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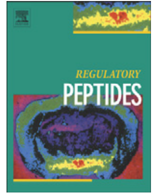

Secretoneurin and the tachykinins substance P and neurokinin-A/B in NMDA-induced excitotoxicity in the rat retina

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ABSTRACT

In a recent investigation using the NMDA-excitotoxicity model in the rat retina, we found that, whereas, following intravitreal injection of NMDA, a time-dependent decrease of the levels of a neuropeptide, namely vasoactive intestinal polypeptide (VIP), was fully counteracted by topical treatment with flunarizine eye drops, the levels of pituitary adenylate-cyclase activating peptide-38 (PACAP-38), another neuropeptide, remained unchanged. The aim of the present study was to find out if NMDA causes reduction in the levels of other neuropeptides such as secretoneurin (SN), neurokinin-A/B (NKA/NKB) and substance P (SP), and if so, whether flunarizine has the ability to counteract this effect or prevent such reduction. The reduction of the levels of SN and NKA/NKB 14 days after intravitreal injection of 2 µl of 100 nmol NMDA into one eye was more pronounced than after 7 days; topical flunarizine had a slight counteracting effect, but could not prevent the decrease in the levels of these peptides. Reduction in SP levels after 28 and 56 days was fully counteracted by flunarizine. By enabling a pronounced influx of Ca^{2+} ions into peptide-expressing cells, NMDA leads to cell death. Since each of these peptides exerts neuroprotective properties in the central nervous system, the drop in their levels caused by acute insult (e.g. NMDA excitotoxicity) or chronic insult (e.g. glaucoma) may cause a breakdown of endogenous neuroprotection in the retina given that these peptides feature neuroprotective properties in the retina as well.
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1. Introduction

Secretoneurin (SN) and the tachykinins are neuropeptides which are present in the central and peripheral nervous system where they act as neurotransmitters and/or neuromodulators. SN is generated *in vivo* by proteolytical processing of secretogranin II (formerly called chromogranin C) [1,2]. This neuropeptide is known to be strongly conserved during evolution [3] and furthermore, in the rat brain [3,4] and also in some peripheral ganglia such as the trigeminal ganglion [5], secretogranin II is almost completely proteolytically processed to SN. SN is widely and distinctly distributed throughout the nervous system (review see [6]) and although a specific receptor has not been found for this peptide so far, it is biologically active as evidenced by a number of biological effects (review see also [6] and [7]).

The tachykinins are a family of neuropeptides which are characterized by a common C-terminal amino acid sequence, in particular Phe-X-Gly-Leu-Met-NH_2 [8], and exert a number of similar biological effects. Substance P (SP) is the best characterized neuropeptide in the body, but in the last three decades, other tachykinins have been characterized, the two main ones being neurokinin A (NKA) and neurokinin B (NKB) [9,10]. The tachykinins exert their effects by interacting with specific NK receptors, namely NK1, NK2 and NK3. SP, NKA and NKB have the highest affinity for, but act preferentially rather than selectively on, the NK-1, NK-2 and NK-3 receptors, respectively [10].

Both SN and the tachykinins SP, NKA and NKB are expressed in the retina. SN is present in amacrine cells in the proximal inner nuclear layer (INL) as well as in cells of the ganglion cell layer (GCL) with dendrites ramifying in sublaminas 1 and 5 of the inner plexiform layer (IPL) in the human retina [11]. Previous studies demonstrated the presence of SP and NKA/NKB in the retina. In the rat retina, SP is expressed primarily by populations of sparsely distributed amacrine cells and displaced amacrine cells that arborize in laminas 1, 3 and 5 of the IPL [12–17]. Some ganglion cells also express SP-immunoreactivity (IR) [18,19]. The other two tachykinins NKA and NKB have also been shown to be present in the retina. In the human retina, NKA was localized to sparse amacrine cells in the proximal INL, to displaced amacrine cells in the GCL with processes ramifying in stratum 3 of the IPL and also to sparse ganglion cells [20]. Staining of NKB, however, was only observed in ganglion cells in the GCL and in the nerve fiber layer (NFL) and double-immunofluorescence revealed cellular colocalization of both NKA and NKB with SP [20].

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Previous research indicated that neurotoxicity in the retina is caused by excessive stimulation of receptors for excitatory amino acids. In particular, glutamate has been shown to act as a neurotoxin which exerts its toxic effect on ganglion cells predominantly through the NMDA receptor (review see [21]). NMDA receptor-mediated toxicity in retinal ganglion cells is dependent on the influx of extracellular Ca^{2+}. The increase in intracellular Ca^{2+} acts as a second messenger that set in motion the cascade leading to cell death [21,22] and the Na^{+}/Ca^{2+} exchanger NCX1 was found to play a role in retinal cell death induced by intravitreal NMDA [23]. Very recently, we evaluated the effect of intravitreal injection of NMDA on the levels of vasoactive intestinal polypeptide (VIP) and pituitary adenylate-cyclase activating polypeptide-38 (PACAP-38) in the rat retina and found a time-dependent reduction of the levels of VIP, whereas the levels of PACAP-38 remained unchanged [24]. Furthermore, topical treatment of the rat eye with flunarizine eye drops counteracted the drop in VIP [24]. Flunarizine was used in our studies because it acts neuroprotectively by inhibiting influx of Na^{+} and Ca^{2+} ions into cells [25]. The aim of the present study was to find out (i) whether there is NMDA-induced reduction in the levels of neuropeptides other than VIP and PACAP-38 and whether the lack of an effect of NMDA on PACAP-38 is an exception, and (ii) whether topical flunarizine can, as in the case of VIP, counteract NMDA-induced reduction in the levels of SN, NKA/NKB and SP, should such a decrease be indeed observed in this study.

2. Methods

2.1. Animals

Male Sprague–Dawley rats ($n = 96$; 160–220 g) used in this study were housed in cages with a dark–light cycle of 12 h each at 23 ± 1 °C. They were fed commercial chow and provided with water ad libitum. All experimental and animal care procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; the animal experiments were approved by the Austrian Ministry of Science.

Each rat received an intravitreal injection of 2 µl of 100 nmol NMDA into the left eye under deep inhalation anesthesia with 1% isoflurane [26]; the untreated eye served as control. To evaluate the efficacy of topical flunarizine treatment, rats were divided in two groups: one group was topically treated with three drops of 2% flunarizine in 50% polyethylene glycol on both eyes two days before NMDA injection and twice daily thereafter until sacrifice; the same protocol was used in the other group, which, however, received topical application of the vehicle alone on both eyes. For the SN- and NKA/NKB-radioimmunoassay (RIA), rats were sacrificed after seven and 14 days (with $n = 6$ at each time point) with an overdose of CO_2. This was done so because neither the long- nor short-term but an intermediate-term effect of NMDA was aimed to be evaluated and because after 7 days amacrine cells certainly should have been lost and after 14 days even ganglion cells. For SP-detection, measurements by RIA were performed from aliquots of samples from our recent study taken 28 and 56 days after NMDA application [24] because there were no sufficient extracts available from the present study. But for SP, it was only of interest to find out whether intravitreal NMDA lowers the levels of SP as well and if so, whether flunarizine counteracts the effect of NMDA and both of these questions could have been answered with the 28 and 56 days time points so that the authors decided not to make further animal experiments. The eyes were enucleated, the anterior segment was removed by cutting circumferentially at the limbus, and the retina separated from the retinal pigment epithelium. It was then weighed and stored at −70 °C. Prior to use, each retinal sample was homogenized in 0.6 ml of 2 M acetic acid, centrifuged (3500 g, 10 min), the supernatant was lyophilized and redissolved in assay buffer.

2.2. SN-RIA

In brief, iodination of SN (Neosystems, Strasbourgh, France) was performed with the chloramine T method to a specific activity of 7.7×10^4 cpm/ng. SN and ^{125}I-SN were used as standard and tracer, respectively. Samples and standards were incubated with the antiserum (dilution 1:18,000) and tracer (10^4 cpm) for 48 h at 4 °C. All dilutions were made with radioimmunoassay buffer [150 mM NaH_2PO_4, pH 7.4, 15 mM NaCl, 0.02% NaN_3, 0.0006% phenol red, 0.1% bovine serum albumin, 0.0006% gelatine]. Bound and free activities were separated by adding 1 ml of dextran-coated charcoal. After15 min incubation at 4 °C, samples were centrifuged for 15 min at 3200 g followed by counting of the supernatant in a γ-spectrophotometer. The antiserum against synthetic rat SN (Sg II 154–186) coupled to keyhole limpet hemocyanin was raised in Chinchilla Bastard rabbits. It reacts equally well with the free peptide and all larger proteins containing the SN sequence including SgII. The antiserum fully cross-reacts with human SN, which differs in only one amino acid from the rat sequence.

2.3. SP- and NKA/NKB-RIA

RIA was performed with specific antisera: SP2 for SP and K12 for NKA/NKB (gift from Theodorsson E., Department of Clinical Chemistry, University Hospital, Linkoping, Sweden). The antibody K12 recognizes both NKA and NKB and other tachykinins including neuropeptide K, NKA (3–10) and NKA (4–10). Non-radioactive peptides were purchased from Peninsula (Peninsula Laboratories, 601 Taylor Way, San Carlos, CA 94070, USA) and radioactively-labelled peptides from Amersham [(^{125}I)-Bolton Hunter-SP; (2-[^{125}I] iodohistidyl^1) neurokinin A; Amersham Biosciences, Münzinger Straße 9, 79111 Freiburg, Germany]. Incubation was performed for 48 h without and a further 48 h with the tracers added (approximately 6000 cpm for SP and NKA/NKB each) with an antibody dilution of 1:10,000 for SP2 and of 1:200 for K12. Separation of bound and free radioactivity was carried out with dextran-coated charcoal. Bound activity was counted by a γ-counter. Under these conditions the detection limit was 0.5 fmol.

2.4. Statistics

Statistical calculation for comparison of controls with treated specimens was performed with the Mann–Whitney *U* test. *P* values <0.05 (*) and <0.01 (**) were considered significant and highly significant, respectively.

3. Results

3.1. SN-like immunoreactivity (SN-LI)

SN was detected in each retinal sample (Fig. 1). SN-LI averaged 16.28 (±1.25) fmol/mg wet weight and 12.98 (±0.56) fmol/mg wet weight in untreated controls after seven and 14 days, respectively. Intravitreal NMDA injection led to a 42.4% (±22.4) decrease of the concentration after 7 days (*) and to a 69.96% (±13.5) decrease after 14 days (**) and both of these values were statistically significant. Topical flunarizine treatment alone had no effect at either of these time points and counteracted the effect of NMDA only partially. Thus, the concentration of SN was 67.1% (±21.95) of controls after 7 days, which was statistically not significant, and 44.45% (±17.85) of controls after 14 days, which remained significantly reduced (**).

3.2. NKA/NKB-LI

Fig. 2 shows the results of the NKA/NKB-RIA. In untreated controls, NKA/NKB-LI averaged 30.68 (±1.46) fmol/mg wet weight after 7 days and 32.14 (±2.7) fmol/mg wet weight after 14 days. In the human retina, RIA performed subsequent to reversed-phase HPLC revealed that

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