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GABAergic SYNAPTIC COMMUNICATION IN THE GABAergic AND NON-GABAergic CELLS IN THE DEEP CEREBELLAR NUCLEI

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Abstract—The deep cerebellar nuclei (DCN) are the final integrative units of the cerebellar network. The strongest single afferent to the DCN is formed by GABAergic Purkinje neuron axons whose synapses constitute the majority of all synapses in the DCN, with their action strongly regulating the intrinsic activity of their target neurons. Although this is well established, it remains unclear whether all DCN cell groups receive a functionally similar inhibitory input.

We previously characterized three types of mouse DCN neurons based on the expression of glutamic acid decarboxylase isoform 67 (GAD67), their active membrane properties and morphological features. Here we describe the GABAergic synapses in these cell groups and show that spontaneous GABAergic synaptic activity can be seen in all three cell types. Since the majority of DCN neurons fire action potentials spontaneously at high frequencies both *in vivo* and *in vitro*, we expected that spontaneous GABAergic synaptic activities mediated by intra-DCN synaptic connections could be uncovered by their sensitivity to TTX. However, TTX had little effect on spontaneous synaptic activity. It seems, therefore that functional GABAergic connectivity within the DCN is sparse and/or weak at least under our experimental conditions. Even though present in all cell types, the spontaneous GABAergic events showed significant differences between the cell types. The synaptic currents in GABAergic cells had lower amplitude, lower frequency and slower kinetics than those of non-GABAergic cells. These differences could not be sufficiently explained by considering only cell size differences or a differential $GABA_A$-receptor α-subunit composition. Rather, the main differentiating factor appears to be the dendritic localization of GABAergic synapses in the GABAergic cells.

Key words: cerebellar nuclei, synaptic transmission, GABAergic.

The deep cerebellar nuclei (DCN) reside at a key location within the cerebellar network. All of the afferent pathways to the cerebellar cortex make collateral connections on to neurons of the DCN, while the main output of the cerebellum is formed by the DCN projection neurons. Despite this anatomical knowledge, the role of DCN in cerebellar computation is largely unclear or controversial. A classic view was that the DCN simply acts as a "relay station" between cerebellar mossy fiber (MF) input and cerebellar output to premotor areas, either directly ("direct pathway") or via the cerebellar cortex ("indirect pathway"). This concept has been challenged by hypotheses which suggest the DCN may act as the substrate of motor memory storage (Lisberger and Sejnowski, 1992; Wada et al., 2007; Ito, 2006). The demonstrations of mechanisms which cause modification of synaptic strength (Telgkamp and Raman, 2002; Aizenman et al., 1998) and active membrane properties (Aizenman and Linden, 2000) support this latter viewpoint. It has also been proposed that "cerebellar memory" is acquired in the cerebellar cortex and subsequently transferred to the DCN for consolidation (Mauk, 1997; Ohyama et al., 2006; Masuda and Amari, 2008; see also Jirenhed et al., 2007). In addition to these possible roles in cerebellar memory, it appears likely that the neuronal network within the DCN processes sensory and motor information in "real-time" but very little is known about the computational functions of the DCN, particularly with respect to the role of specific cell types. Indeed, morphological and electrophysiological studies have revealed that the DCN consists of diverse neuronal populations (Chan-Palay, 1977; Chen and Hillman, 1993; Aizenman et al., 2003; Czubayko et al., 2001; Molineux et al., 2006; Uusisaari et al., 2007), with both glutamatergic and GABAergic neurons forming projecting and local connections (Teune et al., 1995).

We have previously defined three types of DCN neurons based on their transmitter content and functional properties (Uusisaari et al., 2007). Importantly, all of the cells studied fired action potentials spontaneously in the absence of synaptic input. The large non-GABAergic neurons (GADnL; presumed excitatory and glutamatergic projecting neurons) are specialized for conveying tonic spiking activity due to their relative linear and non-adaptive current-frequency relationship. Conversely, the smaller non-GABAergic (GADnS; presumed glutamatergic) and GABAergic (GAD+) neurons exhibit a more pronounced frequency adaptation and lower maximal frequencies during sustained firing. These appear to be more suited for processing phasic signals (Uusisaari et al., 2007).

At present, it is generally assumed that all DCN cells are uniformly contacted by Purkinje neuron synapses that mainly reside on their soma or proximal dendrites (Chan-Palay, 1977; De Zeeuw and Berrebi, 1995) whereas the glutamatergic afferent input from MFs and climbing fibers (CF) is conveyed via more distal synapses, away from the soma (Chan-Palay, 1977). In addition, non-projecting (lo-

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Abbreviations: ACSF, artificial cerebrospinal solution; CF, climbing fiber; DCN, deep cerebellar nuclei; EPSC, excitatory post-synaptic current; GAD, glutamic acid decarboxylase isoform 67; GADnL, large non-GABAergic neuron; GADnS, small non-GABAergic neuron; GFP, green fluorescent protein; IO, inferior olive; IPSC, inhibitory post-synaptic current; K-S, Kolmogorov-Smirnov; MF, mossy fiber; NBQX, 6-nitro-7-sulfamoylbenzo(f)-quinoxaline-2,3-dione; PiTX, picrotoxin; PSC, post-synaptic current; SB, SB-205384; TTX, tetrodotoxin; τ, time constant.

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cal) DCN cells are known to exist (Chan-Palay, 1977; Wassef et al., 1986) but the properties of local DCN synapses are even less understood than those formed by the extrinsic axons.

The topography of the corticonuclear projection makes studying the connections between individual Purkinje and DCN neurons in slices difficult, since the majority of the axons between cerebellar cortex and nuclei are cut during slicing. Previous studies on inhibitory synapses in the DCN have therefore been conducted using spontaneous inhibitory post-synaptic potentials (IPSCs) as an indirect measure for cortical input to the nuclei, or on evoked IPSCs via white-matter stimulation (Anchisi et al., 2001; Ouardouz and Sastry, 2006; Pedroarena and Schwarz, 2003). However these studies targeted only loosely-defined cell groups and no information is available on whether any of the synaptic properties differ between glutamatergic and GABAergic DCN neurons. A recent study (Alonso-Espinaco et al., 2008) explored the expression of Kv3 potassium channels on GABAergic and glutamatergic synaptic terminals on the glutamic acid decarboxylase isoform 67 (GAD) positive and negative cells of the DCN, but the functional properties of those synapses are so far unclear.

In the preset study we take advantage of the green fluorescence protein (GFP)-labeling of GABAergic cells in the GAD67-GFP mouse line (Tamamaki et al., 2003) as well as the previously-described division into GADnL, GADnS and GAD+ cells to address this issue, and find that the properties GABAergic synapses in GAD+ and GAD− cells are, in fact, very different.

EXPERIMENTAL PROCEDURES

Slice preparation

The acute DCN slice is a notoriously difficult preparation that allows systematic patch-clamp studies in juvenile animals only. Young mice between postnatal days 14 and 21 from a GAD67-GFP knock in line (Tamamaki et al., 03) were anesthetized with halothane and decapitated. To ensure that all animals had reached the same minimal developmental stage, only animals with fully open eyes were used. The cerebellum was quickly removed and mounted for sectioning with a vibratome (Leica VT100S, Leica Microsystems, Nussloch, Germany) using ceramic blades (Campden Instruments, Loughborough, UK) in ice-cold standard physiological solution ["artificial cerebrospinal solution" (ACSF)] containing (in mM) NaCl, 124; KCl, 3; KH_2PO_4, 1.2; $MgSO_4$, 1.9; glucose, 20; $NaHCO_3$, 26; and $CaCl_2$, 2, and gassed with 95% O_2/5% CO_2. Two to three coronal slices (thickness 300 μm) of the cerebellum containing the DCN were allowed to recover for >1 h at room temperature (22–24 °C) and used for recordings during the next 4 h. No significant differences were observed in animals of different ages. Experimental protocols were approved by the RIKEN Experimental Animal Committee and conducted in compliance with the Guidelines for the Use of Animals in Neuroscience Research (The Society for Neuroscience, Washington, DC, USA) and all measures to reduce the amount of animals used and their suffering were taken.

Electrophysiological recordings

Slices were transferred to and anchored with a platinum bar fitted with a mesh in a submerged-type chamber mounted on a Leica DMLFSA microscope equipped with differential interference contrast (DIC) optics and whole-field epifluorescence imaging (Till Photonics, Gräfelfing, Germany). Slices were superfused with gassed ACSF (2–3 ml/min, at 23–24 °C). In some experiments recording temperature was increased to +34 °C using an inline solution heater (Warner Instruments, Hamden, CT, USA). At all times the temperature of the bath solution was monitored simultaneously with the electrophysiological signals, and experiments with unstable temperature conditions were not included in the analysis.

Borosilicate glass patch electrodes (2 mm outer diameter) were filled with a high-Cl^- intracellular solution containing (in mM) KCl, 136; NaCl, 10; Hepes, 10; EGTA, 0.2; Mg-ATP, 4; Na-GTP, 0.4; glutathione, 5; biocytin, 8; pH adjusted to 7.3 with KOH (280 mOsm). Electrodes filled with this solution had resistances between 1.5 and 2.5 MOhm; the calculated E_{rev} for chloride is 0 mV, and liquid junction potential was calculated (using the tool from PClamp; Molecular Devices, Union City, CA, USA) as 1.7 mV (and not corrected for). In the intracellular solution used for the current-clamp recordings (Fig. 2 and Supplemental Fig. 1) KCl was replaced with K-gluconate.

Whole-cell patch-clamp recordings were performed using an Axon 700B Multiclamp amplifier and pClamp acquisition software (Molecular Devices). The sampling frequency was 5 kHz. After whole-cell configuration was established cells were held at −65 mV.

Since spontaneous IPSCs prevent the precise measurement of C_m (as described in Uusisaari et al., 2007), at the end of the normal recording gabazine (10 μM) was added to the bath to block the IPSCs before the measurements were taken. The cells were classified as large GAD-negative (GADnL), small GAD-negative (GADnS) or GAD-positive (GAD+) based on the presence or absence of fluorescent labeling and the limit of 100 pF as the boundary between GADnL and GADnS cells (see Uusisaari et al., 2007).

Spontaneous IPSCs were seen in every recorded GADnL cell. Only 2 of the 38 GADnS cells recorded showed no spontaneous PSCs in whole-cell configuration and those recordings were discarded. In GAD+ cells the post-synaptic current (PSC) amplitude was sometimes so small in relation to the noise level that analysis may have been unreliable, hence these recordings were also omitted.

Drugs

Most of the experiments were performed in the presence of 20 μM 6-nitro-7-sulfamoylbenzo(f)-quinoxaline-2,3-dione (NBQX, Tocris, Bristol, UK) and 50 μM D-2-amino-5-phosphonopentoate (D-APV, Tocris) to block AMPA- and NMDA-receptor-mediated synaptic currents. Additionally, in some experiments SR 95531 (gabazine, 10 μM, Tocris), picrotoxin (PiTX, 100 μM, Sigma-Aldrich, Japan), tetrodotoxin (TTX, 200 nM-1 μM, Tocris), zolpidem (200 nM, Sigma-Aldrich) or SB-205383 (1 μM, Tocris), was bath-applied.

Data analysis

Electrophysiological data were analyzed using Clampfit 9.2 (Molecular Devices) and MiniAnal (Synaptosoft, Decatur, GA, USA) software. Post-synaptic events were identified and their amplitude and frequency analyzed using detection algorithms in the MiniAnal software for GADnL and GADnS cells, but GAD+ cells were analyzed manually (due to the low amplitude of the latter events). The values for 10–90% rise time and decay time constants (τ) were calculated for individual events using the appropriate tool in Clampfit, while carefully selecting only events where the baseline or shape of the event was not distorted by other events or noise. Statistical analysis of all parameters was performed using OriginPro 7 software (OriginLab, Northampton, MA, USA). All data are presented as means±S.E.M. unless otherwise specified; for statistical significance, Student's *t*-test, ANOVA or Kolmogorov-Smirnov (K-S) was used as applicable.