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Title: In Rhesus monkeys, the region around the medullo-spinal central canal is a zone enriched with astrocytes and microglia that only contains CSF-contacting neurons.

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[Abstract]

In spinal cord and medulla, ependymal cells (EC) are organized in a monolayer forming the central canal (cc). In rodents, this region, also known as a stem cell niche, was shown to contain cerebrospinal fluid-contacting neurons (CSF-cNs). These neurons are GABAergic and because of their chemo- and mechanosensory properties they would represent a novel sensory system intrinsic to the central nervous system. In primates, little is known about these neurons and more generally about the region around the cc. Here, using immunohistochemical approaches, we investigated the organization of the cc region and CSF-cN properties in *Macaca mulatta* (Rhesus (Rh.) monkey). In contrast to rodent, we observe along the whole medullo-spinal axis a large zone around the cc delimited by long radial fibers originating from EC that is enriched with astrocytes and microglia but largely devoid of neuronal elements except for CSF-cNs. These primate CSF-cNs share with rodent CSF-cNs similar morphological and phenotypical features with a largely immature profile. Our data suggest that they extend their axons in the longitudinal axis to form fiber bundles close to the cc and we further show that CSF-cNs receive GABAergic and serotoninergic but not glutamatergic and cholinergic synaptic contacts on their soma and dendrite. Taken together our results reveal in Rh. monkey a specific organization of the region around the cc potentially forming a buffer zone between CSF from parenchyma where CSF-cNs would play a crucial role in the detection of CSF signals and their transmission to the central nervous system, a role that would need to be further investigated.

[Introduction]

Along the medullo-spinal axis, a monolayer of ependymal cells (EC) forms the central canal (cc) wall and separates cerebro-spinal fluid (CSF) from parenchyma. Ependymal cells selectively express vimentin with markers of stem cells (nestin, Sox2, 3CB2 and FoxJ1) (Meletis et al., 2008; Hamilton et al., 2009; Marichal et al., 2012) and this region is therefore considered as a stem cell niche where reparatory and regenerative processes have been reported following lesion. Interestingly, close to and within the EC layer (ECL) neurons in contact with the CSF are also found and exhibit a characteristic morphology and phenotype that is conserved in all vertebrates (Agduhr, 1922; Bruni and Reddy, 1987; LaMotte, 1987; Stoeckel et al., 2003; Vígh et al., 2004; Marichal et al., 2009; Djenoune et al., 2014; Orts-Del'Immagine et al., 2014) . These CSF-contacting neurons (CSF-cNs) are bipolar GABAergic cells, they extend a single large dendrite toward the cc ended with a protrusion or 'bud' in contact with the CSF and their axon regroup in the ventral region of the spinal cord to form large bilateral horizontal fiber bundles (Stoeckel et al., 2003; Djenoune et al., 2017; Jurčić et al., 2021; Gerstmann et al., 2022). CSF-cNs selectively express the polycystin kidney disease 2-like 1 (PKD2L1) ion channel, a member of the transient potential receptors (TRP) superfamily with sensory properties (Djenoune et al., 2014; Jalalvand et al., 2014; Orts-Del'Immagine et al., 2014; Petracca et al., 2016). In adult animals, they further exhibit an incomplete neuronal maturity state with a persistent expression of doublecortine (DCX) and the homeobox transcription factor Nkx6.1, two markers of immature neurons and neuroblasts, paralleled by a low expression of neuronal nuclear protein (NeuN), a marker of mature neurons (Sabourin et al., 2009; Kutna et al., 2013; Orts-Del'Immagine et al., 2014, 2017). At the functional level, CSF-cNs were shown to exhibit a spontaneous unitary current activity carried by PKD2L1 (Orts Del'Immagine et al., 2015; Jalalvand et al., 2016a; Sternberg et al., 2018; Orts-Del'Immagine et al., 2020) and modulated by changes in extracellular pH and osmolarity (chemoreception) (Orts Del'Immagine

et al., 2015; Jalalvand et al., 2016a) as well as CSF flux and spinal cord bending (mechanoreception) (Böhm et al., 2016; Jalalvand et al., 2016b; Sternberg et al., 2018; Orts-Del'Immagine et al., 2020). Therefore, CSF-cNs were suggested to represent a novel sensory system intrinsic to the central nervous system (CNS). This sensory function was confirmed in behavioral study in the zebrafish larva where CSF-cN selective activation led to a state-dependent modulation of locomotor activity (Fidelin et al., 2015; Hubbard et al., 2016) and more recently in the mouse where CSF-cNs appear to participate in the modulation of postural motor control (Gerstmann et al., 2022).

In non-human primates only little information is available about the region around the spinal cord cc (Becker et al., 2018). Nevertheless, in Macaca , EC in the lateral part of the cc were shown to be multiciliated while in the ventral and dorsal region they are uni- or biciliated, express nestin and are capable of proliferating (Alfaro-Cervello et al., 2014). CSF-cNs were also observed in this model (LaMotte, 1987; Alfaro-Cervello et al., 2014; Djenoune et al., 2014) and were shown to express PKD2L1 and markers of GABAergic neurons (Djenoune et al., 2014) or the vasoactive intestinal peptide (VIP) (LaMotte, 1987).

Therefore, to better understand the whole organization of the cc region as well as CSF-cN properties and phenotype along the antero-posterior axis in primates dedicated studies are necessary. Here, we show that in *Macaca mulatas* or Rhesus monkey (Rh. Monkey in the rest of the text), in contrast with rodents, the parenchyma is separated from the cc by a large hyponeuronal zone containing long radiating EC fibers and enriched with astrocytes and microglia forming a peri-ependymal zone (PEZ). This PEZ is observed along the whole medullo-spinal axis with conserved properties and the only neurons present in this zone are CSF-cNs. Further, CSF-cNs exhibit similar morphological properties as in rodents and show expression of immaturity markers but lack the classical maturity ones. Their axon forms horizontal fiber bundles around the cc while GABAergic and serotoninergic, but not glutamatergic or cholinergic,

synaptic contacts are observed on CSF-cN soma and dendrite. Taken together our results indicate the presence in Rh. monkey of a zone around the cc where astrocytes and ependymal cell fibers would act as a buffering zone for circulating bioactive signals between CSF and parenchyma, while CSF-cNs would play a crucial role in the detection of these signals and their transmission to the central nervous system, a role that would need to be further investigated.

[Results]

1. The region around the central canal forms a hyponeuronal astroglial zone.

We first analyzed the organization of the region around the Rh. monkey central canal (cc) at the cervical (C7/C8) spinal cord (SC) level and look for the distribution of EC and fibers, neuronal cell bodies, dendrites, and axons as well as astrocytes. Labelling against vimentin (Vim) indicates as expected the presence of EC only around the cc (Fig. $1A_1$-D_1) with their cell body forming the cc wall or ependymal cell layer (ECL) (Fig. $1A_2$-D_2). In agreement with previous work (Alfaro-Cervello et al., 2014), we observed that vimentin positive fibers originating from the ECL extend radially within the white matter (WM) over long distance (~100-150 µm on both side) (Apkarian and Hodge, 1989) to form a large zone around the cc (Fig. $1A_2$-D_2), called peri-ependymal zone (PEZ) in the rest of the text. Immunolabelling against the neurofilament H (NFH, non-phosphorylated form; Fig. $1A_3$) or neurofilament 160 kDa (NF-160, Fig. $1B_3$) reveals a strong immunoreactivity (IR) in the whole grey matter (GM) and a more diffused one in the white matter (WM) (Fig. $1A_3$ and B_3). Surprisingly, only rare neuronal elements are visible in the PEZ (Fig. $1A_3$-A_4 for NFH and B_3-B_4 for NF-160). The merge images on the right confirm this atypical organization and indicate a neuron-free or hyponeuronal PEZ around the cc (Fig. $1A_4$-B_4). Note in B3, the presence in the dorsal region of the SC of numerous NF-160 positive puncta corresponding to longitudinal axonal projection to or from supra-spinal structures. The absence

of mature neuron in the PEZ is further illustrated by immunolabelling against the neuronal nuclear protein (NeuN) indicating that neuronal cell bodies are only present outside the PEZ (Fig. 1C_3-C_4).

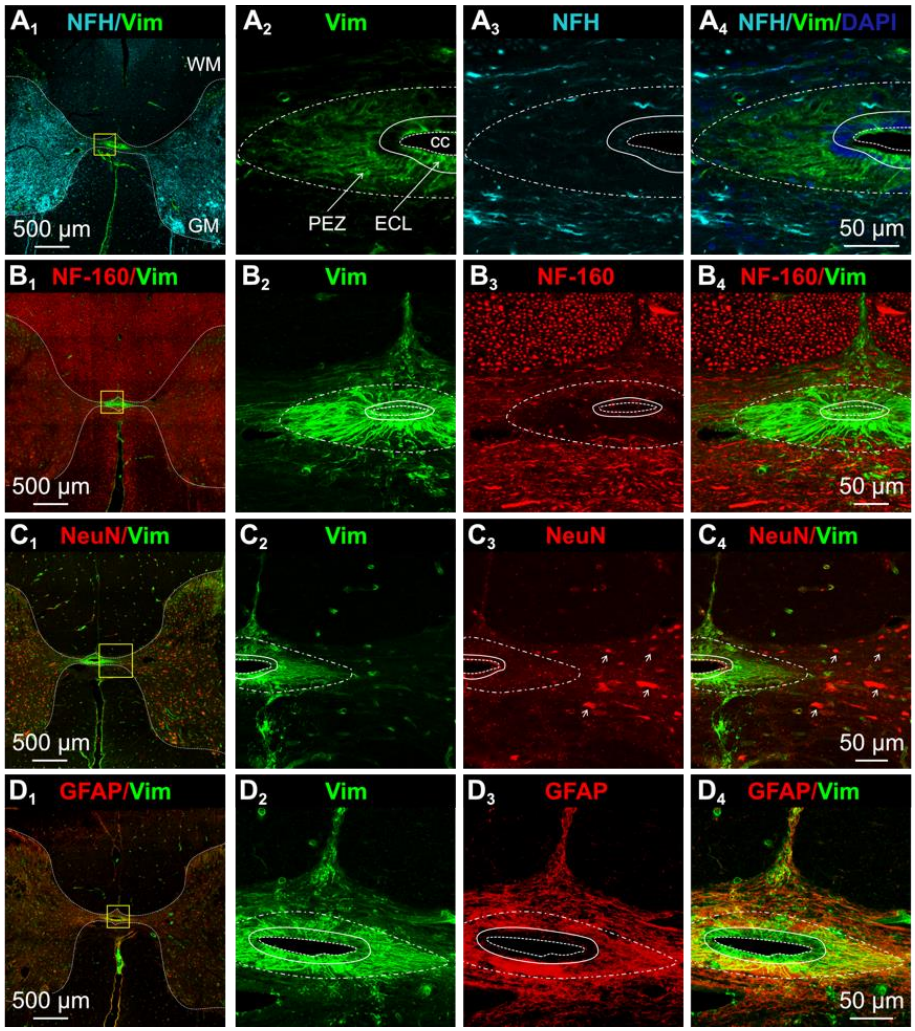

Figure 1: The region around the central canal is enriched with astrocytes and ependymal cell radiating fibers with only few neuronal elements. (**A1-D1)** Low magnification micrographs illustrating the distribution around the central canal of vimentin (Vim), a marker for ependymal cells (EC) and fibers, along with neuronal markers (NFH, A1 and NF-160, B1), neuronal nucleus protein (NeuN, C1) and astrocytic markers (GFAP, D1) in transverse thin sections obtained from the caudal cervical segment of the Rh. monkey spinal cord. (A2-4) High magnification images from A1 for the region around the central canal (cc) indicating the presence of long radiating vimentin positive fibers originating from soma in the ependymal cells layer (ECL) and extending to form the peri-ependymal zone (PEZ, A2). (A3) Immunolabelling against NFH to reveal neuronal structures around the cc. (A4) Merged image showing the distribution of vimentin and NFH along with DAPI, a nuclear marker. Note the presence around the cc of the PEZ delineated by vimentin radiating fibers and largely devoid of axonal projection. **(B2-4)** High magnification images from B1 showing the distribution of Vim (B2) as well as NF-160 to label axons (B3). The merged image in B4 illustrates the organization of axonal fibers in the grey matter and around the PEZ. Note the absence of NF-160 positive fibers within the PEZ as well as the presence of numerous puncta in the dorsal region of the white matter corresponding to longitudinal axonal projection. (**C2-4)** High magnification images from C1 of the Vim (C2) and NeuN (C3, arrows in C3 and C4), distribution around the cc. The merged image in C4 confirms the absence of mature neurons within the PEZ. **(D2-4)** High magnification images from D1 for the distribution of Vim (D2) and GFAP (D3). The merged image in D4 indicates the presence of a region around the cc that is delineated by vimentin positive fibers and enriched with astrocytes. Sections are oriented with the dorsal region on the top and the regions illustrated in high magnification images corresponds to those indicated with yellow boxes in the low magnification images on the left. Color code for the labels on top of images corresponds to the illustrated markers. Dashed lines in A1 to D1 delineates the grey matter (GM) from the white matter (WM) with the dorsal and ventral horns. Scale bars are shown in the images left and right. Dashed lines: cc; Full lines: ependymal cell layer; Doted-dashed lines: PEZ.

Finally, we looked for the astrocyte distribution around and within the PEZ and our data indicate (Fig. 1D) a strong immunolabelling against the glial fibrillary acidic protein (GFAP) within the

vimentine immunoreactive PEZ (Fig. $1D_3$ and $1D_4$). Taken together our results show that in Rh. monkey the region around the cc is in a hyponeural zone enriched with astrocytes and delimited by fibers radiating from EC cell bodies.

2. CSF-contacting neurons are the only neurons present with the Periependymal zone.

In previous studies in mice, we reported that CSF-cNs do not present a high expression level for NeuN and that classical neuronal maturity markers are absent, while microtubule associated protein type 2 (MAP2) immunolabelling prove to be a very efficient marker to visualize these neurons in medullo-spinal tissues (Orts-Del'Immagine et al., 2014, 2017; Jurčić et al., 2021). We therefore performed a set of experiments using antibodies against MAP2 to look for CSF-cNs distribution and morphology around the Rh. monkey cc. In transverse sections at the low cervical level (C7/C8), we observed a dense MAP2 immunolabelling present in the whole grey matter that surround a region highly immunoreactive against vimentin corresponding to the PEZ (Fig. $2A_1$). However, in contrast to immunolabelling against NFH or NF-160, MAP2 IR is also observed within the PEZ that selectively and exclusively labels CSF-cNs (Fig. $2A_2$). These MAP2 positive CSF-cNs present the characteristic morphology with a small round soma (arrows) from which a large dendrite (arrowheads) projects to the cc ending with a protrusion in the lumen (asterisks). Although, in Rh. monkey CSF-cN morphology is similar to that observed in rodents (LaMotte, 1987; Djenoune et al., 2014; Orts-Del'Immagine et al., 2014; Jurčić et al., 2021), they are present within the ECL, beneath it or can be localized further away from the cc with dendrite extending over longer distances (> 50 µm) (Fig. $2A_2$, see also Fig. 4 and 5). Finally, CSF-cN cell bodies are surrounded by vimentin positive radial fibers and their dendrites project to the cc along these fibers (Fig. $2A_3$). We further performed double immunolabelling analyses against NFH and MAP2 or β3-tubuline (Tuj1; β3-Tub), a marker against cytoskeletal microtubules in the somato-dendritic and axonal compartments and confirm that the PEZ represent an hyponeural region where only CSF-cNs are present (Figure $2B_1$) and indicate that

β3-tubuline also allows to selectively visualized CSF-cNs within the PEZ (Fig. 2B$_2$). Next, we looked for the microglia distribution within the spinal cord tissue and reveal a strong IR against the ionized calcium-binding adaptor molecule 1 (IBA1), a selective marker for microglia (Sasaki et al., 2001), in the parenchyma (Fig. 2C). Interestingly, we also observe dense IBA1 IR within

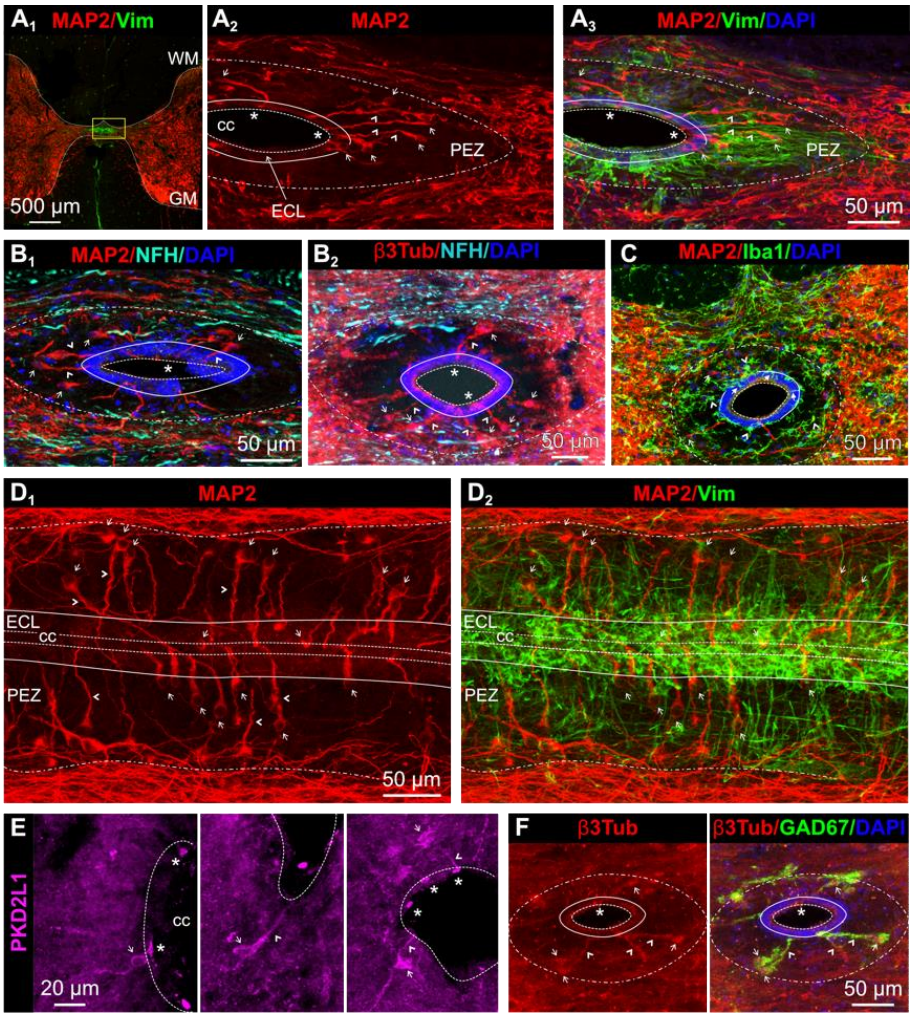

Figure 2: The PEZ contains CSF-contacting neurons, the only neurons present in this region.
(**A1**) Low magnification micrographs illustrating the distribution of vimentin (Vim), to reveal ependymal cells and fibers, and MAP2, a marker for the somato-dendritic compartments, in one transverse thin section obtained from the caudal cervical segment of the Rh. monkey spinal cord. Note that MAP2 is intensively expressed in the whole GM. (**A2**) MAP2 immunolabelling reveals the presence of neurons in the PEZ. These neurons exhibit the characteristic morphology of CSF-contacting neurons (CSF-cNs), with a small soma (arrows) localized around the cc and projecting a single large dendrite (arrowheads) ended with a round protrusion or bud (asterisks) in the cc lumen. Note also the intense MAP2 immunolabelling outside the PEZ, in the GM. The merged image in **A3** shows that CSF-cNs (MAP2) are the only neurons present in the PEZ and extend their dendrites along the Vim-positive fibers. DAPI: nuclear marker. (B) MAP2/NFH co-immunolabelling (**B1**) confirming that CSF-cNs are restricted to the NFH negative hyponeuronal PEZ. (**B2**) β3-tubuline (β 3-Tub), a marker for somato-dendritic and axonal compartments, along with NFH immunolabelling allows CSF-cN visualization in the PEZ devoid of NFH IR. (**C**) Co-immunolabelling against MAP2 and IBA1, a marker for microglial, illustrating the presence of microglial cells in the parenchyma, the PEZ (arrowheads) close to CSF-cNs (arrows) as well as around the cc with projection (filled arrows) within the ECL. (**D**) Horizontal thin section obtained at the level of the cc (dashed lines) of a cervical spinal cord section and immunolabelled against MAP2 (D1 and 2) and Vim to reveal CSF-cNs and ependymal cells or fibers (D2), respectively. Note that MAP2 immunolabelling indicates the distribution in the antero-posterior axis of CSF-cNs within the PEZ along vimentin positive fibers. (**E**) Micrographs illustrating PKD2L1 selective expression in the soma (arrows), dendrite (arrrowheads), and bud (asterisk) of CSF-cNs in transverse thin sections prepared from macaque cervical spinal cord. (**F**) Transverse thin section (caudal cervical level) immunolabelled against β 3-Tub (Left) and GAD67 (Right), a selective marker of GABAergic neurons. For abbreviations see Figure 1. The region illustrated in the high magnification images in A2 and A3 corresponds to that indicated with a yellow box in A1 (left). Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

the PEZ and around the cc where IBA1 positive fibers can be visualize crossing the ECL towards the lumen (full arrows in Fig. 2C) or close to MAP2 positive CSF-cN fibers. The PEZ is therefore a region delimited by vimentin positive radial fibers enriched with astrocyte and microglia where the only neurons present are CSF-cNs. Longitudinal sections further confirm this unique PEZ organization delimited on its edge by the parenchyma (strong IR against MAP2;

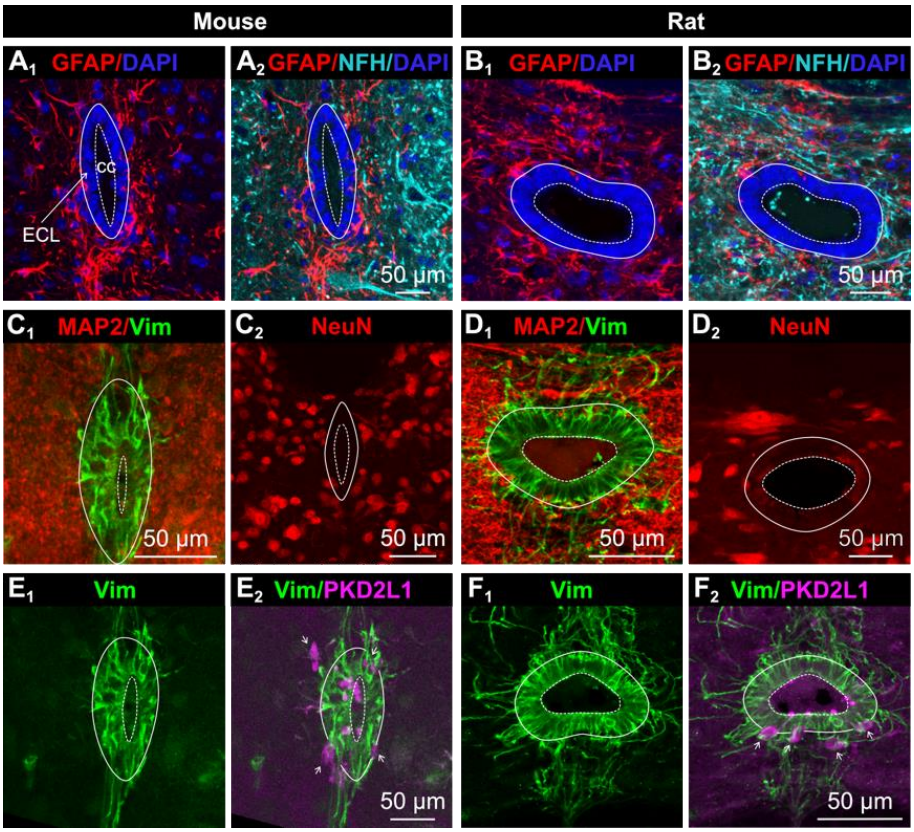

Figure 3: In rodents the ependymal cell layer is contiguous to the parenchyma
Organization of the region around the cc in sections obtained from mouse (**A**, **C** and **D**) and rat (**B**, **D** and **F**) spinal cord. (**A, B**) Images of the astrocyte distribution (GFAP; A1 and B1) along with nuclear labeling (DAPI). (A2 and B2) Merged micrographs showing the distribution for the immunolabelling against GFAP and NFH. Note that in rodent the neural parenchyma is contiguous to the ECL (compare with Figures 1 and 2). (**C, D**) Images of the immunolabelling against Vim (C1 and D1) and PKD2L1 (C2 and D2). Note that CSF-cNs selectively express PKD2L1 and are present within or close to the ECL in direct contact with the parenchyma.
(**E, F**) Images of the immunolabelling against MAP2 and Vim around the cc (E1 and F1) and against NeuN (E2 and F2). The images confirm the absence of a PEZ in rodent illustrated by the close apposition of MAP2 and Vim labelling and the presence of NeuN immunolabelling close to the cc. For abbreviations see Figure 1. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images. Arrows: CSF-cNs. Figure 4: In macaque, the PEZ organization is conserved along the whole rostro-caudal axis of the central canal. (A1-E1) Micrographs at low magnification illustrating the anatomy of the medulla and the spinal cord (cervical to sacral) along the antero-posterior axis in transverse sections with the characteristic organization of the grey matter at each level. (A2-E2) High magnification images of the region around the cc from the sections illustrated from A1 to E1 (yellow squares) and showing the immunolabelling against NFH, GFAP and DAPI. (A3-E3) Representative images for the PEZ organization with the presence of CSF-cNs around the cc and labelled with MAP2. (A4-E4) Images showing the co-immunolabelling against β3-Tub and MAP2 to reveal axonal (β3-Tub only) as well as somato-dendritic compartments (β3-Tub and MAP2). For abbreviations see Figure 1. Arrows point to CSF-cNs. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

Fig. 2C_1), while MAP2 positive CSF-cNs are distributed at high density within the PEZ along the cc with their dendrite projecting perpendicular to the longitudinal axis (Fig. 2D_1) parallelly to vimentin positive fibers originating from EC cell bodies (Fig. 2D_2). Next, in agreement with earlier reports in fish and mouse (Djenoune et al., 2014; Orts-Del'Immagine et al., 2014; Jurčić et al., 2021), CSF-cNs selectively express PKD2L1 (Fig. 2E) and are GABAergic (GAD67, Fig. 2F). To confirm whether the organization observed in Rh. monkey for the region around the cc is specific to this animal model, we repeated a set of experiments in SC sections obtained from mice and rats. In contrast to the data obtained in Rh. monkey, we did not observe a specific PEZ in rodent tissues rather the cc appears directly in contact with both astrocytes (GFAP, Fig. 3A_1 and 3B_1) and neuronal elements (NFH, Fig. 3A_2 and 3B_2). Further, in rodent vimentin positive radial fibers extend from the ECL within MAP2 positive elements in the parenchyma (Vim, Fig. 3C_1 and 3D_1 and MAP2, Fig. 3E_1 and 3F_1) and surround PKD2L1 positive CSF-cNs (PKD2L1, Fig. 3C_2 and 3D_2). Finally, and in contrast to the situation observed in Rh. monkey, immunolabelling against NeuN in rodent tissues reveals the presence of neurons contiguous to the cc (Fig. 3E_2 and 3F_2). Altogether, these results indicate that, in Rh. monkey, CSF-cNs exhibit similar morphological and phenotypical features than in rodent. However, in this primate model, the region around the cc in characterize by a unique PEZ where CSF-cNs are the only neurons present.

3. PEZ and CSF-cN organization and properties along the rostro-caudal axis of the central canal.

To determine whether the unique PEZ organization observed at the cervical level is conserved along the whole cc axis, we performed histological and quantitative analysis of the regions around the cc from the medulla to the sacral segments (Fig. 4A_1 to 4E_1). Our results indicate that the PEZ anatomy is conserved at all medullo-spinal segments and that neuronal elements identified with immunolabelling against NFH surround a large zone around the cc that

is selectively enriched with astrocytes (GFAP, Fig. 4A_2 to E_2). When considering the PEZ and cc orientation and shape in the segments of interest, we noted that both extended in the dorso-ventral axis at the medulla, mid-cervical as well as in the lumbar and sacral segments (Fig. 4). In contrast, at the high-cervical (Fig. 4B), low-cervical (Fig. 1) and thoracic (Fig. 4C) levels, the PEZ and cc were rather oriented in the horizontal axis. We next looked for the presence of CSF-

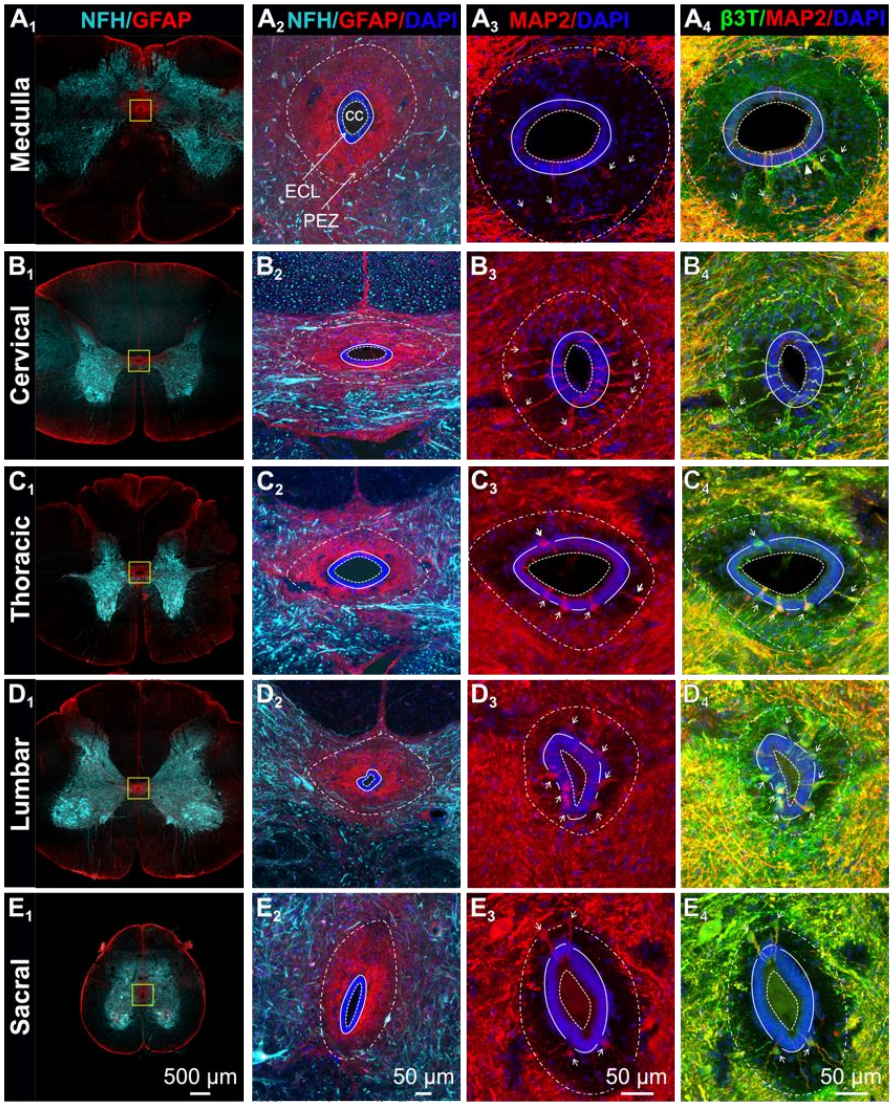

Figure 4: In macaque, the PEZ organization is conserved along the whole rostro-caudal axis of the central canal.
(**A1-E1**) Micrographs at low magnification illustrating the anatomy of the medulla and the spinal cord (cervical to sacral) along the antero-posterior axis in transverse sections with the characteristic organization of the grey matter at each level. (**A2-E2**) High magnification images of the region around the cc from the sections illustrated from A1 to E1 (yellow squares) and showing the immunolabelling against NFH, GFAP and DAPI. (**A3-E3**) Representative images for the PEZ organization with the presence of CSF-cNs around the cc and labelled with MAP2. (**A4-E4**) Images showing the co-immunolabelling against β3-Tub and MAP2 to reveal axonal (β3-Tub only) as well as somato-dendritic compartments (β3-Tub and MAP2). For abbreviations see Figure 1. Arrows point to CSF-cNs. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

cNs in the PEZ in the same segments using staining against MAP2, and our data confirm first that CSF-cNs are present in the PEZ along the whole rostro-caudal axis of the cc but more importantly that they are the only neuronal population in these regions (Fig. $4A_3$-E_3). When looking more closely at CSF-cN distribution in the different segments, our results suggest a differential organization. At the medullar and cervical levels, CSF-cNs are mainly found within the PEZ (Fig. $4A_3$ and $4A_4$) at various distance from the ECL, while in cervical segments they are largely localized more caudally, from thoracic to sacral segments, CSF-cNs are mostly found near or within the ECL or in a dorso-ventral position (Fig. $4C_3$ to $4E_3$ and $4C_4$ to $4E_4$).

According to previous reports, the whole spinal cord size and grey matter shape vary along the different rostro-caudal spinal cord levels (Apkarian and Hodge, 1989) and this observation is confirmed by our results (Fig. 4). To further characterize the organization of the PEZ in our study, we performed a quantitative analysis for the PEZ and cc anatomy as well as of CSF-cN distribution (Fig. 5 and Table 1). First, we measured from the medullar to the sacral levels the PEZ width and we indicate that it is the largest around 80 µm at the medullar and caudal cervical levels (Med and cCerv; Fig. 5A and Table 1A). The PEZ width is smaller at the rostral and mid-cervical (around 50 µm; rCerv and mCerv) and further decreases at more caudal segments to be around 30 µm (from thoracic to sacral segments). Next, we analyzed the cc area (Fig. 5B) at the same levels of interest and found the higher values at the medulla (Med) and sacral (Sac) levels (around 7×10^3 μm^2). In more caudal regions between the rostral cervical and lumbar segments, the cc area ranges from 3 to 5×10^3 μm^2 with no significant differences (Figure 5B and Table 1B; see Figure legend for details). We further analyzed the CSF-cN density (Figure 5C and Table 1C; number of CSF-cNs/10 µm tissue depth) along the rostro-caudal axis of the cc and found the largest cell density at the mid and caudal cervical levels (Figure 5C; cell number between 10 and 15) compared to the other segments where the density is on average around 8 CSF-cNs (Figure 5C and Table 1C; see Figure legend for

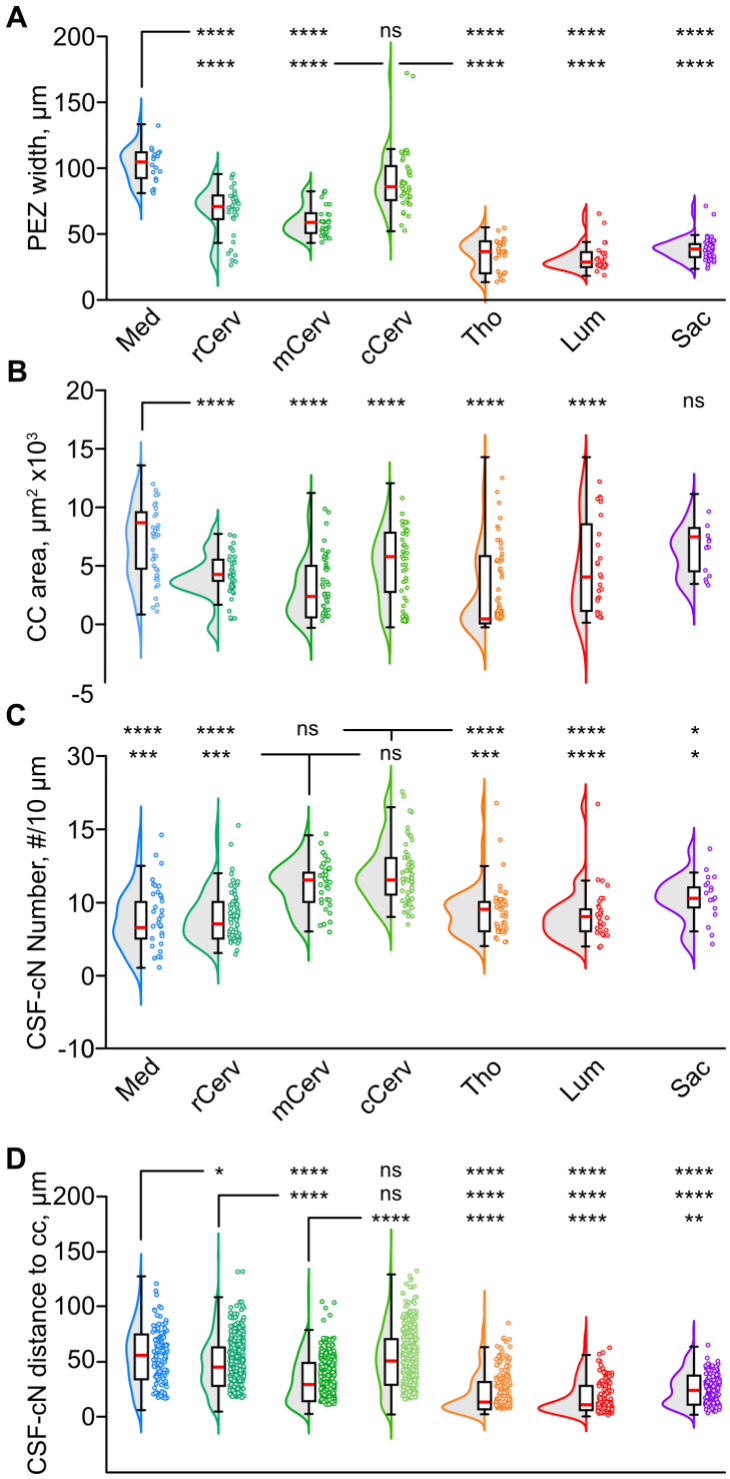

Figure 5: Properties of the peri-ependymal zone (PEZ) and CSF-cNs distribution along the medullo-spinal axis.
Summary graphs for the quantification of the PEZ width (**A**), the cc lumen area (µm2 x 103, **B**), the number of CSF-cNs (**C**) and their distance to the cc (**D**) at the medulla (Med), the rostral, mid and caudal cervical zone (rCerv, mCerv and cCerv), the thoracic (Tho), the lumbar (Lum) and the sacral (Sac) segments. For each graph, whiskers boxplot are presented with superimposed violin plots for the data point density as well as their distribution. Statistical significance was analyzed using a non-linear multi-effect test analysis: (A) $\chi2 = 621.84$, df = 6 and $p(\chi2) < 2.2 . 10\text{-}16$;(B) $\chi2 = 56,11$, df = 6 and $p(\chi2) = 2.763 . 10\text{-}10$; (C) $\chi2 = 113.36$ df = 6 and $p(\chi2) < 2.2 . 10\text{-}16$; (D) $\chi2 = 416.11$ df = 6 and $p(\chi2) < 2.2.10\text{-}16$. Pairwise post-hoc comparison using Sidak method: *, < 0.05; **, < 0.01; ***, < 0.001 and ****<0.0001. And see Table 1

details). Finally, regarding their distance to the cc (*i.e.* the length of their dendrite), our measurements indicate that CSF-cN cell bodies are on average at 50 µm from the cc in the medulla and cervical regions, *i.e.* the most rostral zones, except at the mid-cervical level where CSF-cNs are significantly closer (Figure 5D and Table 1). In more caudal segments, CSF-cNs are found closer to the cc although with a larger variability and an average distance around 20 to 25 µm (Figure 5D and Table 1). In the more anterior segments (Med and Cerv), CSF-cNs could be found at distance as high as 100 µm right at the edge of the PEZ (see Fig. $2A_2$ and $2B_1$), however at all levels, CSF-cNs are distributed in the whole PEZ as well as within the ECL (intra-ependymal). Note that due to the maker used (MAP2) to visualize CSF-cNs and perform the quantification, one cannot exclude the presence of CSF-cNs within the parenchyma. Altogether, our results indicate, and confirm earlier reports, that the PEZ and cc anatomy varies along the rostro-caudal axis with the widest PEZ and larger cc observed mostly in the anterior segments. This anatomy is accompanied by CSF-cNs at a higher density and larger distance to the cc for the same anterior segments.

4. In Rh. monkey, CSF-cNs present a similar phenotype as in rodents and exhibit an intermediate neuronal maturity.

In rodents, CSF-cNs are known to exhibit a low/intermediate neuronal maturity state (Orts-Del'Immagine et al., 2014, 2017; Petracca et al., 2016; Jurčić et al., 2021) characterized by the absence or low levels of NeuN (a marker for mature neurons) and the expression of doublecortin (DCX, a marker of immature neurons) along with Nkx6.1 expression (a homeobox transcription factor expressed in neuronal cells at early developmental stages and only conserved in some neural progenitors of the ventral spinal neuroepithelium in adult animals) (Sabourin et al., 2009; Kutna et al., 2013; Orts-Del'Immagine et al., 2014, 2017). To date, little is known about CSF-cN phenotype and maturity state in Rh. monkey. We reported above the absence of NeuN immunolabelling within the PEZ (see Figure 1C) but to further explore CSF-

cNs maturity state in Rh. monkey, we performed a set of immunohistofluorescence experiments using classical markers. Here, we carried out double immunostaining against MAP2 (to visualize CSF-cNs) and NeuN to assess its level of expression selectively in CSF-cNs. Our results

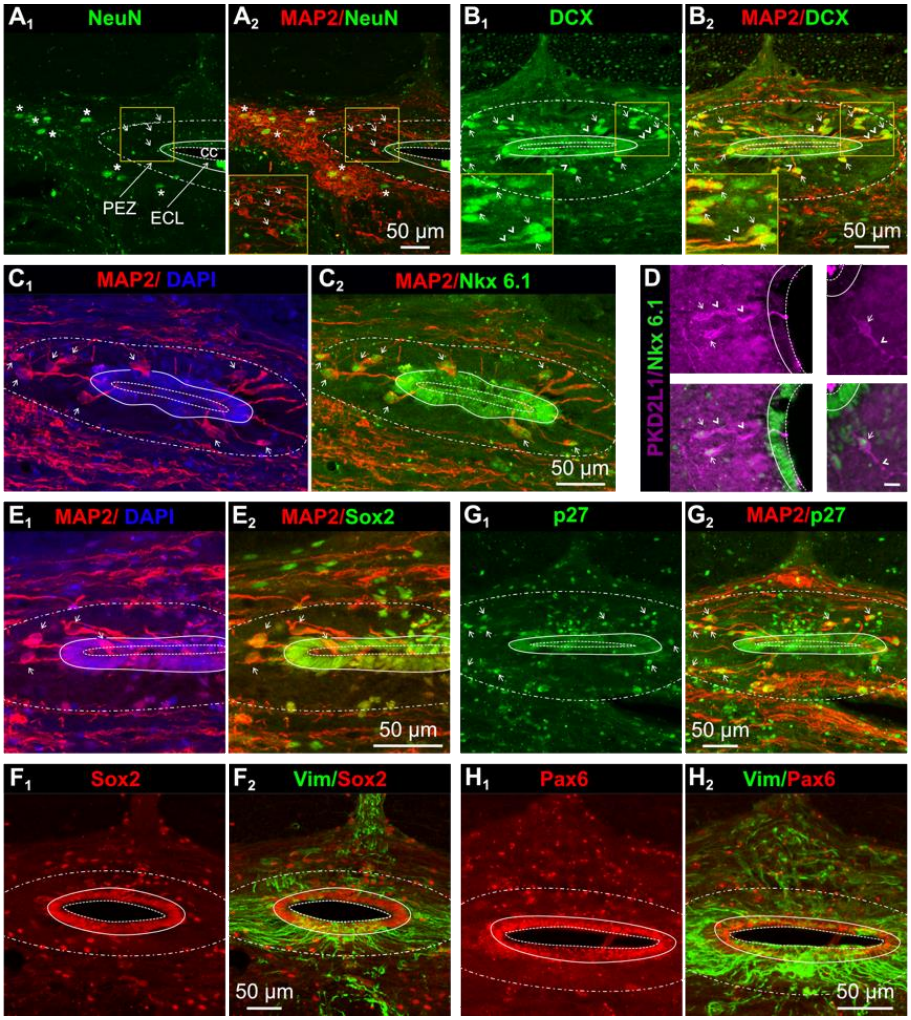

Figure 6: In macaque, CSF-cNs exhibit features of immature neurons
(**A**) Representative micrographs illustrating immunolabelling against NeuN alone (A1) and with MAP2 (A2). Asterisk: NeuN positive neurons in the parenchyma; Arrows: CSF-cNs; Inset in A2: enlargement for the zone around the cc highlighted with a yellow box to illustrate at higher magnification that CSF-cNs are NeuN negative (compare A1 and A2). (**B**) Images illustrating immunolabelling against doublecortine (DCX) alone (B1) and with MAP2 (B2). Arrows: CSF-cNs; Arrowheads: CSF-cN dendrites; Inset in B2: enlargement for the zone around the cc highlighted with a yellow box to illustrate at higher magnification DCX/MAP2 co-expression at the somatic and dendritic levels in CSF-cNs (compare B1 and B2). (**C**) Representative micrographs illustrating immunolabelling against MAP2 alone (C1) and with Nkx6.1 (C2). Arrows: CSF-cNs; Note that Nkx6.1 is expressed by all CSF-cNs and by EC. (**D**) Immunolabelling against PKD2L1 alone (magenta, Top row) and with Nkx6.1 (green, Bottom row) in transverse section prepared from cervical spinal cord. The data confirms that all CSF-cNs selectively express Nkx6.1. (**E-F**) Micrographs illustrating the immunolabelling against MAP2 alone (E1) or Sox2 alone (F1) as well as co-immunolabelling against Sox2 and MAP2 (E2) or Sox2 and Vim (F2). Note the high Sox2 IR of EC around the cc (E2 and F1) as well as Sox2 selective expression in CSF-cNs (Arrows in E). (**G-H**) Micrographs illustrating the immunolabelling against p27 alone (G1) or Pax6 alone (H1) as well as co-immunolabelling against p27 and MAP2 (G2) or Pax6 and Vim (H2). All CSF-cNs and ependymal cells express p27 while Pax6 is only present in ependymal cell. Note the presence in the PEZ of Pax6 positive cell bodies.Illustrated sections were prepared from spinal cord cervical segments. For abbreviations see Figure 1 and color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

confirm the absence of NeuN IR within the PEZ (Fig. 6A$_1$) and further indicate that CSF-cNs in Rh. monkey do not express NeuN (Fig. 6A$_2$ and see inset). We next looked for DCX (Fig. 6B)

and Nkx6.1 (Fig. 6C) expression in CSF-cNs and our data reveal that, in Rh. monkey, CSF-cN do express both immaturity markers. The selective Nkx6.1 expression in CSF-cNs is further confirmed by the co-immunolabelling against PKD2L1 and Nkx6.1 observed in this neuronal population (Fig. 6D).

The immature phenotype for CSF-cNs is further supported by the staining against Sox2, a stem cell marker, observed in the nuclei of this neuronal population only although at an apparent lower level than in EC (Fig. 6E and F). We next looked for expression of p27Kip1, a marker of cell cycle arrest known to be expressed in neuroblasts and observed high IR in Rh. monkey CSF-cN nuclei while it appears lower in EC (Fig. 6G). In contrast, the pattern determinant Pax6 was expressed in Vimentin positive EC but not in CSF-cNs (Fig. 6H). Altogether, our data indicate that in Rh. monkey CSF-cNs do not express maturity markers but rather several proteins classically present in non-mature neurons or neuroblasts.

5. CSF-cN axonal projection and synaptic contacts.

Here, we explored the projection path of putative CSF-cN axon in the spinal cord tissue as well as looked for synaptic contacts on CSF-cNs. Since our data indicate that CSF-cN axon are not labelled with NFH or NF-160, two classical axonal markers (see Fig. 1 and 2), we performed our analysis using double immunolabelling against β3-tubuline (β3Tub) and MAP2. Indeed β3Tub is an element of cytoskeletal microtubules, present both in the somato-dendritic and axonal compartments, while MAP2 only labels soma and dendrites. This approach, therefore, allows to distinguish the somato-dendritic from the axonal compartments and to visualize axonal projection arising from CSF-cN cell bodies within the PEZ (Fig. 7). Our data on transverse spinal cord sections reveals puncta or patches within the PEZ and adjacent to the ventral ECL that are MAP2 negative but β3Tub positive and therefore most likely correspond to axons that were cut in the section process (white arrowhead in Fig. $7A_{1-2}$ and Fig. 4).

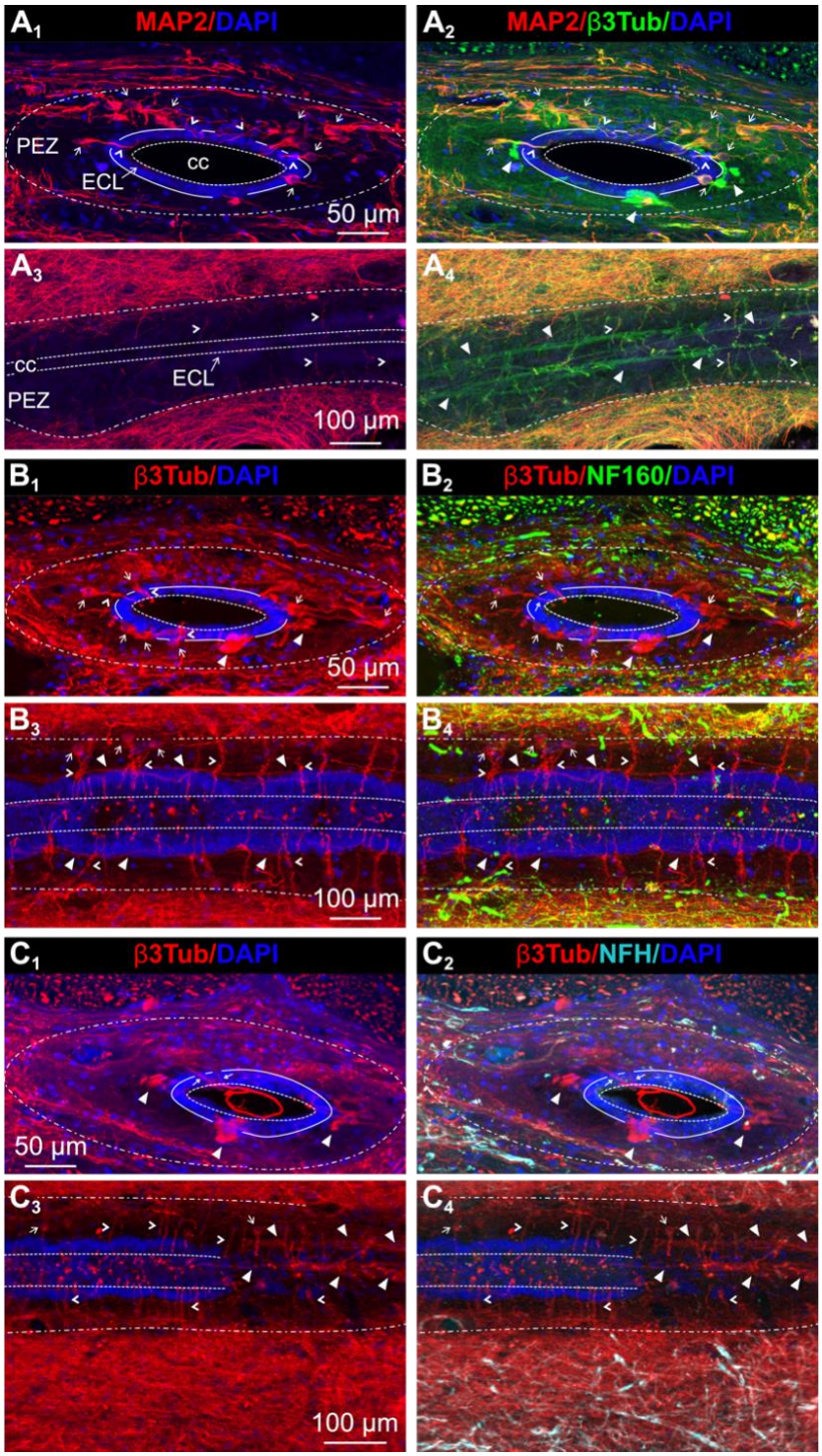

Figure 7: CSF-cN axons appear immature and to project within the PEZ in the longitudinal axis.
(**A**) Representative images illustrating the immunolabelling against MAP2 alone (A1) and with β3-Tub (A2) in a transverse section or in longitudinal sections (A3 and A4). MAP2 labels CSF-cN somato-dendritic compartments while β3-Tub also reveals their axonal projection. In A2, note in the PEZ the presence of β3-Tub positive but MAP2 negative patches (arrowheads) as well as, in A4, of β3-Tub positive longitudinal fiber bundles (arrowheads) that might correspond to CSF-cN axonal projections. (**B-C**) Representative images illustrating the immunolabelling against β3-Tub alone (B1, B3, C1 and C3) and co-immunolabelling against β3-Tub and NF-160 (B2 and B4) or NFH (C2 and C4) in transverse (B1, B2, C1 and C2) or longitudinal sections (B3, B4, C3 and C4). Note in the PEZ, the presence of β3-Tub positive patches and longitudinal fiber bundles (arrowheads) that are neither NF-160 nor NFH positive. In C3 and 4, the section cut with an angle allows visualizing both the CSF-cN cell bodies at the level of the cc (Left) as well as the β3-Tub positive longitudinal fiber bundles (Right) at a more dorsal level. For abbreviations see Figure 1. Small arrows: CSF-cN soma; arrowheads: CSF-cN dendrite; filled arrowheads: axonal projection. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

We next analyzed MAP2/β3tub immunolabelling on longitudinal sections across the cc region to confirm this observation and as expected, we could observe MAP2 positive/β3Tub positive CSF-cNs soma and dendrites within the PEZ (Fig. $7A_{3-4}$) but also MAP2 negative/β3Tub positive rostrocaudal fibers that were either isolated or aggregated as fiber tracts (white arrowhead in Fig. $7A_4$ and see also B-D). To confirm our results, we repeated on transverse and longitudinal sections experiments using double immunolabelling against β3Tub and NFH (Fig. 7B) or NF-160 (Fig. 7C). As previously reported, we observed high NF-160 (Fig. 7B) and NFH (Fig. 7C) IR mainly outside the PEZ however the β3Tub positive fibers/ patches were never NF-160 or NFH positive (Fig. 7B and C). Note on the transverse section illustrated in Figure $7B_2$ occasional NF-160 positive elements present in the ventral and dorsal PEZ as well as the dense NF-160 positive punta in the dorsal region of the spinal cord that correspond to ascending or descending axonal projection tracts. Our results, in agreement with the intermediate maturity state reported for Rh. monkey CSF-cNs (see above) and the previous studies in rodents (Orts-Del'Immagine et al., 2014, 2017), suggest that their axon do not express mature axonal markers and would project in the longitudinal axis within the PEZ.

To test whether CSF-cNs receive synaptic contacts despite their low maturity state, we analyzed the presence of synaptophysin labelling on MAP2 positive CSF-cNs and explored the phenotype of the identified contacts (Fig. 8). Our results for the cervical segments indicate the presence of synaptophysin positive puncta along CSF-cN dendrites (Fig. 8A) within the PEZ and on their soma but not intra-ependymally. In the thoracic and lumbar regions, most CSF-cNs are intra-ependymal and in these segments, the synaptophysin positive synaptic contacts are mainly observed on CSF-cN cell bodies (Fig. 8B).

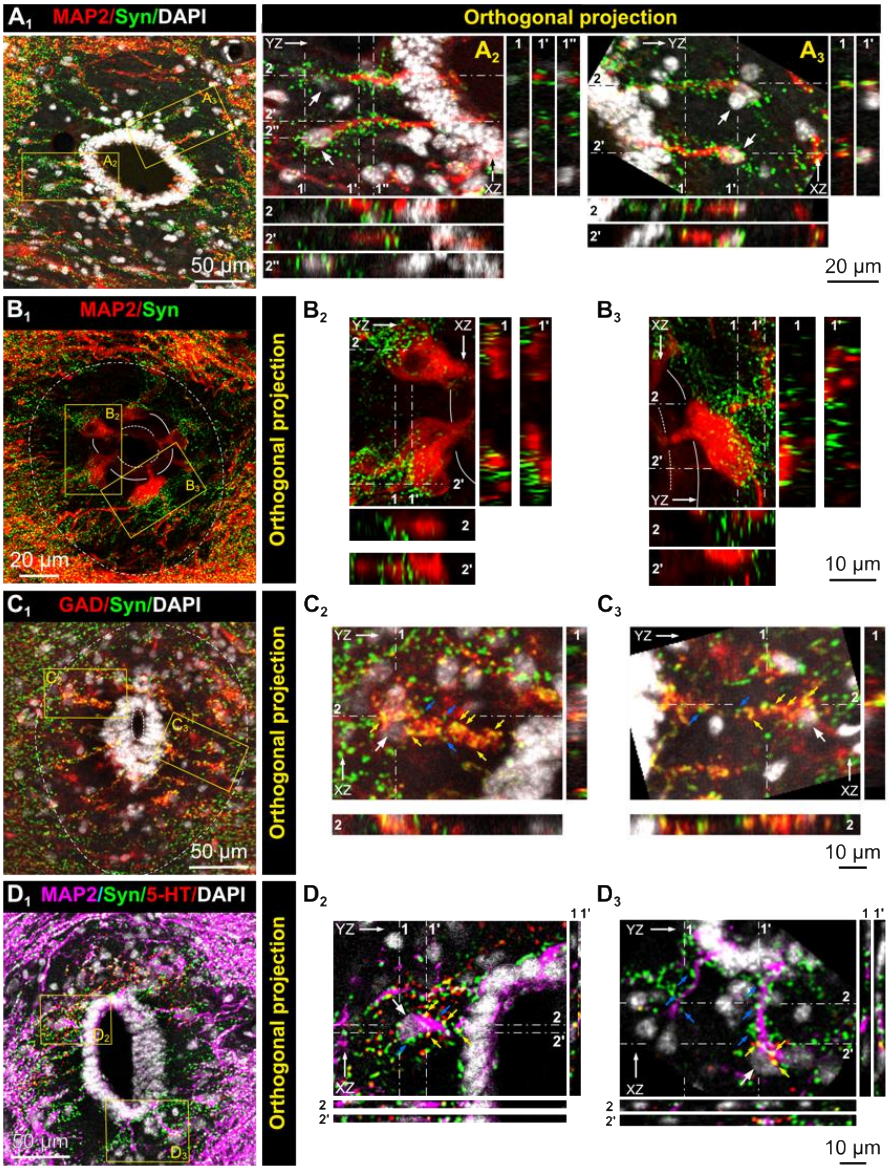

Figure 8: CSF-cN neurons receive somato-dendritic GABAergic and serotoninergic synaptic contacts.
(**A-B**) Transverse sections illustrating MAP2 and synaptophysin (Syn) co-immunolabelling in cervical (A1) and thoracic (B1) segments and representative synaptic contacts on CSF-cNs around dendrites. (**C**) GAD67 and Syn co-immunolabelling (cervical SC) showing the presence of GABAergic synaptic contacts (C1) around CSF-cN dendrites (C2-3). (**D**) MAP2, serotonin (5-HT) and Syn co-immunolabelling showing the presence of 5-HT positive synaptic contacts (D1) around CSF-cNs cell bodies and dendrites (D2-3) mainly in the dorsal region of the SC. In **A-D**, Enlargements for the regions highlighted with yellow boxes in A1-D1 and the respective orthogonal projections (along the YZ axis: 1 and 1' and the XZ axis: 2 and 2', see labels). White, blue, and yellow arrows indicate CSF-cN soma, syn positive and GAD (**C**) or 5-HT (**D**) positive puncta, respectively. For abbreviations see Figure 1. White arrows: CSF-cN or cholinergic soma; Yellow arrows: synaptic contacts; Arrowheads: cholinergic puncta or fibers. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

The orthogonal projections illustrated in Figures 8A_2 and B_2 further support this anatomical organization and indicate that the synaptophysin positive contacts form dense IR puncta along the dendrites and surrounding the whole structure or are present on the soma. To specify the nature of the synaptic contact CSF-cNs receive, we tested the colocalisation of synaptophysin with markers of cholinergic (ChAT), GABAergic (GAD67), glutamatergic (vGlut1) and

serotoninergic (5-HT) neurons. GAD67/synaptophysin double immunolabelling reveals that numerous synaptophysin positive elements contacting CSF-cNs were GABAergic (Fig. 8C_{1-2}). Similarly, MAP2/5-HT/synaptophysin immunolabelling shows that 5-HT fibers are present in the PEZ, mainly in its dorsal part and that they are often colocalized with synaptophysin on CSF-cN soma and dendrites (Fig. 8D_{1-3}). In contrast, although we observe ChAT or vGlut1 positive fibers or soma in the parenchyma with some rare fibers arriving in the PEZ, we never visualize on CSF-cNs synaptophysin positive structure that were also IR for ChAT or vGluT1 (Data not shown). To summarize, CSF-cNs do receive synaptic contacts that are restricted on the somato-dendritic compartment in the PEZ but not within the ECL. These contacts appear to be GABAergic and serotoninergic but not glutamatergic nor cholinergic.

[Discussion]

Our analysis of the cc region in Rh. monkey highlights the presence of a large periependymal zone delimited by radial fibers originating from ependymal cells and enriched with astrocytes and microglia. Further, the PEZ is so-called hyponeural with the sole presence of CSF-cNs. This peculiar organization of the region around the cc that extend throughout the entire medullo-spinal axis appears specific to Rh. monkey and represent a major distinction with that observed in rodents. Nevertheless, in Rh. monkey CSF-cNs show a conserved morphology and exhibit an immature phenotype in agreement with the reports in rodents. We finally indicate that these neurons receive on their somato-dendritic compartment synaptic contacts from GABAergic and serotoninergic terminals but not cholinergic or glutamatergic ones.

1. CSF-cNs specific properties are conserved in Rh. monkey

In agreement with previous studies (LaMotte, 1987; Alfaro-Cervello et al., 2014; Djenoune et al., 2014), we show that CSF-cNs are present around the cc in Rh. monkey. Although they are only poorly and partially detectable with PKD2L1 antibodies, they are clearly labelled with antibodies against MAP2 and β3-Tub. A similarity between rodent and Rh. monkey CSF-cNs is also their GABAergic phenotype as indicated by GAD67 IR (Orts-Del'Immagine et al., 2014; Djenoune et al., 2017; Jurčić et al., 2021). As in mice, CSF-cNs in Rh. monkeys have a small ovoid shape and harbor a dendrite that project toward the cc, with a terminal bud in contact with the CSF. We show moreover that they are present in Rh. monkey along the whole medullospinal axis at various distances from the cc depending on the rostrocaudal level. Thus, the CSF-cNs are the more far from the cc at rostral levels (medulla and cerv levels) and the nearest at the thoracic and lumbar levels. Rostrocaudal and dorsoventral variations in CSF-cNs distribution and phenotype have also been described previously in rodents (Orts-Del'Immagine et al., 2014, 2017), suggesting a functional heterogeneity of these cells. CSF-cNs in the medulla and cervical regions were generally much more distant from the cc in Rh. monkey than in mice, although a population of distal PKD2L1+ neurons have been recently described in the mice spinal cord (Jurčić et al., 2021) but in the ventral region.

A common feature of CSF-cNs in all analyzed vertebrates is their incomplete maturity. Thus, in Rh. monkey alike in rodents, although CSF-cNs exhibit properties of classical neurons, as MAP2 expression, synaptic inputs, neurotransmitter production (GAD67+), CSF-cNs do not express NeuN, NF-160 and NFH classically found in mature neurons. In contrast, they express proteins characteristic of neuroblasts or immature neurons such as DCX, Nkx6.1, Sox2, p27Kip1, as previously shown in rodents (Stoeckel et al., 2003; Sabourin et al., 2009; Kutna et al., 2013; Orts-Del'Immagine et al., 2014, 2017). Some characteristics of juvenile neurons have also been observed in mice by electrophysiological recordings (Jurčić et al., 2019). Although it has been shown in rodents that CSF-cNs are not generate by post-natal or adult neurogenesis

(Marichal et al., 2009; Kutna et al., 2013), they may conserve some features and functions of juvenile neurons during the adult stage. Such unique characteristic for CSF-cNs has been related to their environment in the ependymal stem cell niche. Thus, in Rh. monkey CSF-cNs are present in the ependymal cell layer corresponding to the PEZ where their dendrites follow the radiating ependymal fibers. Such preferential link between CSF-cNs and ependymal cells/fibers has also previously been showed in rodents, even for distal PKD2L1+ neurons that are mostly aligned along the ventral ependymal fibers (Jurčić et al., 2021).This unique feature for CSF-cNs, together with their low-mature phenotype, reinforce the hypothesis for a function as a constituent of the stem cell niche in addition to their putative chemosensory and mechanosensory function. It further raise the question whether they could be activated following spinal cord injury and recruited in regenerative processes. To date this is an unresolved issue.

2. A specific astroglial peri-ependymal compartment for CSF-cNs in primates

In rodents, CSF-cNs are mostly located within the ECL or just beneath (subependymal zone) in an area containing astrocytes but also other types of neurons (Djenoune et al., 2014; Orts-Del'Immagine et al., 2014). In contrast, here we show that CSF-cNs in Rh. monkey occupy a specific and wide hyponeuronal peri-ependymal zone (PEZ) which is almost completely devoid of other neuronal elements as revealed by NeuN, NFH and NF-160 and even MAP2 immunolabelling. Due to this specific anatomical zone for CSF-cNs, MAP2 can be considered as a suitable marker for these neurons in primates since it allows revealing almost the whole population of CSF-cNs distant from 0 to 150 μm to the cc. In addition to CSF-cNs soma and dendrites, this specific PEZ zone also contains a dense synaptic innervation on CSf-cN somato-dendritic compartment. This innervation appears mainly GABAergic and could represent at least partially recurrent synaptic contacts between CSF-cNs as recently demonstrated in mice

(Gerstmann et al., 2022; Nakamura et al., 2023). Beside this GABAergic innervation, However, our data indicate that CSF-cNs mainly in the dorsal PEZ may also be contacted by serotoninergic fibers. Concerning the CSF-cN axonal projection in RH; monkey, our data suggest that they would run within the PEZ in a rostro-caudal and horizontal orientation. This is supported by the presence within the PEZ of fibers that were β3-Tub+ but negative for labbeling against of NFH/NF-160. Further, this organization appears different from that observed in mice, in which axon project toward the ventral fissure (Stoeckel et al., 2003; Jurčić et al., 2021; Gerstmann et al., 2022; Nakamura et al., 2023).

Paralleled to the absence of neuronal elements (except CSF-cNs), the Rh. monkey PEZ was enriched with astrocytes in agreement with the study from Alfaro-Cervello and colleagues (2014). A high density of astrocytes around the ECL was also observed in mice (Jurčić et al., 2021), suggesting that these cells may play a role in the interface between the CSF and neural parenchyma. Moreover, we found that the Rh. monkey PEZ contains also numerous microglial cells, with some microglial fibers surrounding and even, in some cases, crossing the ECL toward the CSF. We do not have functional evidence for such an organization, but we can hypothesize they may contribute to the control CSF diffusion/composition and regulate the communication between CSF and parenchyma. CSF-cNs might interact with astrocytes and microglia and further serve to integrate CSF content to convey information about CNS physiological state. This possibility would need to be address in future dedicated studies.

The different cell organization of the cc environment between rodents and Rh. monkey (absence and presence of the PEZ, respectively) raises the question of the anatomical evolution of this PEZ region in different primate phylum and also whether these data can be translated to humans. In contrast to other mammalian species, the cc is generally occluded and disassembled in humans, although it is generally patent in infants and in the adult medulla and most rostral cervical region (Yasui et al., 1999; Alfaro-Cervello et al., 2014; Garcia-Ovejero et

al., 2015; Becker et al., 2018). In the case of occlusion, the cc is replaced by a highly vascularized cell ‘amas’ constituted of bi- and multiciliated ependymal and glial cells (Alfaro-Cervello et al., 2014; Becker et al., 2018). The cell organization of the peri-cc region (putative PEZ) remains however to be characterized. Moreover, based on electron microscopic studies (Alfaro-Cervello et al., 2014), CSF-cNs have not yet been detected in humans, although we can notice the presence of β3-Tub immunoreactive processes in the cc region (Garcia-Ovejero et al., 2015; Becker et al., 2018). Further immunohistochemical investigations using specific neuronal markers will be necessary in human SC and medulla samples, notably with a patent cc, to putatively identify CSF-cNs and to specify the cc PEZ organization relatively to non-human primates.

In conclusion, we report here an in-depth anatomical study for the region around the primate central canal that appears to be also present in human. The functional ground for this unique organization compared to rodents is unclear and would need to be further characterized. One could hypothesize that it represents a buffer zone to control diffusion of active molecules from the CSF towards the parenchyma with CSF-cNs as intrinsic sensory neurons that would serve to sense signals and convey the information to regulated the CNs homeostatic state.

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[Material & Methods]

Animals

Experiments were conducted on 5 adult *Macaca mulatta* (Rh. Monkey; weight between 2 and 7 Kg). Housing condition and all experiments were conducted in conformity with the rules set by the EC Council Directive (2010/63/UE) and the French “Direction Départementale de la Protection des Populations des Bouches-du- Rhône (DDPP13)”. To comply with the 3Rs rule, animal at the end of experimental protocols carried out in the institute for other projects receive a lethal overdose of anesthetic. Once death was confirmed, they were perfused transcardially first with phosphate buffer solution (PBS 0.1M) and subsequently with 4% paraformaldehyde (PFA) in PBS. We further used 3 mice (6 weeks old) and 3 rats (150 g) that were anesthetized following intraperitoneal injection of a prior to intracardiac perfusion with PBS-4% PFA (Project License Nr: APAFIS 17596; 2018111919329153. N.W). Medulla and spinal cord were immediately collected, separated in the segments of interest samples (medulla, cervical, thoracic, lumbar, and sacral levels), post-fixed in PBS-4% PFA at 4°C overnight, cryoprotected for several days in 30% sucrose at 4°C and finally frozen in isopentane (−40°C).

Immunohistochemistry

Transverse and longitudinal thin sections (30 µm) were obtained using a cryostat (Leika CM3050) and collected serially in 24-well plates containing 0.1M PBS. For further conservation, section were transferred in a cryoprotecting solution at -20°C until use. For immunohistochemistry, sections were washed in PBS, incubated first 1h in PBS containing 0.3% Triton X-100 (Sigma) and then in a blocking solution containing 3% horse or goat normal serum and 1% bovine serum albumin (BSA). Sections were incubated overnight at 4°C with primary antibodies in an incubation medium (PBS with 3% horse or goat normal serum). Sections were then washed at least 3 times in PBS and incubated for 2h with secondary

antibodies conjugated to either AlexaFluor 488, 594, or 647 (Life Technologies; See Table 1 for details). At the end of the incubation, sections were washed in PBS serveal times. When more than one primary antibody was used in an immunohistofluorescence labeling experiments, the primary antibodies were applied sequentially. Sections were mounted on polylysine coated slides and coverslipped with a mounting medium for fluorescence microscope preparation (generally after DAPI staining). To assess the selectively of the observed immunolabelling, in each set of experiments, some sections were treated in the absence of primary antibodies (negative control). Each experiment was performed on tissues obtained from 2-3 animals and analyzed on 3-6 sections for each segment of interest and for each animal (for one SC region). The following primary antibodies were used: β3 tubuline/TUJ1 (Sigma, T2200, rabbit, 1:500; Biolegend, 801213, mouse, 1:500), ChAT (Millipore AB144P, goat, 1:100), DCX (Santa Cruz SC-8066, goat, 1:100), GAD67 (Millipore MAB5406, mouse, 1:500), GFAP (Sigma, G3893, mouse, 1:500; DAKO, Z0334, rabbit, 1:500), 5-HT (Immunostar 20079, goat, 1:500), Iba-1 (Wako 019-19741, rabbit, 1:800), MAP2 (Sigma M-1406, mouse, 1:600), NeuN (Millipore MAB377, mouse, 1:800), NF-160 (Sigma, N5264, mouse, 1/1000), NFH non-phosphorylated SMI32 (Biolegend, 801702, mouse, 1:500), Nkx6.1 (Dev.Stud. Hybrid.Bank F55A10, mouse, 1:100, gift of Dr. JP Hugnot), p27Kip1 (Epitomics, 2187, rabbit, 1/200), Pax6 (Santa Cruz, sc-53108, mouse, 1:100), PKD2L1 (Millipore AB9084, rabbit, 1:700), Sox2 (Santa Cruz E4, sc-365823, mouse, 1:200), Synaptophysin (Epitomics, 1485, rabbit, 1/500), V-Glut (Millipore ABN1647, rabbit, 1:200), Vimentine (Millipore AB5733, chicken, 1/700). Most of these antibodies (NeuN, GFAP, Vim, β3-Tub, DCX, Nkx6.1, MAP2, PKD2L1) have been previously used in our studies in mice (Orts-Del'Immagine et al., 2014; Jurčić et al., 2021) and were validated in macaque by the labelling observed in similar cell populations as in rodent and positive labelling for NeuN, MAP2, ChAT, β3-Tub, NFH, NF-160 and synaptophysin in neurons and neuronal elements present in the same section away from the cc. Vimentin, GFAP, IBA-1

immunofluorescence were detected as expected in ependymal cells, astrocytes microglial cells. The antibodies Sox2, Pax6, p27Kip1 were validated by the expected immunolabelling of the cc ependymal stem cell population. Selectivity of the immunostaining was confirmed by the absence of fluorescence Experiments conducted.

Image acquisition and analysis

Sections were observed on a confocal laser scanning microscope (CLSM; Zeiss LSM700) equipped with solid state fiber optic lasers and single plane images or stacks of images were acquired with a 0.5-1x digital zoom using a 20x objective (numerical aperture, NA: 0.8 for an optical thickness of ~2 µm). Images were acquired at optimal resolution of 1024×1024 pixels. When using 2-4 fluorochromes with different excitation/emission spectra, images were acquired sequentially for each channel (488 nm, green; 555 nm, red; 405 nm, blue; 639 nm, magenta) and the filters and photomultiplier tubes settings chosen to optimize images and avoid signal crosstalk. Images were analyzed and prepared using ZEN 2009 light Edition (Zeiss software), ImageJ 1.45 (NIH) and Corel Photo-Paint. For better visualization, contrast and brightness were adjusted in images used for the figures. Images illustrated at low magnification were obtained using the ZENN 2009 mosaic routine (from 4 x 4 to 6 x 6 fields of view) while high magnification images were obtained from z-projection of 5 to 12 images in a stack. For quantification analysis of the cell density, $MAP2^+$ CSF-cNs were counted manually in each image and the total cell number divided by the section thickness (10 µm stack depth). For the analysis of peri-ependymal zone width, cc area (10^3 x μm^2), CSF-cN distance to the cc, the measure were carry out using ImageJ. At each medullo-spinal level, the analysis was performed in 3-4 animals on at least 6 different sections per animal.

Statistical analysis

All data presented in Figure 5 are expressed as mean ± SD and represented as with whisker boxplots using the Tukey's method as well as with “violin” plots to illustrate data point density and the data point distribution. In whisker plots, for each data set, the median and the 25th to the 75th percentiles (lower and upper limits of each bar, respectively) are calculated. Next, the interquartile distance (IQR) is determined as the difference between the 25th and 75th percentiles, and the whiskers limits, or “inner fences” calculated as the 75th percentile plus 1.5 times IQR and the 25th percentile minus 1.5 times IQR. All data with value either higher or lower than the inner fences are represented as individual data points and considered as outliers (circles on the plots). On one hand, we compared on parameter (PEZ width, CC area, CSF-cN number or distance to the cc) across several medullo-spinal segments, here labeled as Region. In one animal, the parameters measured in each Region are assumed dependent from the organization of the other regions, and the variable region represents a dependent factor. On the other hand, the same variables represent independent factors when considered in different animals. Therefore, the statistical analyses required using a mixed effect model taking into account in a hierarchical way both dependent and independent variables as well as the interaction between the variables. Finally, the choice of animals used for this study was done randomly among all those available; therefore, it is necessary to implement the statistical model with a factor corresponding to this random effect. The statistical analyses were carried out using the R Studio 3.3.1 statistical software (R Studio Team, 2015) in the R environment and the “non-linear mixed effect (nlme version 3.1–128)” package (Pinheiro et al., 2021). The null hypothesis was set as the absence of difference between the data among each group. The nlme model was tested by using an analysis of the variance (ANOVA), and a subsequent post-hoc Sidak test for multiple pairwise comparison was used. We provide the values for $\chi 2$, the degree of freedom (number of parameters −1, dF) and the p-values for the given fixed factor tested. Statistical differences were considered as significant for $p < 0.05$.

[Figures Legends]

Figure 1: The region around the central canal is enriched with astrocytes and ependymal cell radiating fibers with only few neuronal elements.

(**A1-D1**) Low magnification micrographs illustrating the distribution around the central canal of vimentin (Vim), a marker for ependymal cells (EC) and fibers, along with neuronal markers (NFH, **A1** and NF-160, **B1**), neuronal nucleus protein (NeuN, **C1**) and astrocytic markers (GFAP, **D1)** in transverse thin sections obtained from the caudal cervical segment of the Rh. monkey spinal cord.

(**A2-4**) High magnification images from **A1** for the region around the central canal (cc) indicating the presence of long radiating vimentin positive fibers originating from soma in the ependymal cells layer (ECL) and extending to form the peri-ependymal zone (PEZ, **A2**). (**A3**) Immunolabelling against NFH to reveal neuronal structures around the cc. (**A4**) Merged image showing the distribution of vimentin and NFH along with DAPI, a nuclear marker. Note the presence around the cc of the PEZ delineated by vimentin radiating fibers and largely devoid of axonal projection.

(**B2-4**) High magnification images from **B1** showing the distribution of Vim (**B2**) as well as NF-160 to label axons (**B3**). The merged image in **B4** illustrates the organization of axonal fibers in the grey matter and around the PEZ. Note the absence of NF-160 positive fibers within the PEZ as well as the presence of numerous *puncta* in the dorsal region of the white matter corresponding to longitudinal axonal projection.

(**C2-4**) High magnification images from **C1** of the Vim (**C2**) and NeuN (**C3,** arrows in **C3** and **C4**), distribution around the cc. The merged image in **C4** confirms the absence of mature neurons within the PEZ.

(**D2-4**) High magnification images from **D1** for the distribution of Vim (**D2**) and GFAP (**D3**). The merged image in **D4** indicates the presence of a region around the cc that is delineated by vimentin positive fibers and enriched with astrocytes.

Sections are oriented with the dorsal region on the top and the regions illustrated in high magnification images corresponds to those indicated with yellow boxes in the low magnification images on the left. Color code for the labels on top of images corresponds to the illustrated markers. Dashed lines in A1 to D1 delineates the grey matter (GM) from the white matter (WM) with the dorsal and ventral horns. Scale bars are shown in the images left and right. Dashed lines: cc; Full lines: ependymal cell layer; Doted-dashed lines: PEZ.

Figure 2: The PEZ contains CSF-contacting neurons, the only neurons present in this region.

(**A1**) Low magnification micrographs illustrating the distribution of vimentin (Vim), to reveal ependymal cells and fibers, and MAP2, a marker for the somato-dendritic compartments, in one transverse thin section obtained from the caudal cervical segment of the Rh. monkey spinal cord. Note that MAP2 is intensively expressed in the whole GM. (**A2**) MAP2 immunolabelling reveals the presence of neurons in the PEZ. These neurons exhibit the characteristic morphology of CSF-contacting neurons (CSF-cNs), with a small soma (arrows) localized around the cc and projecting a single large dendrite (arrowheads) ended with a round protrusion or bud (asterisks) in the cc lumen. Note also the intense MAP2 immunolabelling outside the PEZ, in the GM. The merged image in **A3** shows that CSF-cNs (MAP2) are the only neurons present in the PEZ and extend their dendrites along the Vim-positive fibers. DAPI: nuclear marker.

(B) MAP2/NFH co-immunolabelling (**B1**) confirming that CSF-cNs are restricted to the NFH negative hyponeuronal PEZ. **(B2)** β3-tubuline (β3-Tub), a marker for somato-dendritic and

axonal compartments, along with NFH immunolabelling allows CSF-cN visualization in the PEZ devoid of NFH IR.

(C) Co-immunolabelling against MAP2 and IBA1, a marker for microglial, illustrating the presence of microglial cells in the parenchyma, the PEZ (arrowheads) close to CSF-cNs (arrows) as well as around the cc with projection (filled arrows) within the ECL.

(**D**) Horizontal thin section obtained at the level of the cc (dashed lines) of a cervical spinal cord section and immunolabelled against MAP2 (**D1 and 2**) and Vim to reveal CSF-cNs and ependymal cells or fibers (**D2**), respectively. Note that MAP2 immunolabelling indicates the distribution in the antero-posterior axis of CSF-cNs within the PEZ along vimentin positive fibers.

(**E**) Micrographs illustrating PKD2L1 selective expression in the soma (arrows), dendrite (arrrowheads), and bud (asterisk) of CSF-cNs in transverse thin sections prepared from macaque cervical spinal cord.

(**F**) Transverse thin section (caudal cervical level) immunolabelled against β3-Tub (Left) and GAD67 (Right), a selective marker of GABAergic neurons.

For abbreviations see Figure 1. The region illustrated in the high magnification images in A2 and A3 corresponds to that indicated with a yellow box in A1 (left). Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

Figure 3: In rodents the ependymal cell layer is contiguous to the parenchyma

Organization of the region around the cc in sections obtained from mouse (**A, C** and **D**) and rat (**B, D** and **F**) spinal cord.

(**A** and **B**) Images of the astrocyte distribution (GFAP; **A1** and **B1**) along with nuclear labeling (DAPI). (**A2** and **B2**) Merged micrographs showing the distribution for the immunolabelling against GFAP and NFH. Note that in rodent the neural parenchyma is contiguous to the ECL (compare with Figures 1 and 2).

(**C** and **D**) Images of the immunolabelling against Vim (**C1** and **D1**) and PKD2L1 (**C2** and **D2**). Note that CSF-cNs selectively express PKD2L1 and are present within or close to the ECL in direct contact with the parenchyma.

(**E** and **F**) Images of the immunolabelling against MAP2 and Vim around the cc (**E1** and **F1**) and against NeuN (**E2** and **F2**). The images confirm the absence of a PEZ in rodent illustrated by the close apposition of MAP2 and Vim labelling and the presence of NeuN immunolabelling close to the cc.

For abbreviations see Figure 1. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images. Arrows: CSF-cNs.

Figure 4: In macaque, the PEZ organization is conserved along the whole rostro-caudal axis of the central canal.

(A1-**E1**) Micrographs at low magnification illustrating the anatomy of the medulla and the spinal cord (cervical to sacral) along the antero-posterior axis in transverse sections with the characteristic organization of the grey matter at each level. (**A2**-**E2**) High magnification images of the region around the cc from the sections illustrated from A1 to E1 (yellow squares) and showing the immunolabelling against NFH, GFAP and DAPI**.** (**A3**-**E3**) Representative images for the PEZ organization with the presence of CSF-cNs around the cc and labelled with MAP2. (**A4**-**E4**) Images showing the co-immunolabelling against β3-Tub and MAP2 to reveal axonal (β3-Tub only) as well as somato-dendritic compartments (β3-Tub and MAP2).

For abbreviations see Figure 1. Arrows point to CSF-cNs. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

Figure 5: Properties of the peri-ependymal zone (PEZ) and CSF-cNs distribution along the medullo-spinal axis.

Summary graphs for the quantification of the PEZ width (**A**), the cc lumen area ($\mu m^2 \times 10^3$, **B**), the number of CSF-cNs (**C**) and their distance to the cc (**D**) at the medulla (Med), the rostral, mid and caudal cervical zone (rCerv, mCerv and cCerv), the thoracic (Tho), the lumbar (Lum) and the sacral (Sac) segments. For each graph, whiskers boxplot are presented with superimposed violin plots for the data point density as well as their distribution. Statistical significance was analyzed using a non-linear multi-effect test analysis: (**A**) $\chi^2 = 621.84$, df = 6 and $p(\chi^2) < 2.2 \cdot 10^{-16}$;(**B**) $\chi^2 = 56{,}11$, df = 6 and $p(\chi^2) = 2.763 \cdot 10^{-10}$; (**C**) $\chi^2 = 113.36$ df = 6 and $p(\chi^2) < 2.2 \cdot 10^{-16}$; (**D**) $\chi^2 = 416.11$ df = 6 and $p(\chi^2) < 2.2.10^{-16}$. Pairwise post-hoc comparison using Sidak method: *, < 0.05; **, < 0.01; ***, < 0.001 and ****<0.0001.

Figure 6: In macaque, CSF-cNs exhibit features of immature neurons

(**A**) Representative micrographs illustrating immunolabelling against NeuN alone (**A1**) and with MAP2 (**A2**). Asterisk: NeuN positive neurons in the parenchyma; Arrows: CSF-cNs; Inset in A2: enlargement for the zone around the cc highlighted with a yellow box to illustrate at higher magnification that CSF-cNs are NeuN negative (compare A1 and A2).

(**B**) Images illustrating immunolabelling against doublecortine (DCX) alone (**B1**) and with MAP2 (**B2**). Arrows: CSF-cNs; Arrowheads: CSF-cN dendrites; Inset in B2: enlargement for the zone

around the cc highlighted with a yellow box to illustrate at higher magnification DCX/MAP2 co-expression at the somatic and dendritic levels in CSF-cNs (compare B1 and B2).

(**C**) Representative micrographs illustrating immunolabelling against MAP2 alone (**C1**) and with Nkx6.1 (**C2**). Arrows: CSF-cNs; Note that Nkx6.1 is expressed by all CSF-cNs and by EC.

(**D**) Immunolabelling against PKD2L1 alone (magenta, Top row) and with Nkx6.1 (green, Bottom row) in transverse section prepared from cervical spinal cord. The data confirms that all CSF-cNs selectively express Nkx6.1.

(**E**-**F**) Micrographs illustrating the immunolabelling against MAP2 alone (**E1**) or Sox2 alone (**F1**) as well as co-immunolabelling against Sox2 and MAP2 (**E2**) or Sox2 and Vim (**F2**). Note the high Sox2 IR of EC around the cc (**E2** and **F1**) as well as Sox2 selective expression in CSF-cNs (Arrows in **E**).

(**G**-**H**) Micrographs illustrating the immunolabelling against p27 alone (**G1**) or Pax6 alone (**H1**) as well as co-immunolabelling against p27 and MAP2 (**G2**) or Pax6 and Vim (**H2**). All CSF-cNs and ependymal cells express p27 while Pax6 is only present in ependymal cell. Note the presence in the PEZ of Pax6 positive cell bodies.

Illustrated sections were prepared from spinal cord cervical segments. For abbreviations see Figure 1 and color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

Figure 7: CSF-cN axons appear immature and to project within the PEZ in the longitudinal axis.

(**A**) Representative images illustrating the immunolabelling against MAP2 alone (**A1)** and with β3-Tub (**A2)** in a transverse section or in longitudinal sections (**A3** and **A4**). MAP2 labels CSF-cN somato-dendritic compartments while β3-Tub also reveals their axonal projection. In **A2**, note in the PEZ the presence of β3-Tub positive but MAP2 negative patches (arrowheads) as well as, in **A4**, of β3-Tub positive longitudinal fiber bundles (arrowheads) that might correspond to CSF-cN axonal projections.

(**B-C**) Representative images illustrating the immunolabelling against β3-Tub alone (**B1, B3, C1** and **C3**) and co-immunolabelling against β3-Tub and NF-160 (**B2** and **B4**) or NFH (**C2** and **C4**) in transverse (**B1, B2, C1 and C2**) or longitudinal sections (**B3, B4, C3 and C4**). Note in the PEZ, the presence of β3-Tub positive patches and longitudinal fiber bundles (arrowheads) that are neither NF-160 nor NFH positive. In **C3** and **4**, the section cut with an angle allows visualizing both the CSF-cN cell bodies at the level of the cc (Left) as well as the β3-Tub positive longitudinal fiber bundles (Right) at a more dorsal level.

For abbreviations see Figure 1. Small arrows: CSF-cN soma; arrowheads: CSF-cN dendrite; filled arrowheads: axonal projection. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

Figure 8: CSF-cN neurons receive somato-dendritic GABAergic and serotoninergic synaptic contacts.

(**A**-**B**) Transverse sections illustrating MAP2 and synaptophysin (Syn) co-immunolabelling in cervical (**A1)** and thoracic (**B1**) segments and representative synaptic contacts on CSF-cNs around dendrites.

(**C**) GAD67 and Syn co-immunolabelling (cervical SC) showing the presence of GABAergic synaptic contacts (**C1**) around CSF-cN dendrites (**C2-3**).

(**D**) MAP2, serotonin (5-HT) and Syn co-immunolabelling showing the presence of 5-HT positive synaptic contacts (**D1**) around CSF-cNs cell bodies and dendrites (**D2-3**) mainly in the dorsal region of the SC.

In A-D, Enlargements for the regions highlighted with yellow boxes in A1-D1 and the respective orthogonal projections (along the YZ axis: 1 and 1' and the XZ axis: 2 and 2', see labels). White, blue, and yellow arrows indicate CSF-cN soma, syn positive and GAD (C) or 5-HT (D) positive puncta, respectively. For abbreviations see Figure 1. White arrows: CSF-cN or cholinergic soma; Yellow arrows: synaptic contacts; Arrowheads: cholinergic puncta or fibers. Color code for the labels on top of images corresponds to the illustrated markers. Scale bars are shown on the images.

[Table]

Table 1: Quantitative analysis of the Peri-ependymal Zone and CSF-cNs properties

A - Peri-ependymal zone Width (µm)						
Med	**rCerv**	**mCerv**	**cCerv**	**Tho**	**Lum**	**Sac**
88 ± 11	59 ± 15	54 ± 9	79 ± 19	33 ± 9 µm	31 ± 8	38 ± 7
S=18	S=36	S=29	S=42	S=27	S=30	S=35
B - Central canal Area (x 10^3 µm^2)						
Med	**rCerv**	**mCerv**	**cCerv**	**Tho**	**Lum**	**Sac**
6.6 ± 3.0	4.0 ± 1.8	2.8 ± 2.4	4.7 ± 2.8	2.7 ± 3.3	4.7 ± 3.9	6.0 ± 2.0
S=37	S=41	S=44	S=44	S=55	S=26	S=13
C- CSF-cN Density (cell number/10 µm tissue depth)						
Med	**rCerv**	**mCerv**	**cCerv**	**Tho**	**Lum**	**Sac**
7 ± 4	8 ± 3	12 ± 3	14 ± 4	9 ± 4	8 ± 4	10 ± 3
S=32	S=57	S=32	S=51	S=42	S=29	S=18
D - CSF-cN Distance to Central canal (µm)						
Med	**rCerv**	**mCerv**	**cCerv**	**Tho**	**Lum**	**Sac**
56 ± 28	47 ± 25	52 ± 29	33 ± 22	22 ± 19	18 ± 16	26 ± 16
n=130	n= 373	n=321	n=469	n=209	n=154	n=171
S=32	S=57	S=32	S=51	S=42	S=29	S=18
S=10 to 22				S=10 to 24		

Med: medulla; r, m and c Cerv: rostral, mid and caudal Cervical; Tho: Thoracic, Lum: Lumbar and Sac: Sacral
Animals: 2 for Med or Sac 3 for all other segments; S: Number of sections; n: number of cells. Data as mean ± SD; *Non-linear multi effect* (nlme) statistical test.

Figure 1 Kastner *et al.*, 2023

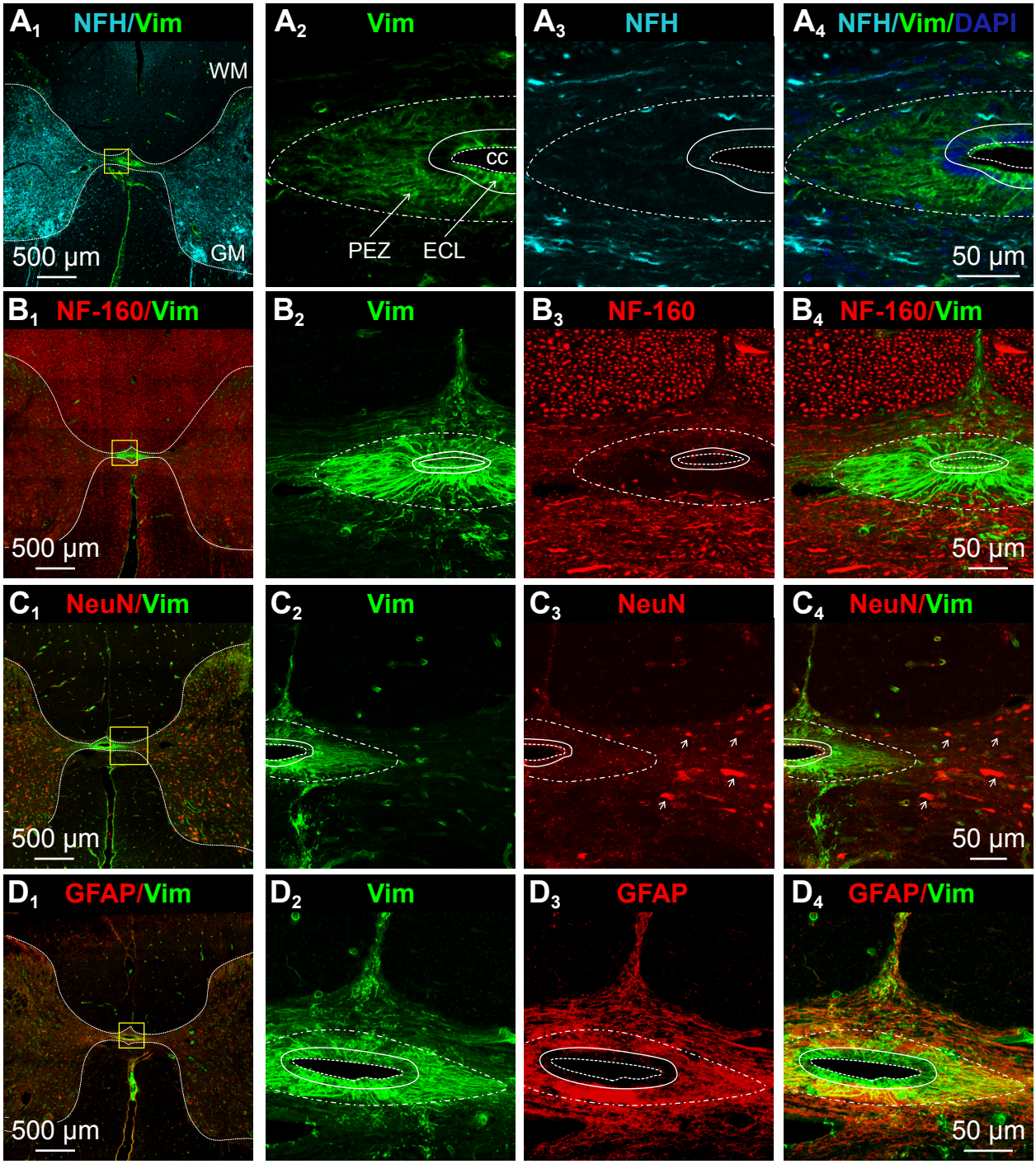


Figure 2 Kastner *et al.* 2023

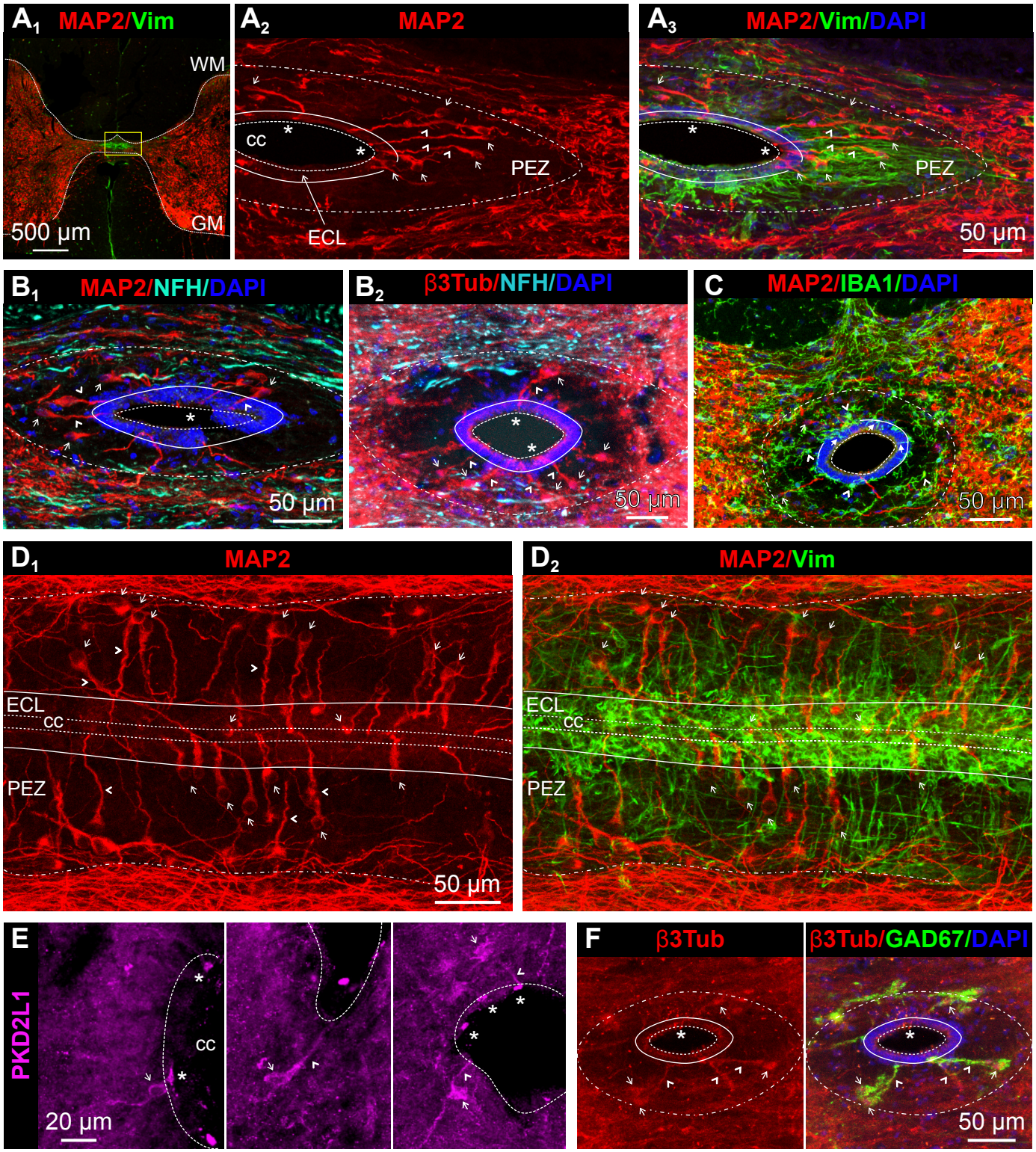
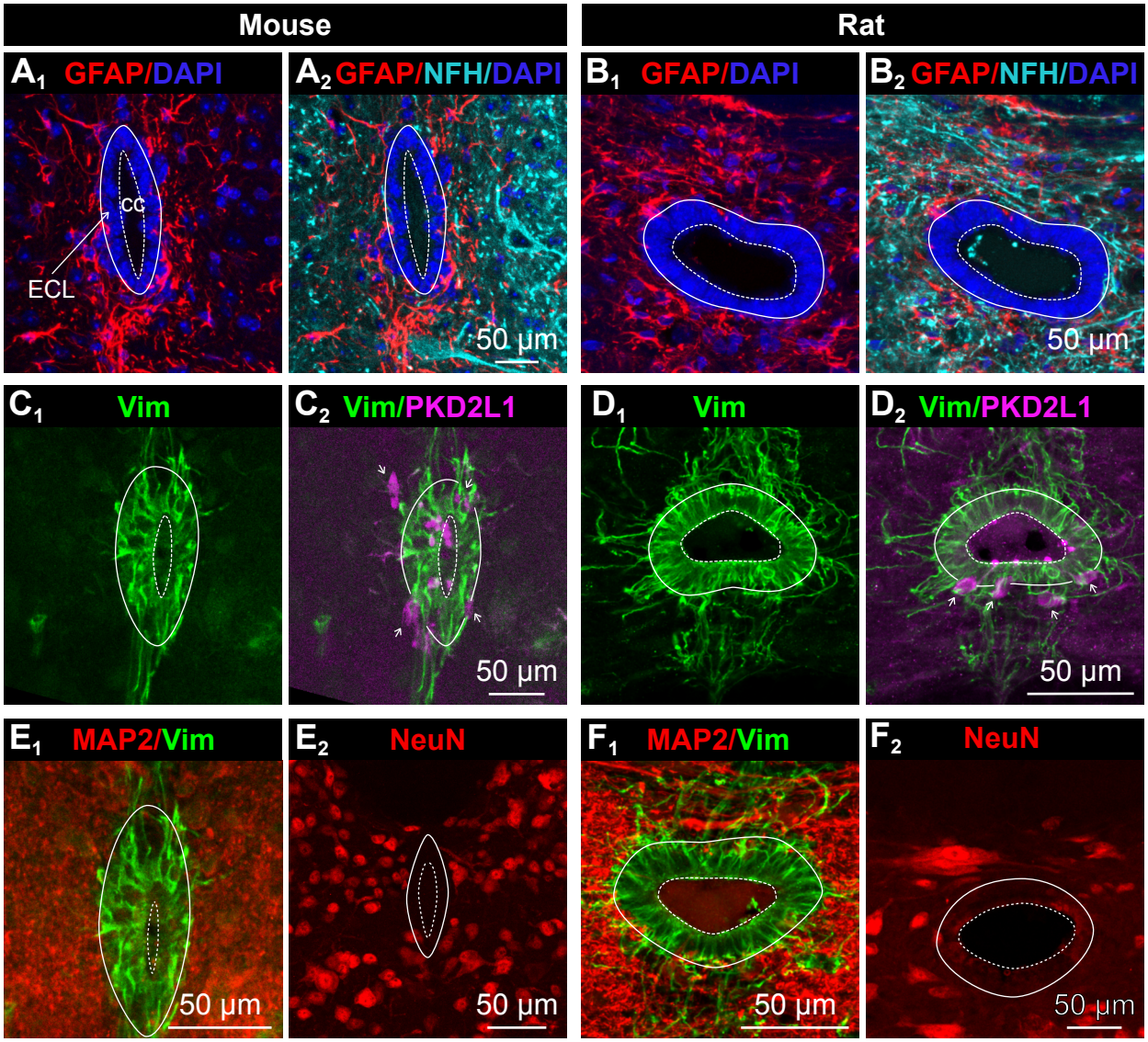


Figure 3 Kastner et al. 2023



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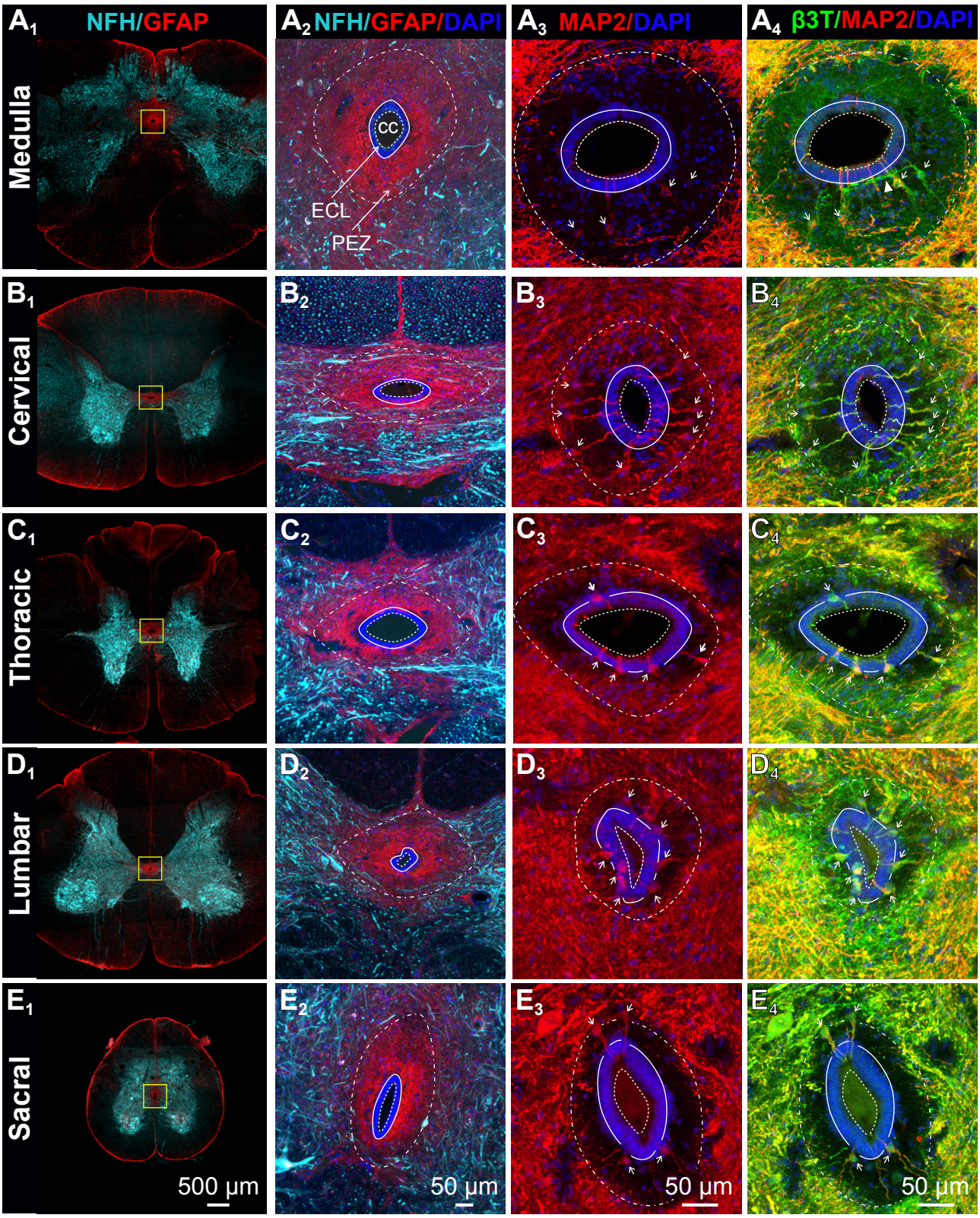

Figure 5 Kastner et al. 2023

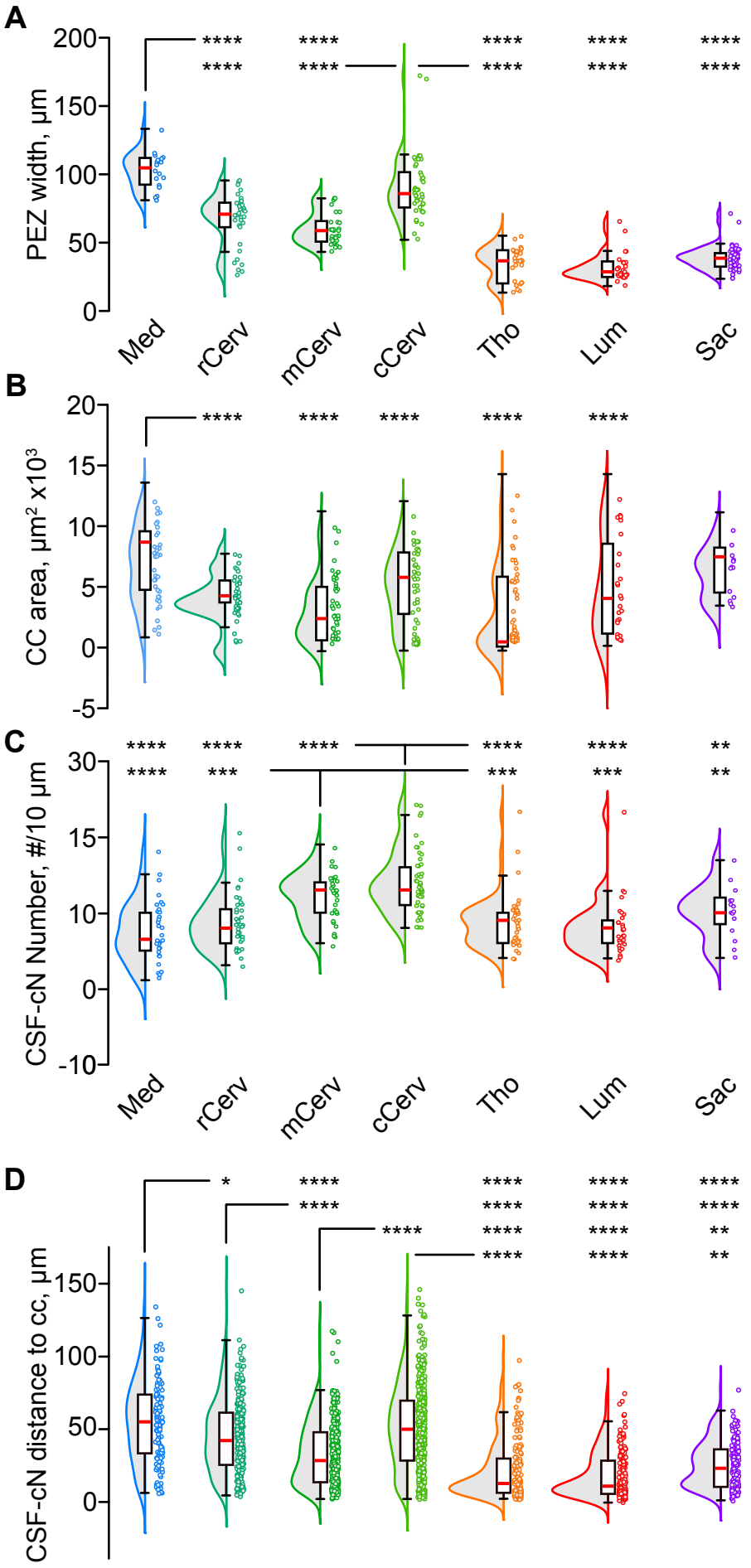

Figure 6 Kastner et al. 2023

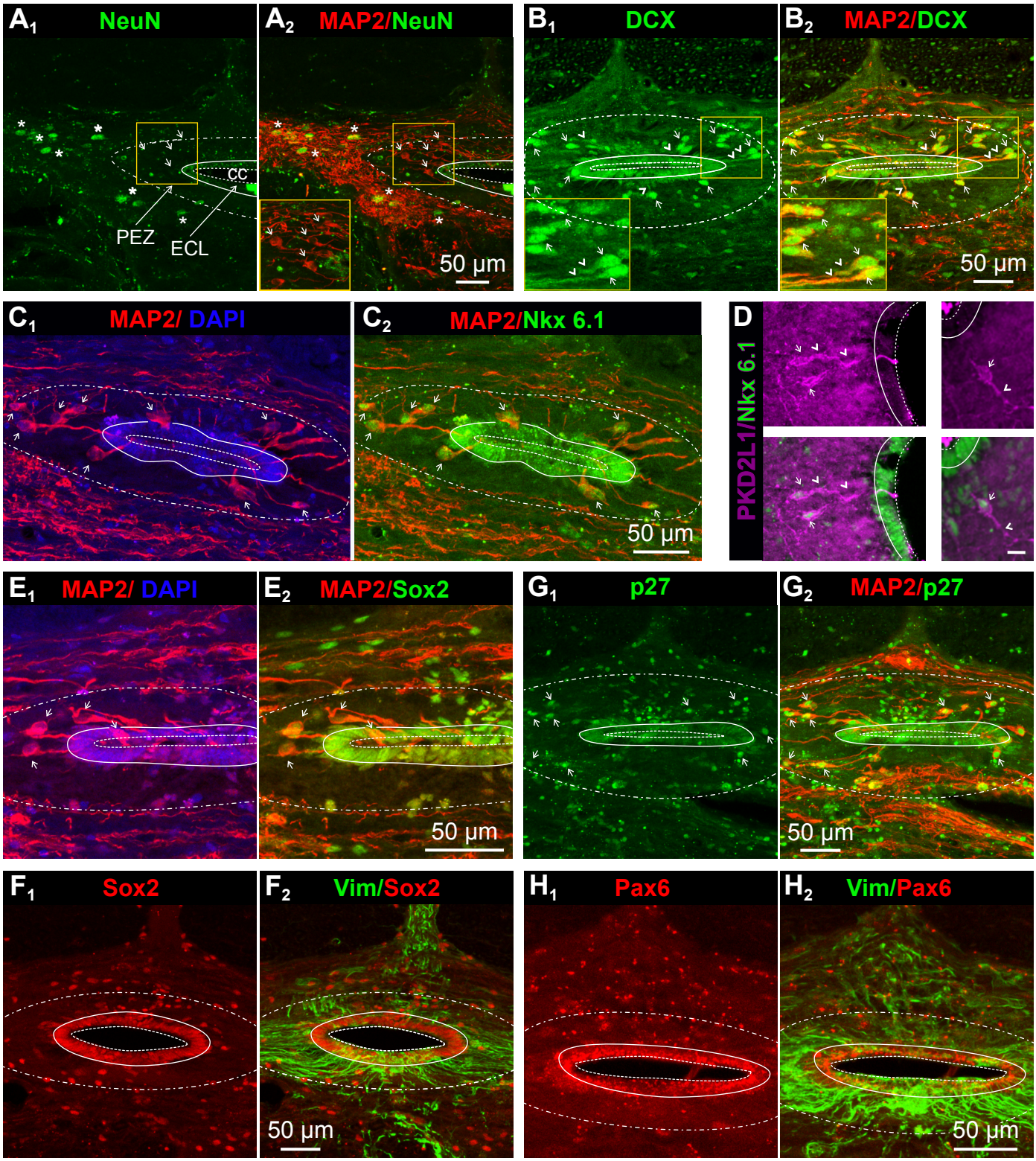

Figure 7 Kastner et al. 2023

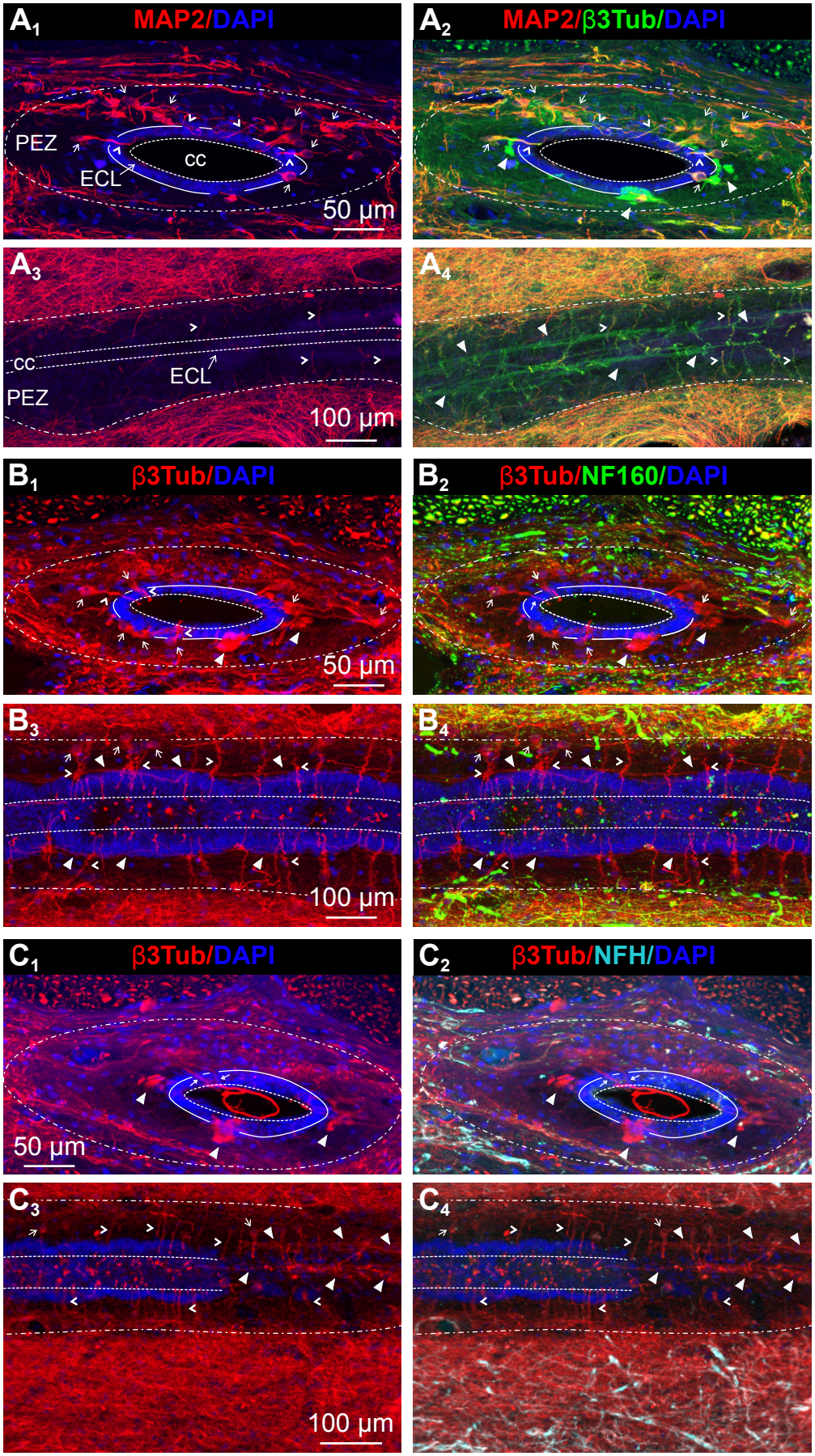
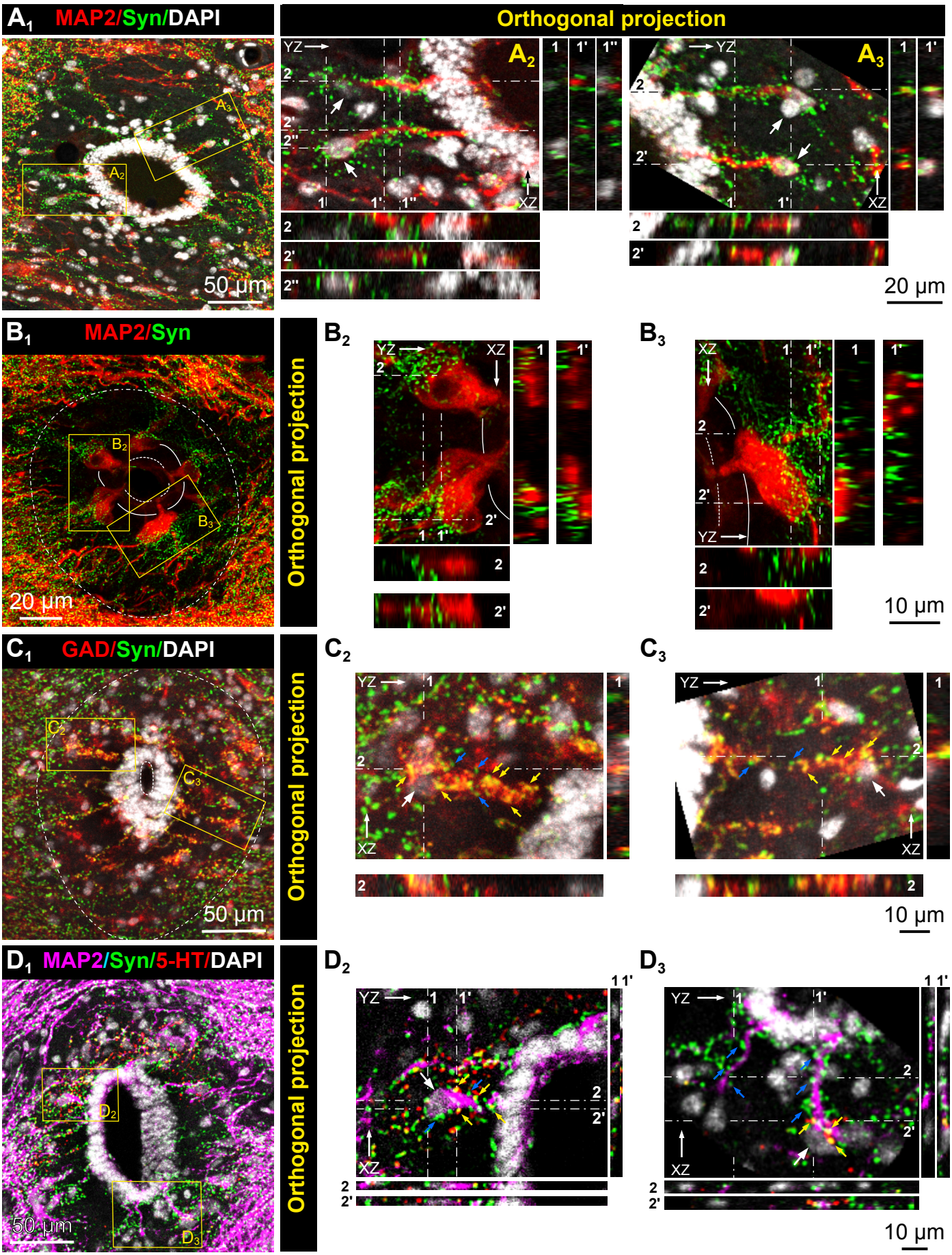
Figure 8 Kastner et al. 2023