexa Fluor fluorescent dyes (Alexa Fluor 546 or Alexa Fluor 488, Molecular Probes, Eugene, OR, USA) at a dilution of 1:200 in 0.1 M PB containing 0.5% Triton X-100 for 1–2 h at room temperature. Then, the slides were coverslipped in a 0.1 M PB–glycerin mixture. Specificity of NK1 immunostaining was evaluated by preadsorbing the antiserum with 10 μM of the peptide fragment $NK1_{393-407}$ overnight at 4 °C. The preadsorbed antiserum was then used in place of the primary antibody. Unspecific immunostaining was detected in the photoreceptor outer segments and occasionally in the neuroblastic layer (NBL), in the inner nuclear layer (INL) and in the ganglion cell layer (GCL) of immature retinas (see Fig. 3B, C).

Double-labeling experiments were performed on wild type retinas during postnatal development. The sections were incubated with a mixture of NK1 and VAChT antibodies in 0.1 M PB