The spinal precerebellar nuclei: calcium binding proteins and gene expression profile in the mouse

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Abstract [1]

We have localized the spinocerebellar neuron groups in C57BL/6J mice by injecting the retrograde neuronal tracer Fluoro-Gold into the cerebellum and examined the distribution of SMI 32 and the calcium-binding proteins (CBPs) calbindin-D-28K (Cb), calretinin (Cr), and parvalbumin (Pv) in the spinal precerebellar nuclei. The spinal precerebellar neuron clusters identified were the dorsal nucleus, central cervical nucleus, lumbar border precerebellar nucleus, lumbar precerebellar nucleus, and sacral precerebellar nucleus. Some dispersed neurons in the deep dorsal horn and spinal laminae 6-8 also projected to the cerebellum. Cb, Cr, Pv, and SMI 32 were present in all major spinal precerebellar nuclei and Pv was the most commonly observed CBP. A number of genes expressed in hindbrain precerebellar nuclei are also expressed in spinal precerebellar groups, but there were some differences in gene expression profile between the different spinal precerebellar nuclei, pointing to functional diversity amongst them.

Key words: Spinal cord - Calcium binding proteins - Precerebellar nucleus – SMI 32 - Genes

1. Introduction

The cerebellum receives afferents from many sources in the hindbrain and spinal cord, collectively known as the precerebellar nuclei [10,28]. In the spinal cord, the dorsal nucleus (D) in T1 to L3, and central cervical nucleus (CeCv) in C1 to C4 are the largest spinal precerebellar nuclei [12]. The lumbar precerebellar nucleus (LPrCb) is present in upper lumbar segments [12,30] and Stilling's nucleus [29] (the sacral precerebellar nucleus - SPrCb)

[1] **List of abbreviations:**

2Cb-10Cb, Lobules 2-10 of the cerebellar vermis; C, cervical; Cb, Calbindin-D-28K; CBPs, Calcium binding proteins; CC, Central canal; CeCv, Central cervical nucleus; Cr, calretinin; Crus1, Crus1 of the ansiform lobule; Crus2, Crus2 of the ansiform lobule; Co, coccygeal; D, Dorsal nucleus; DD, Deep dorsal horn; FG, Fluoro-Gold; IC, Inferior colliculus; L, lumbar; LBPr, Lumbar border precerebellar cells ; LPrCb, Lumbar precerebellar nucleus; PFl, Paraflocculus; PM, Paramedian lobule; Pv, Parvalbumin; S, sacral; Sim, Simple lobule; SMI 32, Neurofilament H non-phosphorylated; Sp6-8, Spinal laminae 6-8; Sp7; SPrCb, Sacral precerebellar nucleus; T, thoracic.

is in sacral segments. Cerebellar projecting neurons in the lateral part of laminae 7-9 in lumbar segments [12] are called the lumbar border precerebellar cells (LBPr). A few cerebellar projecting neurons are dispersed in laminae 4-6 (deep dorsal horn neurons, DD) and laminae 6-8 (Sp6-8 neurons) [15].

Electrophysiological studies on CeCv and D have been reported [11,15,24], but there are few reports on the location of spinal neurons that project to the cerebellum, and none on their calcium binding proteins (CPBs) and patterns of gene expression. Calbindin (Cb), calmodulin, calretinin (Cr), and parvalbumin are involved in cell signaling, calcium uptake and transport, cell motility, and intracellular calcium acceptance [2,22,25]. Pv is often associated with GABAergic neurons in the forebrain, whereas Cb and Cr are associated with both excitatory and inhibitory neurons [5,16,26]. Pv-positive neurons are mostly Golgi type II interneurons, but Cb-positive neurons are primarily Golgi type I [6]. We have in addition used a neurofilament marker (SMI32) that is frequently used to define anatomical boundaries in the brain, and which is present in the hindbrain precerebellar nuclei [21,23].

CBPs and SMI 32 are present in the hindbrain precerebellar nuclei of the mouse [31] and rat [23]; the lateral reticular, pontine, and inferior olive nuclei were found to contain Cb, Pv, and SMI, whereas the external cuneate and reticulotegmental nuclei contained Pv and SMI only. CBP expression has been studied in the spinal cord of the mouse [14], rat [25], cat [3], and human [8], but the distribution of CBPs in the spinal precerebellar nuclei has not been specifically reported in any mammalian species.

Virtually all neurons in the hindbrain mossy fiber precerebellar nuclei of the mouse express a number of genes in common (*B2m, Depdc6, Dgat2, Zic1, Ctsz,* and *Slc17a7*) [9]. However,

we are unaware of a comparable report of gene expression in the spinal precerebellar nuclei of any mammal.

In this study we have investigated the presence of CBPs and SMI in the spinal precerebellar neurons using retrograde tracer injections in the cerebellum. We have also compared the expression of genes in the hindbrain and spinal precerebellar nuclei.

2. Materials and methods

2.1. Animals and surgery

We studied C57BL/6J mice (8-10 weeks, 21-25 g, n=13). All protocols were approved by the UNSW Animal Care and Ethics Committee (approval number 11/75A).

Mice were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) for surgery. A pressure injection of Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) in 5% in distilled water (100 nl) was made into the cerebellar vermis, and another in the right hemisphere (Fig. 1a) using a Hamilton syringe guided by a Kopf stereotaxic instrument.

<Figure 1>

On the 7th day after surgery, mice were euthanized and perfused with 4% paraformaldehyde. Spinal cords were post-fixed for 4 h, cryoprotected in 30% sucrose, and 40 μm transverse sections were cut with a Leica CM 1950 cryostat. Selected sections were mounted and reacted with antibodies.

2.2. Immunofluorescence and image analysis

The sections were incubated in a blocking solution containing 1% bovine serum albumin and 10% goat serum for 1 h, followed by incubation with primary antibodies (see details in Table 1) for 48 h at 4°C. Subsequently, sections were treated with fluorescent conjugated secondary antibodies (Table 1) for 2 h at room temperature, and coverslipped with fluorescent mounting medium. The specificity of the antibodies was determined by comparison with that from earlier publications [23,31], and by reacting the tissue with normal serum instead of specific primary antibodies. No positive reaction was observed in any of the sections for which the primary antibodies were not used.

<Table 1 >

Spinal cord sections were examined for FG signal and immunofluorescence using a fluorescent microscope (Olympus, BX51). Images were captured using an attached camera (Zeiss, AxioCam HRc). The locations of cell clusters were identified using the mouse spinal cord atlas [30]. Segments are identified as cervical (C), thoracic (T), lumbar (L), sacral (S), and coccygeal (Co).

2.3. Cell counting and measurement

The number of FG-labeled neurons was assessed in every fourth section from a case with extensive retrograde labeling (Fig. 1.b1-b4). The number of FG-labeled cell profiles was counted and the total corrected with the Abercrombie formula [1], using average cell thickness measured in Nissl stained cells from the same region in each case.

The proportion of FG-labeled neurons that also contained Cb, Cr, Pv, or SMI 32 immunoreactivity was determined by two observers simultaneously viewing a microscope monitor using AxioVision 4.6.3. The cell was recorded as co-labeled only when the two observers agreed.

Images of FG-labeled neurons in the spinal precerebellar nuclei were captured at the focus level where cells showed the largest diameter. Neurons within each region of interest were randomly selected (n=15-49 depending on the size of neuronal population) for the measurement of the largest diameter of the neuronal soma (Image J, NIH). The results are presented as mean ± S.E.M.

2.4. Analysis of gene expression

Using the website of The Allen Institute for Brain Science (http://www.brain-map.org/, on 08-16/01/12) [17], we examined the spinal expression patterns of genes previously found in the hindbrain mossy fiber precerebellar nuclei (*B2m, Depdc6, Dgat2, Lgals1, Zic1, Ctsz,* and *Slc17a7*) [9] and in the inferior olive (*Rgs4, Zfp533, Pou4f,* and *Stac2*).

3. Results

Retrograde labeled neurons were found in the dorsal nucleus (lamina 5 in T1-L3), CeCv (lamina 7 in C1-C4), LBPr (laminae 7-9 in L1-L6), LPrCb (lamina 7 of the L1-L6), and in SPrCb (lamina 7 in S1-Co3). Labeled neurons were scattered through the deep dorsal horn and Sp6-8 throughout the spinal cord. The dorsal nucleus contained the largest population of cerebellar projecting neurons.

FG-labeled neurons ranged in size from 14.0 to 39.5 μm (Fig 2). The neurons were mostly oval, piriform, or multipolar, except that the majority of D neurons were spindle-shaped (Figs. 3, 4).

<Figures 2 and 3>

Cb-, Cr-, Pv-, and SMI 32-immunoreactivity was found in all spinal precerebellar nuclei. Pv

positive cells were frequently co-localized with retrograde tracer, but the proportion of Cb- or Cr-labeled neurons that projected to the cerebellum varied from one spinal precerebellar nucleus to another. SMI 32 was often co-localized with the retrograde tracer (Table 2).

<Table 2>

<Figure 4>

All of the genes of interest (*B2m, Depdc6, Dgat2, Lgals1, Zic1, Ctsz, Slc17a7 Rgs4, Zfp533,* and *Stac2)* were expressed in D. *Rgs4*, *Stac2,* and *Zfp533* were expressed in all spinal precerebellar nuclei, whereas *Pou4f1* was only expressed in CeCv and D (Table 3).

<Table 3>

4. Discussion

4.1. CBP and SMI 32 expression

The expression of CBPs and SMI in the spinal precerebellar nuclei is similar to that seen in majority of the hindbrain precerebellar nuclei. All spinal cord precerebellar nuclei expressed three types of CBPs (Cb, Cr and Pv) and SMI 32. In the rat, Pv, Cr, and calmodulin are present in CeCv [25], and Pv and Cb neurons are widespread in the spinal gray matter [4]. We found Pv to be the most common CBP in the mouse spinal precerebellar nuclei. Pv is present in inhibitory neurons in some parts of the brain [5,16,26], and is associated with glycine inhibitory neurons in the spinal cord [13], implying that Pv might signal that spinal precerebellar neurons are inhibitory. However, Pv is also present in the hindbrain precerebellar nuclei, which are glutamatergic and excitatory, making it unlikely that Pv is an

indicator of inhibitory function in spinal precerebellar neurons. A substantial number of cerebellar projecting neurons expressed SMI 32. SMI 32 is related to the maintenance of large neurons with myelinated axons [21], consistent with its presence in the long myelinated axons of the spinocerebellar tracts.

CeCv neurons in the rat were both excitatory and inhibitory [24]. In the present study, the vast majority of CeCv precerebellar neurons contained Pv, but as noted above, we cannot assume that Pv precerebellar neurons are inhibitory. The dorsal nucleus contained a substantial proportion of cerebellar projecting neurons expressing Pv (77%), Cb (38%), or Cr (42%). Cr expression in D was previously reported in the mouse [30].

The majority of precerebellar neurons in SPrCb express Pv, and many of them express Cr. This pattern differs from that of CeCv, which could indicate a functional difference. By contrast, Edgley and Grant [7] showed that SPrCb and CeCv neurons are similar – both are monosynaptically excited by group I muscle afferents. Precerebellar neurons in LPrCb and SPrCb contain similar proportions of different CBP expressing cells. LPrCb and SPrCb are continuous rostrocaudally, and they may also be functionally homogeneous.

Finally, we found that more than half of the cerebellar projecting neurons in LBPr expressed Pv, Cr, or Cb, suggesting this nucleus may have diverse functions.

4.2. Gene expression in the mouse spinal precerebellar nuclei

Rgs4, *Stac2*, and *Zfp533* were expressed in the major hindbrain precerebellar nuclei and all the spinal precerebellar nuclei. *Depdc6* was present in all the mouse spinal precerebellar nuclei, with the exception of CeCv. *Dgat2*, and *Pou4f1* that were found only in CeCv and D. *Slc17a7*, which is present in all major hindbrain mossy fiber precerebellar nuclei, was found

only in D. Because they have similar gene expression profiles, the hindbrain and spinal precerebellar nuclei presumably have many functional similarities. However, the variation in gene expression among the spinal precerebellar nuclei raises the possibility of biological differences.

4.3. New anatomical data on the extent of the spinal precerebellar nuclei

The LPrCb was identified by Watson et al. [30] in upper lumbar segments in the mouse (L1-L3) and a similar nucleus was identified in rats in a retrograde tracing study [18]. In the present study, we found that LPrCb neurons were present throughout the lumbar spinal cord (L1-L6). SPrCb has previously been found in S1-S4 segments [20, 30], but we found that the mouse SPrCb extended from S1 to Co3.

4.4. The functions of the mouse spinal precerebellar nuclei

The hindbrain precerebellar nuclei are involved in a variety of functions, including sensory processing, motor functions, and control of respiration [27]. Our CBP results indicate there may be variation in function among the spinal precerebellar nuclei. CeCv neurons receive primary afferent inputs from the labyrinth and muscle spindles of deep dorsal neck muscles, and project to the contralateral cerebellum [12], whereas the dorsal nucleus relays proprioceptive sensory signaling from the ipsilateral hindlimb [11]. SPrCb neurons in the S2-S3 spinal cord segments receive proprioceptive afferents from muscle spindles [12] and are involved in spatial orientation of the tail [19]. The functions of LPrCb and LBPr are not known.

Conflict of interest

The authors state no actual or potential conflict of interest.

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Figure Legends

Fig. 1. Cerebellar injection sites (a) and regions infiltrated with FG (b).

Fig. 2. Maximum diameter of precerebellar neurons (a); markers expressed indifferent sized neurons as % (b).

Fig. 3. Labeled neurons in spinal cord after FG cerebellar injections (a); neurons in the deep dorsal horn (DD) and laminae 6-8 (b).

Fig. 4. Co-localization of Cb, Cr, Pv, and SMI with FG retrograde label. On the left are typical locations of precerebellar nuclei (a1-d1). On the right are Cb, Cr, Pv, and SMI neurons, and their co-localization with FG (a2-5, b2-5, c2-9, d2-5). Co-localized neurons are indicated with arrows.

Table Legends

Table 1 Primary and secondary antibodies for detection of CBPs and SMI.

Table 2 The number of FG-labeled neurons (column a), and the percentages of double-labeled neurons (columns b).

Table 3 Gene expression in the hindbrain and spinal precerebellar nuclei.