Histol Histopathol (1995) 10: 85-89

Histology and Histopathology

# Localization of NADPH-diaphorase activity in the pancreatic ganglia of the young chick

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**Summary.** NADPH-diaphorase activity was localized in pancreatic ganglia of the young chick. At 1 day post-hatching, 60% of the neurons in the pancreatic ganglia were NADPH-diaphorase positive. In each neuron, the NADPH-diaphorase labelling was localized mainly in the cytoplasm of the cell body and its proximal processes, but not in the cell nucleus. There was a gradation in the labelling for the enzyme, with some neurons being heavily labelled while others were lightly to moderately labelled. At 7 days post-hatching, 100% of the pancreatic neurons showed NADPH-diaphorase activity and the average size of the NADPH-diaphorase positive neurons had also increased. By 14 days post-hatching, all the neurons present were heavily labelled for NADPH-diaphorase activity. Some of the labelled nerve processes traversed long distances and finally terminated on other ganglia as well as on the exocrine acinar or endocrine cells. It is concluded that this increase in NADPH-diaphorase/NOS activity in the pancreatic neurons is possibly correlated to the increase in modulation of neurotransmission in the young chick.

**Key words:** NADPH-diaphorase, Ganglia, Pancreas, Chick

## Introduction

Although numerous studies have been made on the mammalian pancreatic ganglia, so far only a few reports are available on the avian species (Kudo, 1971; Watanabe and Yasuda, 1977; Hiramatsu et al., 1988). Recently, the topographical and numerical distribution of ganglion cells in the chick pancreas has also been reported on (Ohmori et al., 1991). Moreover, various peptides have been localized in the intrinsic ganglia and nerves of the chick pancreas (Hiramatsu and Watanabe, 1989; Salakij et al., 1992). Recently, the localization of NADPH-diaphorase activity has been used as a marker for nitric oxide synthase activity in the autonomic nervous system by various workers (Grozdanovic et al., 1992; McNeill et al., 1992; Nichols et al., 1992; Hassall et al., 1993; Santer and Symons, 1993; Tanaka et al., 1993). In these studies, there is substantial evidence that nitric oxide could be modulating neurotransmission in the intrinsic neurons of the autonomic ganglia. The present investigation attempts to localize NADPH-diaphorase activity in the pancreatic ganglia of the young chick.

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## Materials and methods

Fifteen young chicks (1 day old, n=5; 7 day old, n=5; 14 day old, n=5) were used for the present study. Each animal was killed with an overdose of ether and then exsanguinated. The pancreas was carefully dissected under a stereo-microscope, washed in Hank's balanced salt solution (HBSS, Life Technologies, Ltd, U.K.) and fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h. The pancreas was then transferred and kept in PBS containing 10% sucrose and stored overnight at 4 °C before being mounted with Tissue-Tek (Miles Inc., Elkhart, Indiana, USA) on a metal chuck and sectioned at 14 μm with a Reichert microtome (model 1800) at -20 °C. The sections were collected on gelatinised slides, washed in PBS (pH 7.4) and then kept at 4 °C before staining for NADPH-diaphorase activity.

### *NADPH-diaphorase histochemistry*

NADPH-diaphorase activity was localized histochemically by a method described by Saffrey et al. (1992). Briefly, tissues were washed in 0.1M TRIS buffer (pH 7.6) for 10 min and then incubated in the dark at room temperature in 0.1M TRIS buffer containing 0.2 mg $ml^{-1}$ nitroblue tetrazolium, 2.7 mg $ml^{-1}$ L-maleic acid, 1.0 mg $ml^{-1}$ ß-NADPH and 0.1% triton x-100. Tissues were incubated for exactly 1 h and then rinsed in excess TRIS buffer to stop the reaction, washed twice in PBS before being mounted in Citifluor mountant (City University, London, UK) and viewed with bright field

illumination using a Carl Zeiss Axioplan Universal microscope (Germany). Controls were performed with the omission of the enzyme substrate ß-NADPH from the incubation medium.

## Results

NADPH-diaphorase activity was localized in the neurons of the ganglia encountered in the chick pancreas at each of the time intervals studied. Control experiments where ß-NADPH was omitted failed to show labelling of the pancreatic neurons.

### *One-day-old chick*

At this stage, the ganglia encountered were small in sections and the NADPH-diaphorase-positive neurons showed marked gradation of staining (i.e. from light to heavy staining) for the enzyme (Fig. 1a). Quantitative analysis showed that 60% of the neurons were labelled for NADPH-diaphorase. Numerous solitary neurons, interspersed amongst the acinar exocrine portion of the pancreas, were also NADPH-diaphorase positive. Most of the labelled neurons contained several processes emanating from them and these were also NADPH-diaphorase positive (Fig. 1b). In each labelled neuron, the reaction product for NADPH-diaphorase localization was found mainly in the cell cytoplasm and its processes, but not in the cell nucleus. In the few larger ganglia observed, an interesting feature was the gradation of labelling observed amongst the neurons present (Fig. 1b). In the smaller ganglia, usually 1-2 neurons were heavily labelled, while the rest were moderately to lightly labelled.

Some of the NADPH-diaphorase-labelled ganglia were present in close proximity to blood vessels (Fig. 1c). These blood vessels contained endothelial cells stained for NADPH-diaphorase activity (Fig. 1c). Amongst the labelled neurons in the pancreatic ganglia, some of them were much larger in size compared to the others. Numerous attenuated ganglia were also found amongst the connective tissues adjacent to the pancreatic lobules. In these attenuated ganglia, the NADPH-diaphorase-positive neurons also appeared attenuated, with the larger neurons being heavily labelled while the smaller ones were lightly to moderately labelled. In other cases, the neurons from one ganglion appeared to communicate with other ganglia via NADPH-diaphorase-positive nerve processes.

### *Seven-day-old chick*

At this stage, the pancreatic ganglia showed an increase in size in sections compared to those observed at 1 day post-hatching. In NADPH-diaphorase-labelled ganglia, 100% of the neurons were labelled and the individual neurons were mostly heavily stained for NADPH-diaphorase activity (Fig. 1d,e). Only a few moderately- to ligthtly-labelled neurons were seen in these ganglia. Also, numerous NADPH-diaphorase-positive processes were observed to traverse across distances of many micrometres in the connective tissues of the interlobular spaces. In some cases, communication was observed between labelled ganglia via their NADPH-diaphorase-positive nerve processes, while in others, communication appeared to be between more than two ganglia (Fig. 1f). NADPH-diaphorase-positive ganglia often lay close to arterioles and venules, which contained heavily-labelled NADPH-diaphorase-positive endothelial cells (Fig. 1e). Some fine NADPH-diaphorase-positive nerve processes were found interspersed amongst the exocrine acinar cells (Fig. 1e) while others were also seen amongst the endocrine cells of the islets of Langerhans.

### *Fourteen-day-old chick*

At this stage, all the neurons encountered were NADPH-diaphorase positive. Numerous large NADPH-diaphorase-positive ganglia were observed in the connective tissues between the lobules of the pancreas (Fig. 1g,h). Ganglia usually contained between 10 to 30 neurons in sections, and all of them were heavily labelled for NADPH-diaphorase activity. Most of these NADPH-diaphorase-positive neurons were polygonal in shape, with round to oval nuclei and contained several processes emanating from their cell bodies. The labelling was concentrated mainly in the cell cytoplasm and the

**Fig. 1. a.** 1-day-old chick. A small ganglion in the interlobular space. Note that of the two neurons present, one is heavily labelled for NADPH-diaphorase activity (arrowhead) while the other is moderately labelled. **b.** 1-day-old chick. A larger ganglion containing several NADPH-diaphorase-positive neurons in section. Observe that some neurons are heavily labelled (arrowheads) while the rest are moderately labelled for NADPH-diaphorase activity. **c.** 1-day-old chick. Two heavily-labelled NADPH-diaphorase-positive neurons (arrowheads) lie adjacent to a blood vessel (BV). Note the labelling of the cell cytoplasm and its proximal processes, but not the cell nucleus. Observe the dense labelling of the endothelial cells of the blood vessel (BV) as well as the mesothelial cells (arrows) lining the pancreas. **d.** 1-day-old chick. A ganglion containing 5 neurons which are heavily labelled for NADPH-diaphorase activity. Note the heavy labelling of the cell cytoplasm, but not of the cell nucleus of the individual neurons. Observe also the heavy labelling of the endothelial cells within a blood vessel (arrow). **e.** 7-day-old chick. A ganglion showing heavy labelling for NADPH-diaphorase found in the interlobular space. Note the heavy labelling of the endothelial cells within the 2 blood vessels (arrows) lying adjacent to this ganglion. Observe a solitary labelled nerve process (arrowhead) amongst the exocrine acinar cells. L, lobule of pancreas. **f.** 7-day-old chick. Three ganglia (G1, G2, G3) found in the interlobular space of the pancreas. Note the heavy labelling of the neurons in each ganglion for NADPH-diaphorase activity. These ganglia are interconnected with each other via labelled nerve processes (arrowheads). **g.** 14-day-old chick. Two large ganglia (G1, G2) are found in the interlobular space of the pancreas. Note that each ganglion consists of at least 10 neurons, which are heavily to moderately labelled for NADPH-diaphorase activity. **h.** 14-day-old chick. A large ganglion (G) showing heavy labelling for NADPH-diaphorase activity in its neurons. Note the labelling in the cytoplasm but not in the nucleus of the individual neurons. Scale bar represents 50 µm for all micrographs.


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G2
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f
G1
G1
G2
g
G
h

processes of the neurons, but not within the cell nuclei (Fig. 1h). Some of the labelled nerve processes traversed long distances away from the ganglia and they either terminated on adjacent ganglia or the acinar exocrine cells. although most of the ganglia were large, a few small ganglia consisting of 1-5 NADPH-diaphorase-positive neurons were also found, sandwiched amongst the exocrine acinar cells.

## Discussion

NADPH-diaphorase activity has been localized in the pancreatic ganglia of the chick from 1 day to 14 days post-hatching. The majority (60%) of the pancreatic neurons show NADPH-diaphorase activity at 1 day post-hatching. By 7 days post-hatching, 100% of the pancreatic neurons are labelled for NADPH-diaphorase activity. This increase in the number of neurons showing NADPH-diaphorase activity could have resulted from cell division, migration or transformation (Altman and Das, 1966). An increase in the number of neurons during postnatal development of other animal species has been documented in the central (Altman and Das, 1966) and peripheral (Gabella, 1971) nervous system. Moreover, increase in the average neural size and reduction in the packing density are also characteristic of the developing visceral neurons (Gabella and Trigg, 1984). In the present study, it appears that the increase in the number of the NADPH-diaphorase-positive neurons in the ganglia with age may be due to the subsequent development of expression of the neuronal marker used in previously negative neurons. It is not known which one of the above-mentioned factors is responsible for the observed increase in NADPH-activity in the post-hatched chicks. The increase in the size of NADPH-diaphorase-positive neurons observed here during growth may be attributed to an increase in neuroplasm associated with the increase in the size of the pancreas. Such a correlated increase in size of neurons in relation to the size of the innervated target organ has been reported in the myenteric ganglia (Gabella and Trigg, 1984). In addition, the elimination of the small-sized neurons as a result of naturally occurring neuronal cell death during postnatal development and their replacement by larger-sized neurons, which have arisen either by division or migration, is equally possible.

Although NADPH-diaphorase activity can be associated with other enzymes, neuronal activity is thought to be due solely to the presence of nitric oxide synthase (NOS), and in the central nervous system, there is coincidence of expression of NOS-immunoreactivity with NADPH-diaphorase activity (Dawson et al., 1991; Hope et al., 1991). In the brain, NOS and NADPH-diaphorase appear to be identical (Dawson et al., 1991; Hope et al., 1991) and this fact has led to an increasing use of NADPH-diaphorase as a marker for neurons expressing NOS activity in the central and peripheral nervous system (Grozdanovic et al., 1992; McNeill et al., 1992; Nichols et al., 1992; Hassall et al., 1993; Santer and Symons, 1993; Tanaka et al., 1993). The presence of a high percentage of NADPH-diaphorase-positive neurons in the chick pancreas (as early as 1 day post-hatching) indicates that NOS in these neurons is capable of synthesizing nitric oxide (NO), a molecule capable of mediating neural transmission (Bredt et al., 1990; Garthwaite, 1991; Moncada et al., 1991). In addition, it is clear that during growth, some of the neurons in the pancreas are either immature or have not been endowed with the phenotypic expression for NADPH-diaphorase/NOS as seen at 1 day post-hatching. It is hereby hypothesized that there is possibly an intrinsic time-related switch-on mechanism in the genetic make-up of these neurons which is involved in the expression of NADPH-diaphorase/NOS activity. By 7 days post-hatching, 100% of the neurons express, NADPH-diaphorase activity albeit with various grades of intensities. This significant increase in the enzymatic activity possibly results from the maturity of the neurons, resulting in the switch on of the time-related genetic mechanism, thereby giving rise to the NADPH-diaphorase expression. The increase in size and number of NADPH-diaphorase-positive neurons may be mediated by some unknown trophic factors present in the microenvironments within the growing ganglia of the pancreas.

A striking observation in the present study is the dramatic increase in the proportion of pancreatic neurons that express NADPH-diaphorase activity with age in the young chick, especially at 7 days post-hatching. Grozdanovic et al. (1992) have reported that 100% of the myenteric neurons in the mouse oesophagus are NADPH-diaphorase positive. Similarly Gershon and Kirchgessner (1986) have also found NADPH-diaphorase activity in 100% of the submucosal neurons in the guinea pig gut. The pancreas, being an outgrowth of the developing gut, has been shown to be innervated by neurons derived from the gut (Kirchgessner and Gershon, 1990; Kirchgessner et al., 1992). The present study has demonstrated a high proportion of NADPH-diaphorase-positive neurons in the pancreas, even at the early stages of post-hatched life in the chick, and these may also be derived from the gut. In view of the findings that NADPH-diaphorase is colocalized with vasoactive intestinal polypeptide, substance P and neuropeptide Y in the pancreatic neurons (Kirchgessner et al., 1993), it is conceivable that NO may be involved in a complex pattern of interrelationship with other neuroactive substances, thereby mediating or modulating their neural functions. It is evident that there is an increase in the NO content in the pancreatic neurons of the growing chick and this may be correlated to an increase in modulation of neurotransmission by NO in the pancreas.

*Acknwoledgements.* This work was supported in part by the Welcome Trust. The author wishes to thank Professor G. Burnstock for the opportunity to work at University College London, England and Mr. E.W. Moules and Ms. Y.G. Chan for some technical assistance and Mrs. M. Singh for typing the final manuscript.

## References

Altman J. and Das C.D. (1966). Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J. Comp. Neurol. 126, 337-390.

Bredt D.S., Hwang P.M. and Snyder S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347, 768-780.

Dawson T.M., Bredt D.S., Fotuhi M., Hwang P.M. and Snyder S.H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in the brain and peripheral tissues. Proc. Natl. Acad. Sci. USA 88, 7797-7801.

Gabella G. (1971). Neuron size and number in the myenteric plexus of the newborn and adult rat. J. Anat. 109, 81-95.

Gabella G. and Trigg P. (1984). Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbit and sheep. J. Neurocytol. 13, 49-71.

Garthwaite J. (1991). Glutamate nitric oxide and cell-cell signalling in the nervous system. Trends Neurosci. 14, 60-67.

Gershon M.D. and Kirchgessner A.L. (1986). Definition of subsets of enteric neurons by the histochemical demonstration of the activities of endogenous alkaline phosphatase and NADPH-diaphorase: colocalization with neuropeptides. Soc. Neurosci. Abstr. 12, 1021.

Grozdanovic Z., Baumgarten H.G. and Bruning G. (1992). Histochemistry of NADPH-diaphorase, a marker for neuronal nitric oxide synthase, in the nervous system of the mouse. Neuroscience 48, 225-235.

Hassall C.J.S., Saffrey M.J. and Burnstock G. (1993). Expression of NADPH-diaphorase activity by guinea-pig paratracheal neurones. NeuroReport 4, 49-52.

Hiramatsu K. and Watanabe T. (1989). Immunohistochemical study on the distribution of vasoactive intestinal polypeptide (VIP) containing nerve fibers in the chicken pancreas. Z. Mikrosk. Anat. Forsch. 103, 689-699.

Hiramatsu K., Watanabe T. and Fujioka T. (1988). The distribution of acetylcholinesterase (AchE)-positive nerves in chicken pancreas demonstrated by light and electron microscopy. Arch. Histol. Cytol. 51, 159-168.

Hope B.T., Michael G.J., Knigge K.M. and Vincent S.R. (1991). Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA 88, 2811-2814.

Kirchgessner A.L. and Gershon M.D. (1990). Innervation of the pancreas by neurons in the gut. J. Neurosci. 10, 1626-1642.

Kirchgessner A.L., Alderberg M.A. and Gershon M.D. (1992). Colonization of the developing pancreas by neural precursors from the bowel. Dev. Dynamics 194, 142-154.

Kirchgessner A.L., Liu M.T. and Gershon M.D. (1993). NADPH-diaphorase (nitric oxide synthase) in the enteropancreatic innervation. Anat. Rec. Suppl. 1, 72.

Kudo S. (1971). Fine structure of autonomic ganglion in the chicken pancreas. Arch. Histol. Cytol. 32, 455-497.

McNeil D.L., Traugh N.E., Vaidya A.M., Hua H.T. and Papka R.E. (1992). Origin and distribution of NADPH-diaphorase positive neurons and fibres innervating the urinary bladder of the rat. Neurosci. Lett. 147, 33-36.

Moncada S., Palmer R.M.J. and Higgs E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109-142.

Nichols K., Krantis A. and Staines W. (1992). Histochemical localization of nitric oxide synthase neurons and vascular sites in the guinea-pig intestine. Neuroscience 51, 791-799.

Ohmori Y., Wakita T. and Watanabe T. (1991). Number and density of intrapancreatic ganglion cells in the chicken. J. Auton. Nerv. Syst. 34, 139-146.

Saffrey M.J., Hassall C.J.S., Hoyle C.H.V., Belai A., Moss J., Schmidt H.H.H.W., Forstermann U., Murad F. and Burnstock G. (1992). Colocalization of nitric oxide synthase and NADPH-diaphorase in cultured myenteric neurones. NeuroReport 3, 333-336.

Salakij C., Watanabe T., Takahasi S.I., Ohmori Y. and Nagatsu I. (1992). Immunohistochemical studies on the intrinsic pancreatic nerves in the chicken. J. Auton. Nerv. Syst. 40, 131-140.

Santer R.M. and Symons D. (1993). Distribution of NADPH-diaphorase activity in rat paravertebral, prevertebral and pelvic sympathetic ganglia. Cell Tissue Res. 271, 115-121.

Tanaka K., Hassall C.J.S. and Burnstock G. (1993). Distribution of intracardiac neurones and nerve terminals that contain a marker for nitric oxide, NADPH-diaphorase, in the guinea pig heart. Cell Tissue Res. 273, 293-300.

Watanabe T. and Yasuda M. (1977). Electron microscopic study on the innervation of the domestic fowl. Cell Tissue Res. 180, 453-465.

Accepted October 5, 1994