Co-localization of neuropeptide Y and noradrenaline in the rat brainstem at cellular and ultra-structural levels

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The central nor/adrenergic (N/Aergic) system has been linked to increased sympathetic outflow from the brain and to hypertension [1]. Neuropeptide Y (NPY) is widely distributed and co-localizes with nor/adrenaline (N/A) in peripheral and central areas, and various studies suggest a role in blood pressure control [2]. However, the extent to which NPY and N/Aergic systems overlap in brainstem nuclei that are key for cardiovascular control is unknown. Such nuclei are the nucleus tractus solitarius (NTS) that harbour the A2 noradrenergic neurones, the caudal ventrolateral medulla (CVLM) containing the A1 noradrenergic neurones, and the rostral ventrolateral medulla (RVLM) where C1 adrenergic neurones reside. Earlier electron microscopy (EM) studies have demonstrated two vesicle populations in central N/Aergic neurones; small clear vesicles (SCV) of 40-60 nm diameter and dense core vesicles (DCV) of 80-120 nm diameter [3]. Nevertheless, cell physiological studies have suggested an additional population of larger vesicles [4]. Whether NPY and N/A are stored in (and therefore likely released from) the same or different vesicle populations, was not known.

The aim of this study was to investigate (i) levels of coexpression of N/Aergic markers and NPY in the rat brainstem, and (ii) the ultra-structure of central N/Aergic neurones in order to characterize their exocytic vesicles, including any NPY content. To this end, we mapped the cellular and sub-cellular localization of NPY and of N/A using dopamine-β-hydroxylase (DbH) and vesicular monoamine transporter 2 (VMAT2) as markers. To address the question whether the distribution of NPY or N/A may change in hypertension, experiments were carried out in both Wistar (WR) and spontaneous hypertensive rats (SHR).

Experiments were conducted in adult (300g) male WR and SHR, in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines. WR and SHR (n = 3) were injected in NTS and CVLM/RVLM with an adenoviral vector expressing GFP (0.5 ul/injection site, 10¹⁰ TFU/ml) that labels specifically N/Aergic neurones in those nuclei [5]. After 2 weeks, WR and SHR (n = 3 each) were perfusion-fixed transcardially with 4% paraformaldehyde and post-fixed for 12-24 hours at 4C in the same fixative. The brainstem was sliced at 40μm with a cryostat. Slices were incubated with primary antibodies (1:400 NPY; kind gift of Dr. Grouzmann, Lausanne, and 1:200 DbH; abcam) in the blocking solution (3% goat serum; 1-2% BSA; 0.1% PBS-TX) at room temperature for 16-24 hrs and then in secondary antibodies (Alexa Fluor 594 and Biotin-Streptavidin-Alexa Fluor 488) in blocking solution for 1-2 hrs at room temperature. Fluorescent images were acquired with a confocal laser scanning microscope (Leica SP1).

For EM, WR and SHR (n = 3 each) were perfusion-fixed transcardially with 4% paraformaldehyde and kept at 1% PFA at 4°C. The brainstem was sectioned on a Vibratome at 200μm and a 1mm tissue puncher was used to extract areas of interest, which were processed for high pressure freezing (Leica EMMACT2). Subsequently, freeze substitution and resin infiltration with Lowicryl HM20 was performed (Leica AFS2). After polymerization of the resin at 50°C, the tissue was sectioned at 100nm and processed for immunogold labelling [6]. The sections were incubated with primary antibodies (DbH 1:40; abcam, VMAT2 1:40; Millipore, and NPY 1:40; kind gift of Dr. Grouzmann, Lausanne) in blocking solution (0.1% BSA in PBS-TX) for 1 hour. This was followed by incubation with secondary gold-conjugated antibodies (10nm and 15 nm) in blocking solution for 30-60 min. Ultrastructure and immunogold labelling was analyzed in a FEI Tecnai12 TEM. Statistical significance was determined according to Student’s unpaired t-test.

Cellular co-localization of DbH and NPY was higher in rostral NTS (81%), CVLM (88%) and RVLM (82%) than caudal NTS (23%). No differences were seen between SHR and WR in any of these areas (Fig. 1). The preservation of both the ultrastructure and antigenicity was satisfactory.
Interestingly, the mean size of labelled N/Aergic vesicles appeared to be larger than previously thought. A main population was found between 175-475 nm (n = 209 labelled profiles). Co-localization, at the ultra-structural level, for N/A and NPY occurred more often, in both NTS and RVLM, in SHR than WR. Between NTS and RVLM there was no significant difference in the same rat strain (Table 1). In comparing the two rat strains, we noticed that co-localization in the RVLM in SHR takes place in significantly larger vesicles than the RVLM in WR (Fig. 3a), whereas no significant difference was seen for the NTS between SHR and WR (Fig 3b). Further, in agreement with previous cell physiological studies [7], in the RVLM of SHR, NPY and N/A are co-localized in significantly larger vesicles than in the NTS of SHR (Fig. 3).

References


Figure 1: a) Representative confocal images of DbH (red) and NPY (green) immunostaining for both WR and SHR in corresponding brainstem nuclei. Scale bar is 50 μm. b) Percentage cellular co-localization of DbH and NPY in WR (green) and SHR (purple) in corresponding brainstem areas.

Figure 2: Representative TEM images of DbH/VMAT2 (blue arrow) and NPY (black arrow) immunostaining, in central N/Aergic neurones, for a) NTS of SHR; scale bars are 500nm and 100nm respectively, b) RVLM of SHR; scale bars are 500nm and 200 nm respectively.

Figure 3: a) Vesicular size distribution of the co-localization of VMAT2 or DbH and NPY in central N/Aergic vesicles in a) RVLM and b) NTS, for WR and SHR. Statistical significance was determined according to Student’s unpaired t-test; note that red bars occupy significantly larger vesicles than purple and yellow ones: RVLM in WR vs. RVLM in SHR p<0.01; NTS in WR vs. NTS in SHR p=0.1; RVLM in WR vs. NTS in WR p=0.12; RVLM in SHR vs. NTS in SHR p<0.01.

Table 1: % co-localization of NPY and DbH/VMAT2 for WR, SHR in NTS, RVLM at the ultra-structural level

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<td>NPY in NAergic vesicles</td>
<td>21%</td>
<td>23%</td>
<td>52%</td>
<td>61%</td>
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