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Characterization of Calbindin Positive Interneurons within the Ventral Horn of the Mouse Spinal Cord

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Introduction

Sensory-motor circuits in the spinal cord integrate sensory feedback from muscles and modulate locomotor behavior. Although we know how the sensory-motor system generally works, the main issue lies in identifying all neurons involved and understanding their interrelationships. Many interneurons contribute to sensory-motor circuits and have been well studied. For example, Renshaw cells (RC) are inhibitory interneurons that prevent motor neurons from over-activity. A distinguishing feature of RCs is that they are the only interneurons within the ventral-most region of the spinal cord expressing the calcium binding protein calbindin (CB). Recent studies have found other subpopulations of ventral horn interneurons outside of the RC area that express CB, but knowledge regarding the function and connectivity of these neurons is limited. We hypothesize CB expression serves a functional purpose for ventral horn interneurons and as well as identifying RCs. Here we compare known characteristics of RCs with other ventral horn interneurons that express CB. We analyze anatomical location; cellular density; expression of neurotransmitters; motor neuron and sensory afferent contacts; expression of calcium binding proteins CB, calretinin and parvalbumin; and premotor neuron identification. RCs are found in the ventral-most region of lamina VII medial to motor neuron pools while other CB positive interneurons are located lateral to pools as well as upper regions of lamina VII close to the central canal. On average, there are ~1.5 RCs per 100µm segment of the spinal cord while other CB positive interneurons are found less frequently (~0.5 cells per 100µm). Although anatomical location and density can indicate functional differences, fractions of the RCs and CB positive neuron populations both utilize glycine as their neurotransmitter and co-express CB and calretinin. Further experiments may suggest CB has a more involved role in spinal interneurons, providing a deeper understanding of the sensory-motor circuit within the CNS.

Table 1. Ventral Horn CB Positive Interneuron Characteristic Checklist

	Renshaw Cells	Big Calbindin (BCB) Cells
Lineage	V1 progenitor group	V1 progenitor group
Neurotransmitter(s)	Glycine, GABA	?
Motor neuron contact	Yes	?
Sensory afferent contact	Yes (until ~P15)	?
Calcium binding proteins	CB, calretinin, parvalbumin	CB, calretinin
Premotor neuron	Yes	?

Methods

Spinal Cord Tissue Preparation

GlyT2-GFP and wild type (WT) mice at ages P7 (post natal day 7), P14, P28 and P45 (n=3-4 per age group) were anesthetized and perfused transcardially with 10ml of phosphate buffered saline (PBS) solution followed by 20ml of 4% paraformaldehyde. For P0 and P7 pups, anesthesia was induced by exposure to ice cold water while P14, P28 and P45 mice received 0.03ml/l, 0.04ml/l and 0.05ml/l of Euthasol respectively. After perfusing, the lumbar spinal cord was dissected and placed in 4% paraformaldehyde for 24 hours then 30% sucrose for another 24 hours. The lumbar region was divided into three sections (thoracic 13- rostral lumbar 3, caudal lumbar 3- rostral lumbar 5, caudal lumbar 5- sacral 1) and frozen in tissue tek for 24 hours. After freezing the tissue, cords were cut on a cryostat into 20µm thick sections.

Immunohistochemistry

GlyT2-GFP sections were stained for calbindin to determine whether CB positive neurons used glycine as their neurotransmitter as well as analyzing the anatomical location and density of CB positive interneurons. WT sections were triple immunolabeled with CB, calretinin and parvalbumin antibodies to test if CB positive cells also co-expressed calcium binding proteins calretinin and parvalbumin. The primary antibodies used and their details are found in Table 2. All primary antibodies were revealed with the appropriate fluorescent donkey anti rabbit/goat/mouse antibody.

Table 2. Primary Antibodies Used in This Study

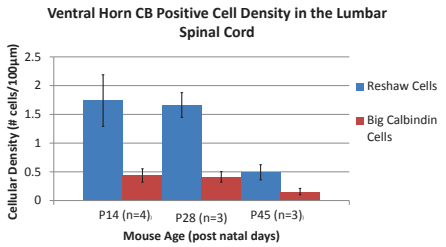
Antibody Name	Type	Host Species	Dilution	Company	Labeling Specificity
Calbindin D28-K	polyclonal	rabbit	1:1000	SWANT	RCs, other CB positive neurons, sensory dorsal neurons
Parvalbumin	polyclonal	goat	1:9000, prediluted at 1:20 with PBS	SWANT	Proprioceptive afferent axons, IN subsets
Calretinin	polyclonal	mouse	1:5000	Zymed	Proprioceptive afferent axons, IN subsets

Image Analysis

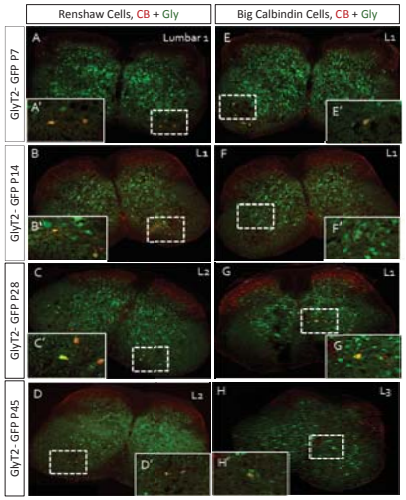
GlyT2-GFP and WT stained sections were observed under the Olympus Epi Fluorescence Spot Microscope and confocal images were taken on the Olympus FV300 scope. Z-stack images were layered over one another to determine CB-glycine and CB-parvalbumin- calretinin overlap. Images were flattened using the Olympus FLOVIEW 1.7 software and adjusted on the Adobe Photoshop CS3 program.

Results

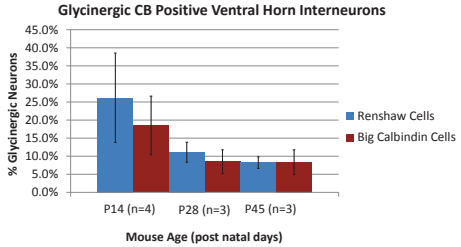
~4X more RCs are found in the lumbar cord than BCB cells.



Both RCs and BCB cells are glycinergic.

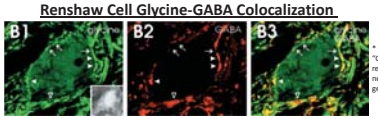


The percentage of glycinergic CB positive neurons decreases with age.



What about CB positive GABAergic neurons?

Renshaw Cell Glycine-GABA Colocalization



* Confocal image taken from Alvarez 2005 "Glycine and GABAA receptor subunits on renshaw cells: relationship with presynaptic neurotransmitters and postsynaptic gephyrin clusters"

- RCs highly express GABA in the embryonic spinal cord but by birth, many RCs switch from GABA to glycine (*Alvarez et al. (2005). Postnatal phenotype and localization of spinal cord V1 derived interneurons. J. Comp. Neurol. 493, 177-192.*)
- Many RCs are surrounded by GABA receptors throughout development but hardly any express GABA within the soma
- Therefore, we believe it is highly improbable for any RCs in this study to express GABA throughout post natal development.
- If BCBs are shown to express high GABA levels, then the differences in neurotransmitter expression may indicate RCs and BCBs are different cell types.

Conclusions

- Larger amounts of RCs are found in the lumbar spinal cord than BCBs during development.
- After P28, the amount of CB positive neurons in the spinal cord decreases by 29% for RCs and 38% for BCBs.
- Throughout development, the percentage of glycinergic RCs steadily decreases while glycinergic BCB levels stop changing after P28.
- The amount of RCs and BCBs reducing glycine and CB expression may be due to the downregulation of CB in the ventral spinal cord.
- Differences in glycine and GABA neurotransmitter expression may reveal differences between RCs and BCBs.

Future Directions

We will be conducting immunohistochemical experiments to evaluate whether all ventral horn CB positive neurons:

- Express GABA
- Receive motor neuron contacts
- Receive sensory contacts
- Express all three calcium binding proteins
- Act as premotor neurons

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Calcium binding proteins	CB, calretinin, parvalbumin	CB, calretinin, ?
Premotor neuron	Yes	?

Acknowledgements

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