Please cite this article in press as: Orts-Del'Immagine A et al. Postnatal maturation of mouse medullo-spinal cerebrospinal fluid-contacting neurons. Neuroscience (2016), http://dx.doi.org/10.1016/j.neuroscience.2016.11.028

Neuroscience xxx (2016) xxx–xxx

POSTNATAL MATURATION OF MOUSE MEDULLO-SPINAL CEREBROSPINAL FLUID-CONTACTING NEURONS

ADELINE ORTS-DEL'IMMAGINE,[a‡] JÉRÔME TROUSLARD,[a] CORALINE AIRAULT,[a] JEAN-PHILIPPE HUGNOT,[b] BAPTISTE CORDIER,[c] THIERRY DOAN,[c] ANNE KASTNER[a†] AND NICOLAS WANAVERBECQ[a*†]

[a] *Université d'Aix-Marseille, PPSN EA 4674, 13397 Marseille, France*

[b] *Université de Montpellier 2, INSERM U1051, Institute for Neuroscience, Hôpital Saint Eloi. Montpellier, France*

[c] *Université d'Aix-Marseille, IMM UMR 7283 CNRS, Marseille, France*

Abstract—The central canal along the spinal cord (SC.) and *medulla* is characterized by the presence of a specific population of neurons that contacts the cerebrospinal fluid (CSF). These medullo-spinal CSF-contacting neurons (CSF-cNs) are identified by the selective expression of the polycystin kidney disease 2-like 1 ionic channel (PKD2L1 or polycystin-L). In adult, they have been shown to express doublecortin (DCX) and Nkx6.1, two markers of juvenile neurons along with the neuron-specific nuclear protein (NeuN) typically expressed in mature neurons. They were therefore suggested to remain in a rather incomplete maturation state. The aim of this study was to assess whether such juvenile state is stable in postnatal animals or whether CSF-cNs may reach maturity at older stages than neurons in the parenchyma. We show, in the cervical SC. and the brainstem that, in relation to age, CSF-cN density declines and that their cell bodies become more distant from the cc, except in its ventral part. Moreover, in adults (from 1 month) by comparison with neonatal mice, we show that CSF-cNs have evolved to a more mature state, as indicated by the increase in the percentage of cells positive for NeuN and of its level of expression. In parallel, CSF-cNs exhibit, in adult, lower DCX immunoreactivity and do not express PSA-NCAM and TUC4, two neurogenic markers. Nevertheless, CSF-cNs still share in adult characteristics of juvenile neurons such as the presence of phospho-CREB and DCX while NeuN expression remained low. This phenotype persists in 12-month-old animals. Thus, despite a pursuit of neuronal maturation during the postnatal period, CSF-cNs retain a durable low differentiated state.

Key words: CSF-contacting neurons, PKD2L1, central canal, brainstem, cervical spinal cord, doublecortin, NeuN, phospho-CREB, PSA-NCAM, TUC4.

INTRODUCTION

In mammalian spinal cord (SC.) and *medulla oblongata* (Me.), the cerebrospinal fluid (CSF) of the central canal (cc) is contacted by several kinds of cell among which ependymal cells (ependymocytes), tanycytes but also a specific population of neurons: the cerebrospinal fluid-contacting neurons (CSF-cNs) (Kolmer, 1921, 1931; Agduhr, 1922; Bruni and Reddy, 1987; Vigh and Vigh-Teichmann, 1998). These neurons mostly GABAergic have a small soma, generally inserted in the sub-ependymal layer lining the cc, from which emerges a short dendrite that ends in the cc with a terminal ciliated protrusion (bud) (Stoeckel et al., 2003; Djenoune et al., 2014; Orts-Del'Immagine et al., 2014; Jalalvand et al., 2016a; Petracca et al., 2016). The role of these CSF-cNs is still poorly understood but they were shown to sense modifications in the composition or pressure/flow of CSF through their bud and their cilia (Vigh et al., 2004; Jalalvand et al., 2016a,b) and therefore to potentially represent sensory neurons. In support to this function, CSF-cNs have been shown to selectively express polycystin kidney disease 2-like 1 protein (PKD2L1 or polycytin-L) (Djenoune et al., 2014; Orts-Del'Immagine et al., 2014; Jalalvand et al., 2016a; Petracca et al., 2016) belonging to the TRP channel superfamily and modulated by changes in extracellular pH and osmolarity (Huang et al., 2006; Orts-Del'Immagine et al., 2012, 2015).

The ependymal cells lining the cc of the adult SC. are able to proliferate and they retain some properties of neural progenitors (Russo et al., 2008; Tanaka and Ferretti, 2009; Hugnot and Franzen, 2011; Marichal et al., 2012). Due to their close vicinity with these ependymal cells, CSF-cNs are considered to be elements of the medullo-spinal stem cell niche. This raises the question of the potential neurogenic properties of CSF-cNs in adults, all the more as the CSF they contact has been shown to be able of stimulating embryonic and even adult neuroge-

*Corresponding author.
E-mail address: nicolas.wanaverbecq@univ-amu.fr (N. Wanaverbecq).

† AK and NW contributed equally to this work.

‡ Present address: University of Texas Health Science Center, San Antonio, TX, United States.

Abbreviations: cc, central canal; cMe., caudal *medulla*; CSF, cerebrospinal fluid; CSF-cN, cerebrospinal fluid-contacting neuron; DCX, doublecortin; DF, degree of freedom; DG, dentate gyrus; DVC, dorsal vagal complex; IQR, interquartile distance; IR, immunoreactivity; MAP-2, Microtubule-Associated Protein 2; Me., *medulla oblongata*; NeuN, neuronal nuclei protein; OB, olfactory bulb; PC, pericanal; Phospho-CREB, Phospho-cAMP Response Element-binding Protein; PKD2L1, polycystin kidney disease 2-like 1 protein; PSA-NCAM, polysialylated neuronal cell adhesion molecule; rMe., rostral *medulla*; SC., spinal cord; spMe., sub-postremal medulla; TUC4, [Turned On After Division]/Ulip/CRMP 4.

http://dx.doi.org/10.1016/j.neuroscience.2016.11.028
0306-4522/

nesis (Lehtinen and Walsh, 2011; Carnicero et al., 2013). In lower vertebrates, the adult ependymal stem cell progenitors give rise to both glial cells and neurons and may thus participate to neuronal regeneration after SC. injury (Tanaka and Ferretti, 2009; Lee-Liu et al., 2013). In such regenerative conditions, CSF-cNs could be generated and may either stay in the sub-ependymal layer as described in lamprey for CSF-cNs (Zhang et al., 2014) or migrate in the parenchyma and differentiate in V2 spinal interneurons as shown in Zebrafish (Kuscha et al., 2012). In the mammalian medullo-spinal axis proliferating ependymal cells are only able to differentiate into glial cell. Neurogenesis can be induced *in vitro* (Kehl et al., 1997), but it is absent or very limited *in vivo* (Horner et al., 2000; Horky et al., 2006; Yang et al., 2006). It was observed under some specific traumatic conditions (Chi et al., 2006; Danilov et al., 2006; Ke et al., 2006) and in the region of the dorsal vagal complex (DVC) in the Me. (Bauer et al., 2005).

Regarding spinal CSF-cNs, they have been shown to be only generated between E13.5 and E16.5 (Marichal et al., 2009; Kutna et al., 2013; Djenoune et al., 2014; Petracca et al., 2016) and to express the transcription factor Nkx6.1 even in adult animals (Sabourin et al., 2009; Orts-Del'Immagine et al., 2014; Petracca et al., 2016). In neonatal mice, CSF-cNs were recently shown to comprise of two distinct populations located either in the dorso-lateral or ventral regions of the cc and exhibiting a different maturity both at a phenotypical and functional level (Petracca et al., 2016). In adult, these neurons display many mature neuronal properties (*e.g.* robust action potential discharge activity, expression of the neuron specific nuclear protein (NeuN), a marker for mature neurons) (Kutna et al., 2013; Orts-Del'Immagine et al., 2014). However, at the same time they retained characteristics of juvenile neurons in terms of cell phenotype (expression of DCX and Nkx6.1) (Stoeckel et al., 2003; Sabourin et al., 2009; Kutna et al., 2013; Orts-Del'Immagine et al., 2014).

These apparent paradoxical observations suggest that CSF-cNs could display an intermediate state of maturity in adult animals. This raises the question whether such low mature state is stable in postnatal animals or if CSF-cNs progressively acquire a more mature state at later stages. Our aim was therefore to analyze, in relation to age, the organization of these CSF-cNs in the cc niche, notably their position and distance to the cc. We also further investigated how CSF-cNs cellular maturation evolved with aging by comparing the expression of immaturity/maturity markers in mice at different ages.

EXPERIMENTAL PROCEDURES

Ethics statement

This study was carried out in strict conformity with the recommendations and rules set by the European Communities Council Directive (2010/63/UE) and the French "Direction Départementale de la Protection des Populations des Bouches-du-Rhône". Prior to any experimental procedure, animals were anesthetized using a mixture of xylazine/ketamine and euthanized (see below). All efforts were made to insure animal well-being and minimize animal suffering and the number of animals used. The experimental procedures have been approved by our local Animal Care Ethics Committee (Comité Ethique de Provence N°14) (license N°13.435 held by JT and N°13.430 by NW). Animals were housed at constant temperature (21 °C) under a standard 12-h light–12-h dark cycle, with food (pellet AO4, UAR, Villemoisson-sur-Orge, France) and water provided *ad libitum*.

Animals

We used PKD2L1-IRES-Cre male and female mice kindly provided by Dr CS Zuker (Howard Hughes Medical Institute, University of California, La Jolla, USA). The numbers of mice used for the present study were: 0–2 postnatal days (p0-2D, $n = 6$); 1, 3 and 12 months (p1M, $n = 3$; p3M, $n = 3$; p12M, $n = 3$, respectively). DCX-GFP transgenic mice (1 animal) containing a BAC-GFP construct (RP23-462G16) were also used and were obtained from Gensat project (http://www.gensat.org/).

Immunohistofluorescence

Mice were anesthetized with an intraperitoneal injection of ketamine (Carros, France) and xylazine (Puteaux, France) mixture (100 and 15 mg/kg, respectively) and transcardially perfused with phosphate buffer solution (PBS at 0.1 M). Subsequently the animals were perfused with 4% paraformaldehyde (PFA) in PBS. Brains and SC. were immediately removed, post-fixed one hour in 4% PFA at room temperature, rinsed in PBS (at 4 °C overnight, ON), cryoprotected for 24–48 h in 30% sucrose at 4 °C and frozen in isopentane (−40 °C).

Forebrain, brainstem (*Medulla*, Me.) and cervical SC. coronal thin sections (40 μm) were obtained using a cryostat (Leika CM3050) and collected serially in twelve-well plates containing 0.1 M PBS. As previously reported (Orts-Del'Immagine et al., 2014), we distinguished the cervical SC. region (antero-posterior stereotaxic coordinates of regions more caudal than −8.50 mm from Bregma; Paxinos Mouse Atlas) and three regions for the brainstem: the most caudal level, caudal *medulla* (cMe., from −8.20 to −7.90 mm), the level including the area postrema, sub-postremal medulla (spMe., from −7.90 to −7.65 mm) and the level corresponding to the opening of the cc into the fourth ventricle, rostral *medulla* (rMe., from −7.65 to −7.35 mm). Only few CSF-cNs were observed (on average < 10 per section at all ages) at the level of the most rostral part of the cc (Fig. 2, Level 4 in Orts-Del'Immagine et al., 2014), in the present study, we therefore essentially focused on CSF-cNs present in the SC., cMe. and spMe levels. Fig. 1A2 illustrates the regions considered in the present study. Rostral brain sections from two p3M mice were analyzed at the level of the dentate gyrus (DG) and of the olfactory bulb (OB).

Please cite this article in press as: Orts-Del'Immagine A et al. Postnatal maturation of mouse medullo-spinal cerebrospinal fluid-contacting neurons. Neuroscience (2016), http://dx.doi.org/10.1016/j.neuroscience.2016.11.028