Research report

Colocalization of tyrosine hydroxylase with oxytocin or vasopressin in neurons of the human paraventricular and supraoptic nucleus

Maria T. Panayotacopoulou ^{a,*}, Frederik C. Raadsheer ^b, Dick F. Swaab ^b^a *Department of Psychiatry, University of Athens, Eginition Hospital, 74 Vassilissis Sophias Ave., Athens 115-28, Greece*^b *Graduate School Neurosciences, Netherlands Institute for Brain Research, Amsterdam, The Netherlands*

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Abstract

In the developing and adult human paraventricular (PVN) and supraoptic (SON) nucleus, a large proportion of neurons contains the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH). In the present study we investigated the possible colocalization of TH with oxytocin (OXT) or vasopressin (VP) in the adult and neonatal PVN and SON. Adjacent paraffin sections were incubated simultaneously with two antibodies: a polyclonal against TH and a monoclonal against OXT or VP and stained with a double peroxidase-antiperoxidase/alkaline phosphatase method. We observed that TH-immunoreactive (IR) perikarya in the human PVN and SON were also positive for OXT or VP. A clear difference between the neonates and adult cases of our sample was observed in the proportion of TH-IR neurons that colocalize OXT or VP. In the neonates the majority of the TH-IR perikarya was also stained for VP, while only few TH-IR neurons were also positive for OXT. The opposite was observed in the adults, where the majority of the double-stained TH-IR neurons colocalizes OXT while only few TH-IR perikarya appear to contain VP. Our study establishes the colocalization of TH with OXT or VP in the adult and neonatal PVN and SON and indicates that antemortem factors such as perinatal hypoxia might increase TH-immunoreactivity of the VP neurons in man.

Keywords: Tyrosine hydroxylase; Human hypothalamus; Paraventricular nucleus; Supraoptic nucleus; Oxytocin; Vasopressin; Perinatal hypoxia; Fetal stress

1. Introduction

Previous immunohistochemical studies indicate that in the adult human paraventricular (PVN) and supraoptic (SON) nucleus a large proportion of neurons contains the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) [25,30]. In the developing human PVN and SON TH-immunoreactive (IR) perikarya appear in the late gestational period, since a large number of TH-IR neurons was found in the full-term neonates, while only few were evident in the preterm ones [31]. Among the full-term infants a large variation was seen with respect to the number of TH-IR perikarya, the larger number being observed in full-term neonates who died of delivery-related asphyxia or hypoxia [31]. Activation of the hypothalamo-neurohy-

pophyseal system during the process of labor was reported by many authors [7,20,29,32] and the observed increased secretion of vasopressin (VP) by the fetus during labor was considered an adaptive mechanism to overcome perinatal hypoxia [32]. Since an increase of TH-immunoreactivity in the VP-producing neurons was observed after experimental manipulations that activate VP-synthesis [24,27,28], we raised the question whether the presence of a larger number of TH-IR perikarya in neonates that died from perinatal hypoxia represents a primary developmental phenomenon, or whether it partially reflects a secondary phenomenon related to the activation of the vasopressinergic systems during the process of labor [31]. The first step in the study of this subject is to investigate whether the TH-IR neurons in the neurosecretory nuclei of the human neonate also synthesize VP.

In the adult human PVN and SON Li et al. [25] estimated that the TH-IR neurons represent 38% and

* Corresponding author. Fax: (30) (1) 7249902.

41%, respectively, of the total neuronal population, and that the majority of these neurons is magnocellular. The same authors applied a double immunofluorescent method to show that only a subclass of the TH-IR perikarya in the adult PVN and SON also contains OXT, whereas no colocalization with VP was observed. They showed, however, that in the SON the majority (77%) of the TH-IR perikarya is concentrated in the lateral subdivision of this nucleus where, as previously reported [13,15], more than 90% of the neurons contain VP. On the other hand they reported that the combined number of TH and VP-IR neurons is higher than the number of Nissl-stained perikarya, and they therefore suggested that some cells may contain both TH and VP, but the double immunofluorescent method they applied was not sensitive enough to demonstrate that [25].

Purpose of the present study was, therefore, to investigate the colocalization of TH, not only with OXT but also with VP, in the adult and neonatal PVN and SON with the application of a double peroxidase-antiperoxidase (PAP)/alkaline phosphatase (AP) technique [9,33] which, due to the utilization of a bridging antiserum, is expected to be more sensitive than the immunofluorescent procedure used in previous experiments [25].

In the present report we demonstrate the colocalization of TH with OXT or VP in the human neurosecretory neurons. We also show that in the neonate TH preferentially colocalizes with VP, while in the adult

cases of our sample TH preferentially colocalizes with OXT.

2. Materials and methods

The material of our study consisted of the brains of 7 full-term infants (from 37 weeks gestational age to 14 months after birth) and 5 adults (ages ranging from 30 to 90 years) with no history of any primary neurological or psychiatric disease. The clinical and pathological data of the cases used are presented in Table 1 (for additional information concerning the infant material see also Table 1 in [31]). The brains were obtained at autopsy with a postmortem delay of 5.5 to 48 h, and fixed in 10% formalin for 19 to 48 days. The hypothalamus was subsequently dissected, dehydrated in graded alcohol, and embedded in paraffin. Serial 6 μ m frontal sections of each subject were stained as follows: section 1 was incubated in a polyclonal anti-VP antibody (Truus, 10.4.1986, 1:1000) [39] to delineate the neurosecretory nuclei. Section 2 was incubated in a polyclonal anti-TH serum (Jacques Boy Institute, France, 1:1000) [30,31] in order to estimate the relative differences in number of the TH-IR neurons among the cases. Sections 1 and 2 were subsequently stained with the PAP method, using diaminobenzidine (DAB) as a chromogen, intensified with nickel ammonium sulphate (Ni) as previously described [31].

Sections 3 and 4 were used for the double PAP/AP method [33] in order to reveal TH+VP or TH+OXT-immunoreactivity in the same section. For that purpose the sections were rehydrated, rinsed in Tris-buffered saline (TBS: 0.05 M Tris buffer with 0.5 M NaCl, pH 7.6 and incubated overnight (at 4°C) simultaneously with two antibodies: a polyclonal against TH (Jacques Boy Institute, France, 1:1000) and a monoclonal against VP (MAB III D7, 1:100 [22] donated by A. Hou-Yu, Columbia University, New York), or a monoclonal against OXT (1:100 for infants and 1:1000 for adults, MAB 1–28 [23], also kindly offered by A. Hou-Yu). The antibodies

Table 1
Clinical and pathological data of the cases studied

Case no.	Age/Sex	Cause of death	Postmortem delay (h)	Fixation (days)	TH-IR neurons in PVN and SON	TH + VP neurons	TH + OXT neurons
89.153.3	37w/M	pneumothorax, streptococcus sepsis, bronchopneumonia, hypoxia	48	30	++	+	±
87.361.3	39w/F	perinatal asphyxia, brain oedema	5.5	24	+++	++	±
88.353.2	40w/M	asphyxia, meconium aspiration	24	33	+++	++	+
86.358.6	40w/F	meconium aspiration, cerebral hypoxia	24	19	++	+	±
87.280.3	40w/M	hypoplastic left heart syndrome, cerebral anoxia	24	35	+	±	±
85.002.3	3m/F	bronchopneumonia, myocarditis, subarachnoidal haemorrhage	24	33	+++	+	++
88.209.2	14m/M	pneumococcal meningitis, sepsis, pericarditis, endocarditis, brain oedema	12	28	+	±	±
81.255	30y/F	acute heart death	24	39	+	–	+
84.248	37y/M	bronchopneumonia, pleuritis	39	35	++	±	++
81.093	42y/M	adenocarcinoma, pneumothorax, bronchopneumonia	22	42	++	±	++
81.032	74y/M	heart failure, bronchopneumonia	13	48	++	±	++
8538	90y/F	breast cancer	6	33	++	±	++

w: weeks of gestation; m: months after birth; y: years; M: male; F: female

Symbols indicative of the relative differences in TH-IR cell numbers: +++: large number; ++: many; +: some; ±: very few; –: none. For additional data concerning the infant cases see also [31].

were diluted in an incubation buffer consisting of TBS (pH 7.6) with 0.25% gelatin and 0.5% Triton X-100. After the incubation in the primary antisera the sections were washed with TBS (2×10 min) and incubated in goat anti-rabbit serum (Betsie, 1:50 in incubation buffer) for 30 min and rinsed with TBS (2×10 min). Subsequently the sections were incubated simultaneously in rabbit PAP (1:1000) and alkaline phosphatase (AP)-labeled goat anti-mouse IgG (H+L) serum (1:50, Kirkegaard and Perry Laboratories, USA) in TBS for 1 h, washed with TBS (2×10 min) and rinsed with 0.1 M Tris-HCl buffer (pH 8.5). The AP was revealed in blue color after incubation of the sections for 30 min in a filtered solution of 2 mg Fast Blue BB base (Sigma) in 10 ml 0.1 M Tris-HCl buffer (pH 8.5) containing 2 mg Naphthol AS MX phosphate (Sigma) and 3 mg Levamisole (Sigma) diluted in 0.2 ml *N,N* dimethylformamide. The sections were then washed with Tris-HCl buffer (0.1 M, pH 7.6, 3×10 min) and the peroxidase was revealed in red color after incubation of the sections for 20 min in a filtered substrate consisting of 25 mg 3-amino-9-ethyl-carbazole (Sigma) in 2.5 ml *N,N*-dimethylformamide diluted in 50 ml 50 mM Na-acetate buffer (pH 4.9) and activated with 20 μ l of 30% H_2O_2 just before use. The sections were finally rinsed with TBS (3×10 min) and coverslipped with Kaisers glycerin gelatin (Merck). The specificity of the monoclonal antibodies MAB 1–28 for OXT and MAB III D7 for VP was previously tested using radiobinding, absorption and immunocytochemical tests [22,23]. The specificity of the immunohistochemical reaction for TH was checked by incubation of adjacent sections in a serum adsorbed with the purified enzyme TH, which was kindly donated by Prof. J. Thibault (College de France, Paris), or with pre-immune serum.

3. Results

The observation of the two adjacent sections stained with DAB-Ni for VP and TH respectively, showed that, within the boundaries of the neuroendocrine nuclei, a considerable population of TH-IR perikarya was present in both the infant and adult cases. Most of the TH-IR neurons within the PVN and SON were magnocellular and stained intensely throughout the entire cytoplasm and processes. Some lightly stained TH-IR neurons were also clearly seen, especially in the SON. In the control sections, i.e. those incubated with the antibody adsorbed with the purified enzyme (TH) or the pre-immune serum, no peroxidase reaction appeared in any cell soma or fiber in the hypothalamus.

The number of TH-IR neurons varied among the cases, as is shown in Table 1. The largest number of TH-IR neurons was observed in two newborns of 39 and 40 weeks of gestation, as well as in an infant who died 3 months after birth. In the above two newborns intensely stained TH-IR perikarya were distributed across the entire PVN and SON, while in the 3-month-old infant the TH-IR neurons in the SON were mainly found in the dorsal part, i.e. the OXT-containing part of this nucleus (for details on the distribution of TH-IR neurons in the infant PVN and SON see Figs. 1 and 2 in [31]).

A limited number of TH-IR neurons was observed in a neonate of 40 weeks of gestation, a 14-month-old infant and a 30-year-old adult. In these cases some

TH-IR perikarya were revealed in the PVN, while in the SON only a few TH-IR neurons could be seen in the dorsal part. In the majority of the adult cases of our sample we observed many intensely stained TH-IR perikarya throughout the PVN and the dorsal part of the SON, while some lightly stained TH-IR neurons were evident in the central part of the SON.

The application of the double PAP/AP method for the simultaneous demonstration of TH + VP or for TH + OXT on the same section showed that TH is localized within both the VP and OXT-producing neurons in the human PVN and SON. With this method a clear difference was observed between infant and adult cases concerning the relative number of the TH-IR neurons that colocalize VP or OXT (Table 1). In the PVN and SON of the neonates the majority of TH-IR perikarya was also stained for VP, whereas only few were also stained for OXT. The opposite was observed in the adult cases of our sample, where the majority of the double-stained TH-IR neurons colocalized OXT, while only few appeared to contain VP. In the 3-month-old infant relatively more TH-IR neurons colocalized OXT than VP.

With the double procedure we used, the TH-IR neurons were revealed in red stained with amino-ethyl-carbazole, the VP or OXT-IR neurons in blue, stained with fast blue, and the double-stained perikarya for TH + OXT or TH + VP in purple or violet. In the adults, the AP-Fast blue reaction for the visualization of the monoclonal antibodies against VP or OXT was quite intense. Therefore it became unnecessary to use a bridging antiserum to intensify this reaction.

In the neonates the immunohistochemical reaction with the monoclonal antibodies against VP and, especially, OXT was lighter than that observed in the adult. In order to ameliorate the staining for OXT in the neonates, a higher concentration of the monoclonal antibody (1:100) was used.

3.1. Colocalization of TH with OXT

Colocalization of TH with OXT was especially clear in the adult cases. Many double-stained TH + OXT positive neurons were found throughout the PVN, in the dorsal part of the SON as well as in the accessory SON. Most of the double-stained TH + OXT neurons in the adult were revealed in purple (Fig. 1). Single-stained TH-IR or OXT-IR neurons were also evident, stained in red or blue, respectively (Fig. 1). In the majority of the infant cases we observed only a limited number of neurons showing a faint double reaction for TH + OXT positive perikarya. Only in the 3-month-old infant did we observe many double stained TH + OXT positive perikarya (Fig. 2).

3.2. Colocalization of TH with VP

In the adult cases of our sample the double PAP/AP method revealed only a limited number of double-stained TH + VP positive perikarya in both the PVN (Fig. 3) and the SON (Fig. 4). In a 30-year-old case in whom a limited number of TH-IR perikarya was found, no double-stained TH + VP positive neurons were visualized.

In the neonates, however, a considerable number of double-stained TH + VP neurons was visualized in the PVN (Fig. 5) as well as in the SON (Fig. 6). In two infant cases (no. 87.280.3 and 88.209.2), both containing only a limited number of TH-IR perikarya, we also observed only a limited number of lightly stained TH + VP positive neurons in the PVN, but not in the SON. In the 3-month-old infant that contained a large number of TH-IR perikarya, some TH + VP positive neu-

rons were evident in both the PVN and SON, although the majority of double-stained TH-IR neurons appeared to colocalize OXT.

4. Discussion

With the application of the PAP procedure a considerable number of TH-IR perikarya was observed, both in the infant and adult PVN and SON, which is indicative of catecholamine synthesis [25,30,31]. Strong individual differences were observed concerning the number and distribution of the TH-IR perikarya, especially in the SON. Similar differences had been reported previously, both in the developing and adult human hypothalamus, and appeared not to be related to the gender of the subjects, nor to postmortem or fixation time [30,31], indicating that antemortem factors may influence the expression of TH-immunoreactivity in neurons of the human neurosecretory nuclei.

The application of the double PAP/AP method for the simultaneous staining of TH + OXT or TH + VP immunoreactivity showed that a population of TH-IR neurons in the human PVN and SON also contains OXT or VP. Interestingly, this double procedure revealed differences between adults and neonates concerning the proportion of TH-IR perikarya that colocalize OXT or VP. Since the numbers of OXT and VP-IR neurons are similar in full-term neonates and adults [16], the differences in number of double-stained neurons might be attributed to a differential expression of TH-immunoreactivity within the OXT and VP-IR neurosecretory perikarya.

In the rat PVN and SON the level of TH-immunoreactivity in the magnocellular neurosecretory neurons is shown to be regulated by afferent inputs [24]. In the rat brain – under normal conditions – only very few TH-IR neurons are detectable in the magno-

cellular subnuclei of the PVN [21,40], while in the SON TH-IR perikarya are only occasionally found [6]. However, after midbrain transection, which disrupts the afferents from the lower brainstem to the PVN and SON, a substantial increase was observed in the number of TH-IR neurons, the distribution of which overlaps that of OXT and VP-producing neurons [24].

In the adult human cases of our sample the majority of double-stained TH-IR neurons was also intensely stained for OXT, while in the neonates only a few, faintly, reacted to OXT as well. Only in the 3-month-old infant numerous intensely stained TH + OXT-IR neurons could be visualized. Based on the above observations, we suggest that either the TH-immunoreactivity in the OXT-producing neurons develops gradually within the first months of life in the human infant or, alternatively, that the concentration of TH and/or OXT in these neurons is below the detection limits of the double labeling technique we applied.

In the neonates of our sample, a lighter reaction was observed when we used the monoclonal antibodies against VP and, especially, OXT, compared to that observed in the adults. In the human fetus radioimmunochemically detectable amounts of VP and OXT are present as early as the 11th and 13th weeks of gestation respectively [4], but the appearance of OXT is delayed in comparison to that of VP [4] due to a delayed post-translational processing of the precursor form [12]. An active secretion of both OXT and VP was reported during delivery [7,20], supporting the hypothesis of an active involvement of the fetus in the process of labor [7,29,38]. Since the immunohistochemically detectable amount of a peptide depicts the balance between production, transport and release, we suggest that the lighter staining for OXT and VP observed in the neonates compared to that of the adults might be attributed either to a limited synthesis of these peptides in the early neonatal period, as

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Fig. 1. PVN of a 74-year-old male (no. 81.032) double-stained for TH + OXT. TH-IR perikarya are stained in red (open arrow), OXT-IR neurons in blue and double-stained for TH + OXT neurons in purple (arrow).

Fig. 2. SON of a 3-month-old infant (no. 85.002.3) double-stained for TH + OXT. Note neurons containing both TH and OXT stained in purple (arrow) but also single-stained TH-IR perikarya in red (open arrow).

Fig. 3. PVN of a 37-year-old male (no. 84.248) double-stained for TH + VP. TH-IR neurons are revealed in red, VP-IR neurons in blue and double-stained TH + VP positive perikarya in violet (arrow).

Fig. 4. SON of a 37-year-old male (no. 84.248) double-stained for TH + VP. Violet neurons containing both TH and VP are evident (arrow) among the blue VP-IR perikarya.

Fig. 5. PVN of a neonate of 40 weeks of gestation (no. 88.353.2) double-stained for TH + VP. Note many double-stained TH + VP positive neurons revealed in purple (arrows).

Fig. 6. SON of a neonate of 37 weeks of gestation (no. 89.153.3) double-stained for TH + VP. Note some perikarya containing both TH + VP revealed in purple (arrow) among the blue VP-IR perikarya. The bar represents 25 μ m.

suggested for the rat PVN and SON [8,41,35], or to the increased secretion of these neurohormones during labor [7,20].

Our findings confirm the previously reported data that a subclass of TH-IR neurons in the adult human PVN and SON also synthesizes OXT [25] and show that the colocalization of TH with OXT is present, albeit in a limited number of neurons, as early as the time of birth. Colocalization of TH with OXT was also reported in the PVN of the rabbit, but not of the rat or mouse, indicating that there are species differences as far as the expression of the TH-immunoreactivity in the neurosecretory perikarya is concerned [36].

Our study provides the first evidence that a population of TH-IR neurons in the human PVN and SON also synthesizes VP. The colocalization of TH with VP was clearly visualized in a considerable number of perikarya in the infants, while in the adult cases of our sample this colocalization was evident only in a limited number of neurons. Thus, the apparent discrepancy between our results and those reported previously for the adult PVN and SON [25] might be attributed to the difference in sensitivity of the applied immunohistochemical procedures. The use of a bridging antibody in the PAP/AP method we used may have increased the sensitivity of the immunohistochemical reaction, thus allowing the visualization of a few TH + VP-IR perikarya in the adult PVN and SON, whereas these did not appear to be present when the double immunofluorescent technique was applied [25].

The highest number of TH + VP colocalizing neurons was observed in the neonates of our sample, indicating an increased expression of TH-immunoreactivity in the VP-producing magnocellular neurons in these cases. A selective increase in the expression of TH-immunoreactivity [24,27] as well as of the mRNA for TH [43,28] was reported in magnocellular VP-producing neurons of the rat after experimental manipulations that activate the synthesis of VP. In the human neonate activation of the neurohypophyseal system was reported by many authors [7,20,29,32]. During the expulsion phase of labor in humans, VP levels reach the highest value ever recorded in any area of the human physiology [7]. After normal delivery, VP levels in cord blood are much higher than after elective caesarian section without labor, indicating an active secretion of VP by the fetus during labor [7]. It has been reported that the increased secretion of VP by the fetus during labor is induced by stress, asphyxia, or rises in intracranial pressure associated with delivery [7,20,32]. It is considered to be an adaptive mechanism intended to redistribute cardiac output to vital organs such as brain, heart and adrenals [32]. Since the highest number of TH + VP colocalizing perikarya was observed in the newborns of our sample, all of whom were delivered vaginally and died of perinatal asphyxia or hypoxia, we

suggest that the large number of TH-IR neurons observed in the PVN and SON of the human neonate [31] might be attributed to an increase of TH-immunoreactivity in the VP-producing neurons, due to or related to the hypoxic conditions during the process of labor.

It has been reported that the PVN and SON are only rarely affected by hypoxia in terms of hypoxic changes as revealed by conventional neurohistological techniques [2]. However, in the present study as well as in our previous one [31], we were able, with the application of immunohistochemical methodology, to demonstrate qualitative changes in the stainability of magnocellular neurons for TH in the PVN and SON of human neonates that died from perinatal asphyxia or hypoxia. Histochemical and *in situ* hybridization studies in experimental animals demonstrated that magnocellular neurons in the PVN and SON respond to various physiological stimuli in a different way, and appear to synthesize and store multiple bioactive substances (among others also TH) and their mRNAs [27,28]. Therefore, the observed increase of TH within the VP-producing neurons in the neonates could be considered as a physiological adaptive response to hypoxia probably subserving effective catecholamine synthesis under hypoxic conditions in labor (see below). The observed heterogeneity among the cases of our sample may thus depict different degrees of activation of this adaptive system.

TH is an oxygen-requiring enzyme catalyzing the initial rate limiting step in catecholamine synthesis, and kinetic properties suggest that oxygen availability may limit the synthesis of catecholamines in the brain [11]. However, TH loses its oxygen dependency during physical stress [11,3], indicating that substrate limitation can be overcome when the neuronal needs for a neurotransmitter are increased [11]. PC-12 pheochromocytoma cells cultured under hypoxic conditions showed an increased TH activity attributed to an increase in enzyme protein [14]. This increase in TH activity and content is considered to be an adaptive response subserving effective catecholamine metabolism under hypoxic conditions [14,34]. *In vivo* hypoxia exerts a stimulatory influence on TH protein level, as well as in TH mRNA in the carotid body and the adrenal gland [37,10,42]. In the neonatal rat brain the rate of tyrosine hydroxylation decreases within the first 30 min of hypoxia [17] but returns to normal levels when the hypoxic period is extended [18] and even shows a twofold increase during the recovery period [19]. Furthermore, perinatal asphyxia in rat pups resulted in a time-dependent increase in number of TH-IR perikarya in the substantia nigra and ventral tegmental area [1]. On the other hand, unilateral hypoxic-ischaemic injury in the neonatal rat brain results in a persistent increase of striatal TH-immunoperoxidase staining of fibers on the injured side [5].

Although it is difficult to dissociate the effect of all the varieties of stress sustained by the human fetus during labor, taking into consideration the above experimental data, we suggest that perinatal hypoxia could be a potent stimulus for an activity-related increase of the TH-immunoreactivity in VP neurons of the human neonate. Other antemortem factors known to activate VP release – such as subarachnoid hemorrhage [26] reported in the case history of the 3-month-old infant of our sample (no. 85.002.3) – could also influence the intensity of TH-immunoreactivity in the VP neurons in man. This hypothesis is supported by the observation of an increased number of TH-IR perikarya in the PVN and SON of this case, part of them also colocalizing VP.

In conclusion, our study establishes the colocalization of TH with both OXT or VP in the human PVN and SON and provides an indication that antemortem factors could influence the expression of TH-immunoreactivity within the classically described neurosecretory neurons in man.

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