Brain Research 1700 (2018) 19–30

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/bres

Research report

Melanin-concentrating hormone (MCH) neurons in the developing chick brain

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ARTICLE INFO

Article history:
Received 17 April 2018
Received in revised form 27 June 2018
Accepted 1 July 2018
Available online 2 July 2018

Keywords:
Melanin-concentrating hormone
MCH
Chicken embryo
Brain development
Hypothalamic periventricular organ
Serotonergic hypothalamic neurons

ABSTRACT

The present study was undertaken because no previous developmental studies exist on MCH neurons in any avian species. After validating a commercially-available antibody for use in chickens, immunohistochemical examinations first detected MCH neurons around embryonic day (E) 8 in the posterior hypothalamus. This population increased thereafter, reaching a numerical maximum by E20. MCH-positive cell bodies were found only in the posterior hypothalamus at all ages examined, restricted to a region showing very little overlap with the locations of hypocretin/orexin (H/O) neurons. Chickens had fewer MCH than H/O neurons, and MCH neurons also first appeared later in development than H/O neurons (the opposite of what has been found in rodents). MCH neurons appeared to originate from territories within the hypothalamic periventricular organ that partially overlap with the source of diencephalic serotonergic neurons. Chicken MCH fibers developed exuberantly during the second half of embryonic development, and they became abundant in the same brain areas as in rodents, including the hypothalamus (by E12), locus coeruleus (by E12), dorsal raphe nucleus (by E20) and septum (by E20). These observations suggest that MCH cells may play different roles during development in chickens and rodents; but once they have developed, MCH neurons exhibit similar phenotypes in birds and rodents.

1. Introduction

Melanin-concentrating hormone (MCH) is a highly conserved neuropeptide found in the posterior hypothalamus of all vertebrates (Croizier et al., 2013; Vallarino et al., 2009). In mammals, MCH neurons spread out mostly over dorsal and lateral areas of the posterior hypothalamus (Croizier et al., 2013). In non-mammalian vertebrates, MCH neurons are mostly found in medial locations in the posterior and dorsal hypothalamus, surrounding the hypothalamic periventricular organ (HPO; also called the circumventricular organ or paraventricular organ; Croizier et al., 2013). In the few species of birds where they have been described (including chickens), MCH neurons are found both close to the HPO and scattered laterally into the lateral hypothalamic area (Cardot et al., 1999).

MCH was originally isolated from salmon pituitaries (Kawauchi et al., 1983); in fish, MCH exerts a major effect on skin pigmentation, making skin paler (Vallarino et al., 2009). This divergence of function in fish is accompanied by the presence of an additional MCH gene expressed by a separate group of neurons that are magnocellular, project to the pituitary gland (Baker and Bird, 2002; Croizier et al., 2013), and release MCH, which acts both as a hypophysiotrophic hormone and a neurohormone (Vallarino et al., 2009).

More generally, MCH neurons appear to be involved in a variety of physiological functions, due to their widespread projection patterns (Diniz and Bittencourt, 2017). Such functions include regulation of the sleep-waking cycle, feeding and energy metabolism, and osmoregulation (Diniz and Bittencourt, 2017; van Dijk et al., 2011). Concerning the latter, the HPO of some fish (Francis et al., 1997) and frogs (Francis and Baker, 1995) also contain monoaminergic neurons that contact the cerebrospinal fluid (CSF), and supposedly monitor its chemical composition or osmolarity (Xavier et al., 2017). Interestingly, mammals lack an HPO (including its associated hypothalamic serotonergic and dopaminergic neurons), but their MCH neurons are involved in osmoregulation (secondarily

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https://doi.org/10.1016/j.brainres.2018.07.001
0006-8993/

to vasopressin stimulation; Yao et al., 2012) and CSF homeostasis (by contacting ciliated ependymocytes of the ventral part of the 3rd ventricle; Conductier et al., 2013).

Judging from a limited number of studies, early detection of fish MCH neurons (a few days before or after hatching of the larva) underlines the importance of MCH for fish skin pigmentation regulation. In fact, MCH magnocellular neurons appear before the parvocellular ones and MCH is detected earlier in larvae living in a light rather than a dark environment (Risold et al., 2009). MCH neurons become detectable rather later in developing terrestrial frogs (at metamorphic climax for both parvo- and magnocellular neurons) and show signs of increased secretory activity when the animal is emerging from the water, supporting a role for MCH in osmoregulation (Francis and Baker, 1995). In rats, MCH neurons are first detected during the second half of embryonic development, after which their number quickly increases. A role for MCH has also been suggested in establishing both feeding behavior and sleep-waking patterns (Brischoux et al., 2001; Steininger et al., 2004). No previous studies exist on developing MCH neurons in bird embryos.

In order to gain primary insight into the mode of development of MCH-containing neurons in the chicken, our initial aim was to discover when MCH cells first become detectable, and to compare their development pattern with the one described for rodents. Our studies relied on immunohistochemistry with a commercial antibody that we first validated in chickens. Embryos were examined at ages ranging between embryonic day (E) 6 (when no MCH staining was detectable in the brain) and E20 (the day before hatching). Post-natal day (P) 1 and P21 chicks were also stained to check for differences between pre- and post-natal brains. Labeled cells were counted using stereological techniques. A second aim of these studies was to directly compare the distribution of MCH neurons with that of H/O neurons on consecutive sections from the same individual animals, to see whether these two neuronal populations spatially overlap, as they appear to do in rodents (Cvetkovic et al., 2004). In other non-mammalian vertebrates, MCH neurons are close to dopaminergic and serotonergic neurons in the HPO (Cardot et al., 1999); adult chickens were previously reported to have a few MCH neurons in the HPO (Cardot et al., 1999). A third aim was to ascertain whether the HPO could serve as the source for MCH neurons, as it does for dopaminergic and serotonergic neurons (Xavier et al., 2017). Finally, the presence and qualitative features of MCH fiber staining was described at different ages. Part of this work was previously published in short form (Pompeiano et al., 2014).

2. Results

2.1. Western blot analysis

Western Blot analysis revealed that the anti-proMCH (PMCH) antibody recognized a band at a molecular weight of about 20 kDa in the diencephalon (Fig. 1, left panel). A lighter band was seen in the pallium (Fig. 1, left panel). In the presence of the blocking peptide, no bands were seen (Fig. 1, center panel). The anti-β-actin antibody recognized a band of about 40 kDa (Fig. 1, right panel) and showed that similar amounts of proteins were loaded for the two tissues.

2.2. MCH neuron developmental pattern

MCH cells were seen only in the posterior hypothalamus, close to and inside the HPO. From the HPO, MCH cells appeared to spread mostly laterally. Some cells were seen a little bit cranially to the HPO anterior end, along the 3rd ventricle (at a coronal level that contains the optic chiasm). Staining of anterior hypothalamic as well as brainstem slices failed to show any labeled cells.

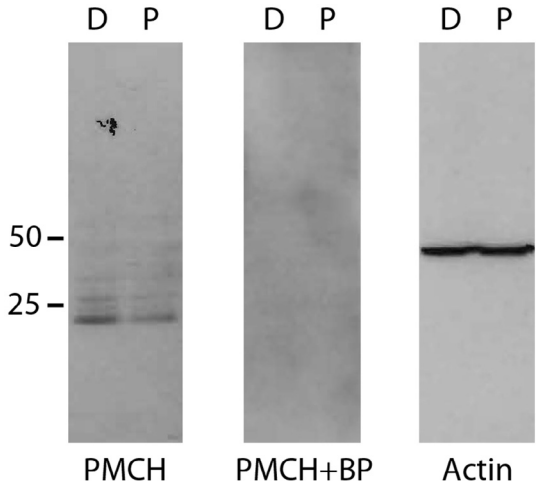

Fig. 1. Western blot analysis of the anti-PMCH antibody in the chick embryo brain. Extracts from an E20 chick embryo diencephalon (D) and pallium (P) were loaded (20 μg of protein). The anti-PMCH antibody binds to a peptide (~20 kDa) which is present in both tissues and is more abundant in D than P (left panel). Preincubation of the anti-PMCH antibody with the blocking peptide (BP) did not yield any band (center panel). An anti-actin antibody revealed that similar amounts of proteins were loaded for D and P (right panel). The same blot was used for all stainings.

Labeled cells were consistently detected in all embryos starting from E9 and were seen in ~5–6 sections in each embryo (cranial-to-caudal spatial extent: ~200–240 μm). No attempt was made to quantify MCH neurons at this age because the relatively low intensity of the staining and the small size of the labeled cells made it difficult to conduct exact cell counts. We quantified labeled cells starting at E10 (Table 1, Fig. 2A). The estimated number of MCH neurons at E10 and E12 was ~1000 and 1850, respectively. A strong and significant increase was seen in cell numbers at older ages with respect to both E10 and E12. The number of MCH neurons was ~5000 and ~6000 at E16 and E20, respectively. A significant decrease with respect to E20 was seen at P1, with ~4300 MCH cells. The cranio-to-caudal spatial extent of the hypothalamic area containing MCH cells (Table 1; Fig. 2B) was about ~1.0 and ~1.4 mm at E10 and E12, respectively. A significant increase was seen at older ages with respect to both E10 and E12. Values of ~1.7, ~1.8, and ~1.6 mm were obtained for E16, E20, and P1, respectively and these values were not significantly different from each other.

2.3. Features of MCH neurons at specific ages

No MCH-positive cells were detectable at E6. At E8, two of the three embryos examined showed a few, very lightly stained cells in a small area with a paramedian location in the dorsal part of

Table 1
Total number of MCH neurons found in the entire hypothalamus, and cranio-caudal spatial extent (in μm) of the hypothalamic area containing MCH neurons at different ages.

Age	n	Labeled neurons (mean ± SEM)	Cranio-caudal extent (μm; mean ± SEM)
E10	4	1006 ± 46	1000 ± 101
E12	4	1850 ± 261	1360 ± 86
E16	5	5271 ± 485	1744 ± 93
E20	5	6306 ± 795	1840 ± 107
P1	5	4293 ± 558	1616 ± 132

n, number of animals; SEM, Standard Error of the Mean.