Abstract The nucleus of the solitary tract (NST) is the major visceral sensory nucleus in the brainstem. The development of the rat nucleus of the solitary tract was followed during late prenatal and early postnatal life in order to determine when subnuclear organization and chemoarchitectural features develop. In Nissl-stained sections, the nucleus of the solitary tract becomes visible as a distinct cluster of cells by about E17. Between E17 and E19, a profound change in the Nissl-stained appearance of the nucleus occurred, so that by E19 all the subnuclei were discernible. Acetylcholinesterase activity in the developing NST showed an early period of rapid differentiation (E15 to E17), while by E19 the basic adult pattern of distribution of this enzyme had already been achieved. The subnuclei of the NST began to show clear differential staining for nicotinamide adenine dinucleotide phosphate diaphorase at about the same time as reactivity for that enzyme first appeared (E19). With respect to calbindin- and calretinin-immunoreactive neurons within the nucleus, many of the chemoarchitectural features associated with these two markers were obvious even by late fetal life. For example, in the central subnucleus, a strongly labelled, dense population of calbindin-immunoreactive neurons was present from E17; while in calretinin-immunoreacted material, this subnucleus was prominent because of its immunonegativity also from E17. Nevertheless, the total number of calbindin- and calretinin-immunoreactive neurons in the NST did not peak until late postnatal life. Tyrosine hydroxylase immunoreactive neurons were visible from E15, began differentiation by E17 and were distributed in a similar pattern to the adult from E19. Substance P immunoreactivity in the NST was also very similar to the adult pattern by E19. Many of these immunochemical and histochemical markers indicate a similar pattern of development, i.e. a rapid period of differentiation until E19, by which time a relatively stable adult-like pattern has been attained. The present findings indicate that many of the cyto- and chemoarchitectural features of this nucleus are present well before birth, by which time the nucleus must serve vitally important functions such as relaying information for control of respiration and the circulation.

Keywords Calbindin · Calretinin · NADPH diaphorase · Tyrosine hydroxylase · Substance P

Introduction

The organization of the adult mammalian nucleus of the solitary tract (NST) has been well studied (Contreras et al. 1982; Altschuler et al. 1989; Estes et al. 1989; Barry et al. 1993; Boissonade et al. 1996), but relatively little information is available on the development of this nucleus and its subdivisions. This is despite the importance of the NST in processing afferent information from a wide variety of systems, which are behaviourally and physiologically important for the neonate, such as the respiratory and digestive systems. In particular, the respiratory afferents in the vagus nerve, concerned with pulmonary stretch receptors, are important for postnatal survival and the central connections of these afferents would be expected to mature quite early.

Altman and Bayer (1980) reported that neurons destined for the NST were born from E11 to E14, with peak neurogenesis occurring at E12. Only a few studies have examined the immunohistochemical development of the NST in any mammal. The mouse NST is strongly immunoreactive for the glycoconjugate CD15 epitope at E14, even though the adjacent and functionally related dorsal motor nucleus of vagus is less immunoreactive, but the precise significance of this is uncertain at present (Ashwell and Mai 1997). Substance P is the only neurotransmitter whose appearance in the developing NST has not been examined. For that marker Sakanaka et al (1982) reported on the development of the substance P neuronal system in the lower brain stem of the rat as a whole,
not dealing specifically with subnuclear distribution. Takemura et al (1996) have used NADPH-diaphorase (NADPH-d) histochemistry to examine the development of the rat brainstem as a whole, and they reported that NADPH-d+ neurons appeared in the rostral lateral division of the NST at E15, although at that stage the medial and caudal lateral divisions were negative. The NADPH-d+ neurons first appeared in the medial and caudal lateral divisions at P4. To date, no information is currently available on the development of other immunohistochemical markers for neuronal groups in the NST (such as calbindin, calretinin, tyrosine hydroxylase).

The general aim of the present study was to determine when the different subnuclear components of the NST began to differentiate in the prenatal rat. This is intended to provide clues as to the earliest age at which circuitry associated with particular parts of the NST can be expected to have reached a functional state. For example, if the cytological and chemoarchitectural features of the central nucleus do not reach a mature appearance until after birth, then it is unlikely that that subnucleus is able to perform its mature function effectively. These data will also provide baseline information on the compartmentation of the NST during development, which can be used to interpret findings on the ingrowth of afferents and synaptogenesis, to be discussed in a subsequent report. The initial part of the analysis in the present paper relied on identification of subnuclei of the NST in Nissl-stained material. This was correlated with data about histochemical reactivity for acetylcholinesterase (AChE), cytochrome oxidase (CO) and NADPH-d (Hope et al. 1991) and immunohistochemical reactivity for tyrosine hydroxylase (TH), calbindin (Cb), calretinin (Cr) and substance P (SP) to try to determine the time course of chemical maturation of individual subnuclei. This information was then interpreted in the light of behavioural and physiological data concerning maturation of visceral afferent systems in the fetal and neonatal rat.

**Materials and methods**

This study was carried out on approximately 120 outbred fetal and neonatal Wistar strain rats, and 18 adult Wistar rats. All procedures were approved by the Animal Care and Ethics Committee of the University of NSW and conform to guidelines prepared by the National Health and Medical Research Council of Australia. For timed matings, the day of finding a sperm-positive vaginal smear was denoted as D (embryonic day) 0. If left to full term, most females gave birth late on E22 or early on E23. The day of birth was denoted as P (postnatal day) 0.

At prenatal ages E13, E15, E17, E19 and E21, mothers were overdosed by a single intraperitoneal injection of phenobarbitone (60 mg/kg). All postnatal animals were perfused as described for the E21 fetuses. Eighteen adult rats (300-350 g) of either sex were also anaesthetised with an overdose of Nembutal (phenobarbitone sodium, 60 mg/kg) delivered by intraperitoneal injection. There were no apparent sex differences in the structure of the NST. The adult animals were perfused as described above.

Some tissue for very young fetal ages (3 animals at both E13 and E15) was embedded in paraffin and sectioned coronally at a thickness of 6 µm before staining with hematoxylin and eosin. All other tissue was cryoprotected by overnight immersion at 4°C in 30% sucrose in 0.1 M phosphate buffer. Brainstems for enzyme and immunohistochemistry were sectioned coronally with the aid of a cryostat at thicknesses of either 30 µm (E15 and E17), 40 µm (E19 to P10) or 50 µm (adult). These frozen sections were stained for Nissl substance or reacted for histo- and immunohistochemical markers. For each marker at each age at least 4, and usually 5 to 6 animals, were studied. For AChE, the technique outlined in Paxinos and Watson (1986) was applied, except that 0.1 M phosphate buffer (pH 7.4) was used in the sodium sulphide solution. Initial incubation in S-acetyltiocysteine occurred at room temperature for 15 h. For CO, the technique of Wong-Riley (1979) was used, with incubation occurring for 24 h at 37°C. In our hands, the technique of Carrive and Paxinos (1994) for NADPH-d was found to be most effective. Sections were incubated in a 1 mg/ml solution of NADPH for 20 h at 4°C.

Immunohistochemistry for TH, Cb, Cr and SP was performed on free-floating sections. The antibody for TH (Incstar) was a mouse monoclonal antibody generated against TH isolated and purified from PC12 cells and was used at a concentration of 1/100,000. The monoclonal antibodies for Cb (diluted 1/16,000) and Cr (diluted 1/64,000) were obtained from SWant. The anti-Cb antibody had been raised in rabbit, while anti-Cr had been raised in mouse. The monoclonal antibody for SP (diluted 1/1,000) had been raised in rabbit and was obtained from Auspep. All primary antibodies were diluted in PBA (0.1 M phosphate buffer, pH 7.4, with 0.1% bovine serum albumin, 2% normal horse serum and 0.2% Triton-X 100). Primary incubation was for 2 days at 4°C on a rotating table. The appropriate biotinylated secondary antibody was applied for 2 h at room temperature, at a dilution of 1/200 in PBA. Peroxidase-conjugatedextravidin (Sigma) was diluted 1/500 and applied for 2 h at room temperature.

Secondary sections were reacted with 0.5 mg/ml 3, 3′ diaminobenzidine in 0.04% nickel ammonium sulphate, with 1 µl of glucose oxidase per ml of final solution. Negative controls for immunohistochemistry were performed by omission of the primary antibody from the initial incubation.

The qualitative aspects of staining intensity and distribution of the material were analyzed from photographs at representative levels through the nucleus. Quantitative aspects, such as the size of the population of Cr and Cb+ neurons and the somata areas of those neurons, were determined using the Magellan 3.1 system (Halasz and Martin 1984), running on a computer-linked Olympus BHS microscope. Sections throughout the entire rostrocaudal extent of the nucleus including all subnuclei were used for the estimation of population size. Every second section in postnatal animals were used for population estimation and somata size. Raw counts were multiplied by the inverse of the proportion of sections used to obtain a raw total population estimate before correction for potential double counting in sectioned material. Somata diameter was also used to calculate correction factors according to the Abercrombie formula (Abercrombie 1946). For that purpose, somata diameter of 100 neurons per age across the rostrocaudal extent of the NST was measured in the vertical dimension (i.e. in the direction perpendicular to the plane of section) under an oil immersion objective (x100) and by using the calibrated scale on the focusing knob of an Olympus BHS microscope to estimate vertical travel. Accuracy of the mechanical focussing control on this particular instrument (an Olympus BH-2) is rated at 0.2 µm by the manufacturer. Optical accuracy involved in judging where the somata boundary lies, was estimated from multiple trials on individual cells to be less than 1.0 µm. The estimates of somata diameter.