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Multiple origins, migratory paths and molecular profiles of cells populating the avian interpeduncular nucleus

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ABSTRACT

The interpeduncular nucleus (IP) is a key limbic structure, highly conserved evolutionarily among vertebrates. The IP receives indirect input from limbic areas of the telencephalon, relayed by the habenula via the fasciculus retroflexus. The function of the habenulo-IP complex is poorly understood, although there is evidence that in rodents it modulates behaviors such as learning and memory, avoidance, reward and affective states. The IP has been an important subject of interest for neuroscientists, and there are multiple studies about the adult structure, chemoarchitecture and its connectivity, with complex results, due to the presence of multiple cell types across a variety of subnuclei. However, the ontogenetic origins of these populations have not been examined, and there is some controversy about its location in the midbrain-anterior hindbrain area. To address these issues, we first investigated the anteroposterior (AP) origin of the IP complex by fate-mapping its neuromeric origin in the chick, discovering that the IP develops strictly within isthmus and rhombomere 1. Next, we studied the dorsoventral (DV) positional identity of subpopulations of the IP complex. Our results indicate that there are at least four IP progenitor domains along the DV axis. These specific domains give rise to distinct subtypes of cell populations that target the IP with variable subnuclear specificity. Interestingly, these populations can be characterized by differential expression of the transcription factors Pax7, Nkx6.1, Otp, and Otx2. Each of these subpopulations follows a specific route of migration from its source, and all reach the IP roughly at the same stage. Remarkably, IP progenitor domains were found both in the alar and basal plates. Some IP populations showed rostrocaudal restriction in their origins (isthmus versus anterior or posterior r1 regions). A tentative developmental model of the structure of the avian IP is proposed. The IP emerges as a plurisegmental and developmentally heterogeneous formation that forms ventromedially within the isthmus and r1. These findings are relevant since they help to understand the highly complex chemoarchitecture, hodology and functions of this important brainstem structure.

Introduction

The interpeduncular nucleus (IP) is a highly conserved structure in all vertebrates, found subpially across the median floor plate of the brainstem at the posterior end of the interpeduncular fossa. The IP receives its major input from the medial habenula (mHb) via the fasciculus retroflexus (Contestabile and Flumerfelt, 1981; Herkenham and Nauta, 1979) and shows widespread projections, both ascending (to limbic structures) and descending (mainly to the raphe nuclei; Groenewegen et al., 1986).

Functional studies on the mHb-IP axis suggest its implication in a variety of brain functions and behaviors such as learning and memory, motor activity, stress, affective states (anxiety, depression, reward phenomena), as reviewed by Klemm (2004) and Hikosaka (2010). The IP consists of several cyto- and chemoarchitectonically distinct cell groups organized in a complex tridimensional structure (Hamill and Lenn, 1984; Hamill et al., 1984; Hemmendinger and Moore, 1984; Ives, 1971). Studies in mammals have addressed the diverse adult neurotransmitter phenotypes of its neurons and its projections to multiple brain areas, largely of the limbic system (e.g., Ferraguti et al., 1990; Groenewegen et al., 1986; Klemm, 2004; Nieuwenhuys et al., 1998; Panigrahy et al., 1998; Shibata and Suzuki, 1984; Shinoda et al., 1988).

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It would be expected that subpopulations of the IP nuclear complex, differentially characterized by their neurotransmitter or peptide typology, or producing distinct projections, should be generated in different progenitor domains during development. However, the literature has not addressed to date the possible correlation of these cytochemical and hodologic subdivisions with embryological heterogeneity.

Concerning its adult topography, the location of the IP is still controversial, and it has been contradictorily attributed either to the midbrain (Hanaway et al., 1971; Panigrahy et al., 1998; Quina et al., 2009), to the rostral hindbrain (Herrick, 1934; His, 1892, 1895; Nieuwenhuys et al., 2008; Puelles et al., 2007; Ziehen, 1906) or even to the diencephalic tegmentum (Bayer and Altman, 2006).

Contemporaneous studies on the origin of spinal cord cell types have shown that positional patterning differences at the level of neuroepithelial progenitors arranged along the dorsoventral dimension of the neural tube wall underlie important aspects of neuronal subtype diversity. This occurs in the context of ventralizing and dorsalizing developmental instructive mechanisms that control differential molecular specification of the neuroepithelium (Briscoe and Ericson, 2001; Dessaud et al., 2010; Jessell, 2000; Shirasaki and Pfaff, 2002).

We have applied the same logic to analyze the developmental construction of the IP. Previous studies had shown that some subpopulations of the adult interpeduncular nucleus express *Pax7* (Stoykova and Gruss, 1994) or *Nkx6.1* (E. Puelles et al., 2001), these being transcription factors known to characterize different DV progenitor domains in the developing hindbrain. On the basis of these studies, our working hypothesis was that this nuclear complex might have multiple dorsoventral origins of its subpopulations.

In this study we focused on the origin and development of diverse molecularly characterized subpopulations of the chicken IP complex. The position of the mature IP subpially across the midline raises the question whether these cells originate locally at the floor plate domain, or migrate in from other longitudinal zones (basal or alar plates). We first addressed the potential existence of distinct progenitor domains for subpopulations of the IP, examining various molecular profiles and locating precisely the respective positions along the dorsoventral (DV) and anteroposterior (AP) axes. To this end, we first analyzed the expression pattern of the previously identified IP marker genes PAX7 and *Nkx6.1*, later adding to the analysis two other IP markers identified by us, *Otx2* and *Otp*. The analysis was performed at several embryonic stages of development, to illuminate apparent migration paths, and comparisons with DV and AP reference marker genes established the relative topography of both the progenitor domains and the migrating cells.

Recognition of specific differential molecular markers for the IP nucleus helped us also to address its controversial topology, using specific transcription factors serving as AP reference markers and quail-chick experimental fate mapping. To date, no other studies have analyzed in detail the development of this complex nucleus in any species. The present data provide the first experimental examination of the anteroposterior (AP) and dorsoventral (DV) origin of IP subnuclear populations in any vertebrate.

We identified four separate progenitor domains whose derivatives variously contribute to the IP complex; these each express differentially one of the analyzed transcription factors. It turns out that the IP integrates neuronal cohorts with separate alar versus basal, or rostral versus caudal, origins. It was possible to trace the migratory route of the respective derivatives into specific parts of the IP complex. Our data provide further evidence corroborating that positional molecular identity of the neural progenitors is an organizing principle underlying phenotypic diversity among the different neuronal subpopulations of a nuclear complex such as the IP. It is expected that present highlighting of developmental molecular diversity at the IP may help understand the alternative chemoanatomic and hodologic properties coexisting within this nuclear complex.

Materials and methods

Animals

All animals were treated according to the regulations and laws of the European Union (86/609/EEC) and the Spanish Government (Royal Decree 223/1998; revised Royal Decree 1021/2005) for the care and handling of animal in research. Fertilized chick (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were incubated in a forced draft incubator at 38 °C. The stage of the embryos was established according to the Hamburger and Hamilton (1951) tables (stages HH). The embryo heads were fixed overnight in cold PFA 4% in PBS, and the brains were dissected before further processing.

Embryos to be sectioned with vibratome were previously embedded in 4% agarose in phosphate-buffered saline solution (PBS) and sectioned 100 μm-thick using a vibratome (data in Figs. 1–4A–D and 5) or, alternatively, dehydrated for paraffin embedding (results in Fig. 4E–L) or cryoprotected for cryostat sectioning (data in Fig. 6). The selected sectioning planes (transverse, horizontal or sagittal) referred specifically to r1, considering the axial incurvation caused by the cephalic flexure (Puelles et al., 2007). The sections were then processed for *in situ* hybridization and/or immunohistochemistry.

Embryos selected to be cryostat-sectioned were cryoprotected in 10% sucrose solution in PBS and embedded in 10% gelatin/10% sucrose solution in the same buffer. The blocks were frozen for 1 minute in −70 °C isopentane cooled on dry ice, and then stored at −80 °C. Cryostat serial sections 20 μm-thick were cut sagittal or transverse to r1, mounted as parallel sets on SuperFrost slides, and stored at −80 °C until used.

Quail-chick chimeras

Homotopic and isochronic quail-chick grafts of r1, r2 or isthmus were performed, using quail embryos as donors. The embryos were operated at stage HH10, and the chimeras where fixed at stages HH36–38 (10–12 days in ovo). The grafts affected one-half of the neural tube, and included different DV extents of the neural tube wall as judged from the dorsal midline, with some attached mesoderm. Some chimeras (such as chimera 1) were fixed overnight in 4% paraformaldehyde in PBS, and the heads were embedded in 4% agarose to obtain three parallel series of 80 μm-thick transverse sections; these were used for different *in situ* probes in comparable adjacent sections. Other chimeras (such as chimeras 2 and 3) were immersed overnight in Clarke's fixative, ulteriorly washed, dehydrated and embedded in paraffin, sectioned 12 μm-thick and mounted in two parallel series. One series was stained with cresyl violet for cytoarchitectural analysis, and the other was immunoreacted with anti-quail antibody (QCPN) (monoclonal mouse anti-quail antibody from the Developmental Studies Hybridoma Bank, Iowa University, Iowa City, IA). The QCPN selectively labels the cell nuclei of quail cells and thereby identifies the grafted tissue (labeled ventricular zone) and its derivatives (labeled mantle zone populations).

RNA probes

Digoxigenin-labeled riboprobes were synthesized from cDNAs kindly provided by other laboratories, as follows: *Dbh* and *ChAT* from H. Rohrer (Developmental Neurobiology, Max-Planck-Institute for Brain Research, Frankfurt, Germany), *Fgf8* from GR. Martin (Dept. of Anatomy and Program in Developmental Biology, University of California, San Francisco, CA USA), *Hoxa2* from R.J. Wingate (MRC Centre for Developmental Neurobiology, King's College London, Guy's Campus, London UK), *Phox2a* from J.F. Brunet and C. Goridis (Ecole Normale Supérieure, Département de Biologie, Paris, France), *Otp* and *Otx2* from A. Simeone (CEINGE Biotecnologie Avanzate,