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Quantitative Comparison Of Vesicular Glutamate Transporters in rat Deep Cerebellar Nuclei

Haian Mao,[a] Salah Hamodeh[a] and Fahad Sultan[a,b]*

[a] *Department of Cognitive Neurology, HIH for Clinical Brain Research, Otfried-Müller-Str. 27, 72076 Tübingen, Germany*

[b] *Department of Integrative Medical Biology, Umeå University, Linnéus väg 9, 901 87 Umeå, Sweden*

Abstract—The excitatory synapses of the rat deep cerebellar nuclei (DCN) were quantitatively analyzed by vesicular glutamate transporter 1 and 2 (vGluT1 and vGluT2) immunolabeling. We calculated the number and sizes of the labeled boutons and compared them between lateral/dentate nucleus (LN/DN), posterior interposed nucleus (PIN), anterior interposed nucleus (AIN), and medial nucleus (MN). The density of vGluT1+ boutons differs significantly within these nuclei. In contrast, the vGluT2+ bouton density is more similar between different nuclei. The phylogenetically newer DCN (LN/DN and PIN) have a 39% higher density of vGluT1+ boutons than the phylogenetically older DCN (AIN and MN). The volume of vGluT1+ boutons does not differ between the DCN, however the average volume of vGluT2+ boutons is larger in MN. In summary, our current results confirm and extend our previous findings showing that the increase in dendritic and axonal wiring in phylogenetically newer DCN is associated with an increase in vGluT1+ bouton density.

Key words: vesicular glutamate transporter, quantitative analysis, bouton density, bouton size, deep cerebellar nuclei.

INTRODUCTION

The deep cerebellar nuclei (DCN) are the main output of the cerebellum and have been implicated to play an important role in motor skill learning (De Zeeuw and Ten Brinke 2015). In addition, the evolution and increase in size and shape complexity in primates of one part of these nuclei (the lateral nuclei and in humans termed the dentate nuclei, LN/DN) has led to continued interest and research (Sultan et al., 2010; Bostan et al., 2013; Tellmann et al., 2015; Hamodeh et al., 2017). In our previous work (Hamodeh et al., 2014), we showed that the neuronal wiring is heterogeneous in the four cerebellar nuclei of the rat. Compared to the phylogenetically older (MN and AIN), the newer DCN (PIN and LN/dentate) have a higher length density of dendrites and Purkinje cell axons. In this study, we asked whether the dendritic wiring increase is also accompanied by an increase in the excitatory synaptic density.

The DCN neurons receive the excitatory glutamatergic synapses from mossy and climbing fibers and inhibitory GABAergic synapses from Purkinje cells. The excitatory synapses are divided into two subsets according to different vesicular transporters (vGluT1 and vGluT2) that are utilized within the presynaptic bouton to load glutamate into the vesicles. These transporters are largely colocalized with vesicles at the presynapse (Bellocchio et al., 1998; Takamori et al., 2000, Herzog et al., 2001, Varoqui et al., 2002). These two subgroups are generally expressed in a complementary pattern in the brain (Ni et al., 1994; Ni et al., 1995; Ni et al., 1996; Aihara et al., 2000; Fremeau et al., 2001, Herzog et al., 2001, Kaneko and Fujiyama 2002; Kaneko et al., 2002; Hartig et al., 2003; Hisano 2003, Fremeau et al., 2004a, b). The cerebellum is among the few brain regions that utilize both classes of transporters, with a small subset of presynapses co-expressing both transporters (Hioki et al., 2003). The climbing fibers from the inferior olive only use vGluT2, while mossy fibers from different precerebellar nuclei use vGluT1, vGluT2 or both (Hisano et al., 2002). VGluT3 is only transiently expressed in the cerebellum during early development (Boulland et al., 2004) and is absent in the adult pre-cerebellar nuclei and cerebellum (Gras et al., 2002; Hisano 2003; Costes et al., 2004; Herzog et al., 2004a).

Previous studies have not quantified the excitatory synapses within DCN. These excitatory afferents are from different precerebellar nuclei and terminate on the proximal and distal dendrites of the DCN neurons (Angaut and Sotelo 1973). The Purkinje cell GABAergic synapses terminate on the somata and proximal dendrites (De Zeeuw and Berrebi 1995). It has been reported that 62% in cat (Palkovits et al., 1977) and 73% in rat (De

*Correspondence to: F. Sultan, Dept. of Integrative Medical Biology, Umeå University, Linnéus väg 9, 901 87 Umeå, Sweden.
E-mail address: fahad.sultan@umu.se (F. Sultan).
Abbreviations: AIN, anterior interposed nucleus; DCN, deep cerebellar nuclei; PB, phosphate buffer; PIN, posterior interposed nucleus; vGluT, vesicular glutamate transporter.

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Zeeuw and Berrebi 1995) of the DCN synapses are Purkinje cell GABAergic synapses, leaving the rest 38% to 27% of the synapses belonging to be excitatory synapses. In this paper, we used vGluT1 and vGluT2 antibodies to label these excitatory synapses in the DCN and studied the four sub nuclei systematically. We compared the vGluT1- and vGluT2-labeled synaptic terminals and found significant differences in vGluT1+ bouton density within the DCN.

EXPERIMENTAL PROCEDURES

Tissue preparation

All animal experiments were carried out in accordance with the Society for Neuroscience and German Law (approved by the Regierungspräsidium Tübingen). Brains from three adult rats were used in this study (body weight 170–300 g and age 6–12 weeks). A mixture of ketamin (20 mg/100 g body weight) and xylazine (2mg/100 g body weight) was used to induce a deep anesthesia. The rats were perfused transcardially by 0.1 M PB and then by 4% ice-cold paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The brain was immediately dissected out of the skull and then cryoprotected in an ascending concentration of sucrose (10%, 20%, and 30% in 0.1 M PB). The cerebellum was cut off and mounted on a freezing microtome with the lateral side facing the platform. Sections of 40 μm were serially cut and stored in 0.1 M PB. Immunofluorescence staining was carried out on free floating sections. Before primary antibody incubation, sections were washed in 0.1 M PB for 3 times, 5 min each and then blocked in 0.1 M PB with 10% horse serum (PAA Laboratories, Coelbe, Germany) and 0.3% Triton X-100 at room temperature for 1 h.

Immunofluorescence

The working concentration and incubation times were tested for each antibody in single stains. The optimized conditions were then used for the multiple stains. We performed a quadruple staining protocol for vGluT1, vGluT2, Purkinje Cell Protein 2 (PCP2), and KCNC3 (Kv3.3). Data of the Kv3.3 were used for a subsequent publication (Mao et al., in preparation). The sections were first incubated with goat vGluT1 (sc-13320, Santa Cruz Biotechnology, Texas) at 1:1000 at 4 °C for 16 h, followed by 3x washes in 0.1 M PB and then incubated with donkey anti goat Alexa Fluor 488 at 1:500 for 2 h at room temperature. The sections were then washed again before incubation with the combination of three antibodies of vGluT2 (1:1000, lot number: 135404, synaptic systems, North Saanich British Columbia), Kv3.3 (1:500, lot number: APC-102, Alomone labs, Israel) and PCP2 (1:200, lot number: sc-137064, Santa Cruz Biotechnology, Texas) at 4 °C for 16 h. The sections were then washed again and incubated with the combinations of the three secondary antibodies: goat anti guinea-pig Alexa Fluor 633 (1:500, A21105, Invitrogen, California), goat anti rabbit Cy3 (1:500, lot number: 81-6115, Invitrogen, California) and goat anti mouse Alexa Fluor 405 (1:500, lot number: A31553, Invitrogen, California) for 2 h at room temperature. Sections were washed and mounted with Mowiol 4–88 (Merck, Darmstadt, Germany) in glycerol on glass slides. The slides were stored at 4 °C.

Image acquisition

Images were acquired with the confocal laser microscope (LSM 510, Carl Zeiss, Jena, Germany). We took overview images of vGluT1 staining for every section with 488-nm excitation and emission band pass filtering 505–550 nm at low magnification of 10× and probe locations were determined by first marking an identifiable origin (x, y coordinates: 0,0) and then determining the other probes location in the DCN region. These were taken at regular intervals of 250 μm × 250 μm from two rats and at 200 μm × 200 μm for the third rat. The location of the origin point was chosen from an easily identifiable structure (i.e., vessels) within a core region of the DCN, however, this location differs from slice to slice and is not related to the structures to be analyzed. For each probe, a z-stack was acquired under the 63× magnification, with a 2× zoom. The fluorophores were excited with 405 nm, Argon or HeNe lasers. We stained and analyzed 16 cerebellar sections (5–6 slices from each rat) and 189 probes were sampled in total (44 from the first rat, 55 from the second rat and 90 from the third rat). Details of antibodies, excitation wavelength and emission filters are listed in Table A.1 (see Appendices).

We also determined tissue shrinkage by taking four random positions within the DCN region for each slice and determining the upper and lower section boarders by the staining signal for vGluT1 at a magnification of 20× dry objective. 64 probes were taken together in 3 rats (16 slices in total). The mean thickness of the slices after mounting was 26um, while the original sectioning thickness was 40um. This corresponds to a shrinkage to 65%, less than what we have observed (to 36%) in our previous studies (Hamodeh et al., 2014).

Classification of the rat DCN was based on the description in the previous paper (Hamodeh et al., 2014).

Data preprocessing

Data were acquired as 8-bit format and exported as such in Zeiss lsm format. Stacks were then deconvoluted using the iterative (n = 10) "blind deconvolution" of AutoQuant X3 (Media Cybernetics, Bethesda, MD) with the maximum likelihood estimation and constrained iteration. After deconvolution data were saved in Autoquant to 12-bit Zeiss tiff format. Data were imported and intensity range adjusted in Imaris (Bitplane AG, Switzerland) to unsigned 8-bit format.

The four antibodies showed slightly different tissue penetration abilities. Therefore, the deconvoluted image stacks were manually cropped in Imaris to eliminate the upper and lower slices that were not well stained. We performed a within voxel colocalization analysis in Imaris using Pearson's coefficient (Costes et al., 2004) and with a threshold of 50/255 for all four channels.