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Research article

An electrophysiological analysis of deep cerebellar nuclei, with particular focus on Kv3 channels

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Deep cerebellar nuclei (DCN) form three symmetrical, bilateral nuclei, deep within the white matter of the cerebellum and represent the sole output of the cerebellum to the central nervous system. Although innervated by spontaneously active inhibitory Purkinje cells, DCN neurons also fire action potentials spontaneously at a relatively high frequency, a property attributed to Kv3 channels. In the present study, an electrophysiological approach was carried out on DCN neurons, the purpose of which was to investigate the properties and elucidate the involvement of Kv3 channels. Using whole-cell patch clamp techniques, electrophysiological recordings from the dentate nucleus were obtained from coronal cerebellar slices of Wistar rats ($P \sim 12$) in both current and voltage clamp modes. Kv3-mediated currents were investigated in the presence of tetrodotoxin (1 μM), by applying low concentration tetraethyl ammonium (TEA) (100 μM). Current-clamp recordings revealed a population of tonically firing neurons, exhibiting a maximum action potential firing frequency of 18.1 ± 6.9 Hz and a steady firing frequency of 14.2 ± 4.8 Hz ($n = 6$). These neurons were also shown to exhibit a mean action potential amplitude of 48.7 ± 9.5 mV, mean half-width of 2.9 ± 1 ms and mean afterhyperpolarization amplitude of 19.6 ± 7.5 mV. Depolarizing sag (indicative of the I_h current), followed by rebound depolarization, was observed in all neurons. Voltage-clamped neurons revealed a voltage-dependent slowly inactivating outward current with a peak amplitude of 1538.5 ± 1096.6 pA ($n = 10$). Application of low concentration TEA (100 μM) reduced the current by a statistically significant 19.4 ± 13.4% ($P < 0.05$; paired *t*-test) and this effect was reversible. Application of a Boltzmann function yielded a $V_{1/2}$ value of 9.9 mV for the TEA-sensitive current. As Kv3 channels have been previously localized in DCN, the results suggest that the TEA-sensitive current (~20% of the total outward current exhibited) is mediated by Kv3 channels.

Key words: deep cerebellar nuclei, Kv3, TEA-sensitive current, whole-cell patch clamp.

Introduction

The cerebellum is located at the hindbrain and operates at an unconscious level maintaining posture, balance and muscle tone.[1] Structurally, it has two major components: the cerebellar cortex, which makes up the grey matter in the cerebellum, and the deep cerebellar nuclei (DCN), situated deep in the white matter of the cerebellum.[2, 3]

DCN form three separable, symmetrical bilateral nuclei in the cerebellum known as the fastigial nucleus, interpositus nucleus and dentate nucleus, all of which can be observed in coronal sections of the posterior cerebellum.[4, 5] These nuclei represent the primary output of the cerebellum which in turn project to the rest of the central nervous system (CNS),[6, 7] while receiving afferent innervation from the cerebellar cortex, pre-cerebellar nuclei and the inferior olive.[6] Three different types of neurons exist within the DCN—large excitatory projection neurons, smaller inhibitory projection neurons and inhibitory interneurons[6, 8]—which can be identified by size of the soma, connectivity and neurotransmitter content.

Although DCN represent the major output from the cerebellum, inhibitory Purkinje cells represent the major output of the cerebellar cortex.[6, 7] These cells receive and integrate signals of error and sensory-motor information before projecting to the DCN,[9, 10] where all DCN neurons are innervated by Purkinje cells.[11] Purkinje cells are tonically active at rest[12] and therefore neurons of the DCN experience tonic inhibition. However, in spite of this, DCN neurons have the propensity to fire spontaneous fast action potentials,[12]

observed as either the more common regular repetitive spiking or, under a hyperpolarizing influence, cyclical bursts of action potentials.[6, 13]

The spontaneously generated repetitive action potentials of the DCN have an amplitude of ~67 mV, a width of ~1.5 ms[7] and an average frequency of 20–35 Hz.[14] This frequency of firing is known as β-oscillations, which are also observed in the primary motor cortex and voluntary muscles in unison.[15] It has been suggested that this β oscillation activity is either propagated or reset by the DCN, thus having a functional implication in motor control.[15, 16]

Spontaneously active DCN neurons are responsive to both hyperpolarizing and depolarizing currents where they have been observed to reduce and increase firing frequency respectively.[7, 14] Further, upon large depolarizing current injections, properties of bursting cells have been shown to convert to regular spiking properties.[17] This suggests neurons of the DCN demonstrate plasticity between firing properties and experience a variety of regulation from different sources. It is this plasticity that allows spontaneous firing to transform synaptic input into spike output.[18]

The firing patterns of neurons can also be, in part, determined by the types and amounts of potassium channels they express as these affect the rates of repolarization and afterhyperpolarization (AHP).[19–21] Using immunohistochemical analysis, voltage-dependent potassium channels containing Kv1.2, Kv1.4 and Kv1.6 subunits have been localized to the cell bodies of DCN neurons.[22] Kv1 channels, therefore, mediate some of the repolarization current in DCN neurons.

Another type of voltage-dependent potassium current that contributes to repolarization in DCN neurons is mediated by Kv3 channels. These channels mediate a fast delayed rectifier-like potassium current,[12] have been localized in DCN neurons,[23] are sensitive to tetraethyl ammonium (TEA) (<1 mM)[24] and are thought to facilitate action potential generation at high frequencies.[25]

In mammals, there are four Kv3 genes that encode four Kv3 pore-forming alpha subunits, Kv3.1–Kv3.4. These form channels that are homolog's of the *Shaw* subfamily of Shaker potassium channels identified in the fruit fly, *Drosophila*. The subunits form homomeric tetramers but have also been shown *in vitro* to form heteromeric channels resulting in a diverse range of kinetics.[20, 25, 26]

Unlike classical potassium channels, Kv3 channels are not open at or near the resting membrane potential and instead have a high activation threshold of approximately −20 to −10 mV.[24, 25] Kv3 channels also have fast activation and deactivation kinetics; the deactivation rate is said to be faster than all known voltage-gated potassium channels.[25, 27, 28] Kv3 channels are, therefore, well suited to help generate repetitive narrow action potentials with short refractory periods that are essential to high-frequency firing.[29] Interestingly, the Kv3 sequence is highly conserved across species, suggesting an importance for these channels in the function of the CNS.[19, 28]

Kv3 subunits are expressed throughout the CNS in varying amounts, particularly in interneurons,[19] and are prominently expressed in the cerebellum.[20] Neurons of the DCN express all four Kv3 subunits with a greater expression of Kv3.1 and Kv3.3 in the cell bodies,[20, 23, 26, 30] especially in larger diameter DCN neurons.[20] Kv3.1 and Kv3.3 subunits are often found co-localized in neuronal populations,[19, 20, 26] suggesting the formation of heteromeric Kv3 channels *in vivo*.

Kv3 channels, therefore, function to facilitate high-frequency firing by repolarizing the membrane without affecting (i) threshold as the channels are not open at the resting membrane potential, (ii) the depolarization phase as the channels are only significantly activated when an action potential has reached its peak and (iii) the refractory period current as the channels deactivate rapidly. All of these properties help keep action potentials short and narrow and, therefore, reduce the amount of sodium channel inactivation allowing the sodium channels to remain in a ready state for the next action potential and, thus, facilitating high-frequency repetitive firing.[25, 31]

Neurons of the DCN are relatively uncharacterized but do fire at high frequency,[13] a property attributed to the presence of Kv3 channels.[25] The aim of this experiment, therefore, was to investigate the electrophysiological properties of DCN neurons in detail, with a particular focus on Kv3 channels to determine the potential contribution of current mediated by these channels. DCN neurons were examined using both current and voltage whole-cell patch clamp techniques to show firing frequency, action potential parameters and potassium current kinetics. Kv3 channels were elucidated using a pharmacological block via application of low concentration TEA, a selective Kv3 channel blocker.[24] This experiment, therefore, attempted to achieve an electrophysiological analysis of DCN neurons and Kv3 channels, which were expected to fire action potentials repetitively and exhibit a reduction in potassium current after TEA block.

Materials and methods

Tissue preparation and solutions

Sixteen Wistar rats, aged ~12 days, were anaesthetized by intraperitoneal injection of urethane (2 g/kg) and transcardially perfused with cold sucrose-based artificial cerebrospinal fluid (sucrose ACSF; mM: sucrose 252, KCl 3, $NaHCO_3$ 24, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2 and D-glucose 10), under the UK Animals Scientific Procedures Act 1986. The rats were then decapitated, and the brain immediately removed and placed in chilled sucrose ACSF (4°C). The brain was dissected to obtain the cerebellum from which coronal cerebellar slices (250 μm) were prepared using a vibratome (Intracel 1000 plus) and placed into a holding chamber of oxygenated (95% O_2 and 5% CO_2) ACSF (mM: NaCl 124, KCl 3, $NaHCO_3$ 24, NaH_2PO_4

1.25, $MgSO_4$ 2, $CaCl_2$ 2 and D-glucose 10). Slices were left in the holding chamber at room temperature (20–23°C) until recordings commenced (10 min to 7 h after slicing). No noticeable differences were observed between slices used and the duration held in the holding chamber.

Electrophysiological recording

During recording, slices were maintained in a glass-bottomed recording chamber (volume ~2 ml) and continuously perfused at 2 ml per min with oxygenated ACSF. Individual neurons were visualized with differential interference optics, using a water-immersion objective (×60 magnification, Olympus LumPlan Fl/IR, Japan) on an Olympus BX51WI light microscope (×10 magnification) at ×600 overall magnification. Recordings were made at room temperature (20–23°C) using electrodes pulled from borosilicate glass capillaries (GC120F-10, Harvard Apparatus, Kent, UK) using a PP-830 upright electrode puller (Narishinge, Tokyo, Japan). Electrodes were pulled to a resistance of 4–7 MΩ and were filled with filtered intracellular solution (mM: KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 10, HEPES 10, ATP-Mg 3 and GTP 0.3, pH 7.3), supplemented with 1–4 mg/ml Lucifer yellow CH disodium salt (Sigma, Poole, UK) and 1% Biocytin hydrochloride 98% TLC (Sigma) to confirm cell identity after recording. Electrode wires were coated in silver chloride before use.

Tight seal (>1 GΩ) whole-cell patch clamp recordings were made using a Digidata 1322A (Axon Instruments, Foster City, CA, USA) and Multiclamp 700A amplifier (Axon Instruments), controlled by Multiclamp 700 Commander software (Axon Instruments) running on a Macintosh (Power Mac G4) computer. All data were visualized and stored by Axograph (version 4.9; Axon Instruments). Data was filtered (4 kHz), and throughout all experiments a Humbug (50 Hz) was used to eliminate background noise.

Electrophysiological recordings were restricted to identifiable larger neurons (5–35 μm diameter) of the dentate DCN, which are likely to include a mixture of GABAergic and glutamatergic neurons.[6] Although all three DCN can be visualized in cerebellar coronal slices, the dentate DCN was used to obtain data due to its overall uniform location throughout different levels of the cerebellum.[32] Where the cells were unstable or did not survive (<20 min whole-cell patch), recordings were excluded from analysis.

Throughout the experiment, neurons under current clamp were held at a holding potential of −60 mV using DC current injection. Depolarizing (10 pA step depolarization current injection of 0–180 pA for 600 ms) or hyperpolarizing (25 pA step hyperpolarizing current injection from 0 to −350 pA for 600 ms) current pulses were applied to ascertain the membrane and firing properties of the neurons. Single action potential properties were determined by applying a brief depolarizing current pulse at threshold (20–50 pA depolarizing current injection for 300 ms).

Neurons under voltage clamp were held at a holding potential of −90 mV, and four 10 mV hyperpolarizing stimuli followed by depolarizing stimuli increasing in amplitude by 10 mV from −90 to 40 mV were applied to the cells to evoke inward sodium and outward potassium currents with subtracted leak current. Tetrodotoxin (TTX; 1 μM) (Sigma) was applied to remove the sodium current and to aid the measurement of the evoked potassium currents. Low concentration TEA (100 μM), a known Kv3 channel blocker,[24] was applied before being washed out. Protocols (100 ms step depolarizing voltage from −90 mV to +40 mV) were run throughout.

Data analysis

Data analysis was carried out using Axograph (version 4.9; Axon Instruments) and Kaleidagraph software (version 3.08; Synergy software), and data is presented as a mean value ± SEM, where n = number of cells. Statistical significance was determined using a Students paired and two sample (equal variance) t-tests as well as a two factor repeated measures analysis of variance (ANOVA). *Post hoc* Bonferroni analyses were used to test for any significance between the treatments. A value of $P < 0.05$ was deemed significant. Action potential parameters—threshold, amplitude, half-width duration (duration between the depolarizing and repolarizing phase of an action potential at half the amplitude), AHP amplitude and AHP duration—were measured from single evoked action potentials. Firing frequency was measured, converted from milliseconds to Hertz using the equation $Hz = (1/(ms/1000))$, and graphed as maximum firing frequency (F_{max}) and steady firing frequency (F_{steady}). The I_h current sag and rebound depolarization (RD) latency was also measured and averaged from all cells.

Peak current was measured as the highest outward current and steady current was measured as the current ~10 ms before the depolarizing stimuli were removed. To determine activation curves, the Boltzmann expression was used to calculate conductance values. The equation used was: $Gv = G_{max}/(1 + \exp(-(V - V_{1/2})/k))$, where G_{max} is the maximum conductance, V the membrane voltage, $V_{1/2}$ voltage at which half the channels are open and k a measure of the slope. TEA-sensitive current was also calculated by digitally subtracting current elicited in TEA from current elicited under control conditions. The Boltzmann expression was also used to calculate conductance values of the TEA-sensitive current.

Results

Electrophysiological data were collected from a total population of 14 visually identified DCN neurons (15–35 μm diameter[6]) in the dentate DCN of Wistar rats. The neurons

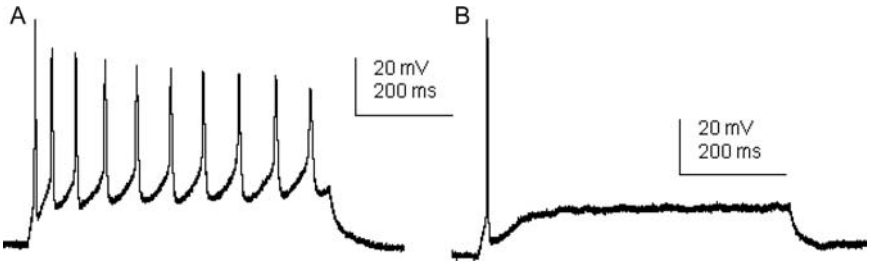

Figure 1. Representative traces of tonic and single firing neurons. (**A**) A tonic firing neuron at the largest depolarizing pulse injection (180 pA for 600 ms). (**B**) A single firing neuron at the largest depolarizing pulse injection (180 pA for 600 ms).

exhibited a mean resting membrane potential of -48.4 ± 4.7 mV and had a mean input resistance of 190 ± 75.1 MΩ. The mean cell capacitance of the neurons was 4.6 ± 2.5 pF and after obtaining the total current produced at the highest membrane potential (+40 mV), the mean current density was calculated as 319.1 ± 104.4 pA/pF. On the basis of firing properties, the total population was divided into previously observed tonic firing neurons,[7, 13] which fire repetitive action potentials in response to depolarizing stimuli (Fig. 1A), and an uncharacterized population of single firing neurons, which fire a single action potential (Fig. 1B). Tonic firing neurons expressed a mean resting membrane potential of -47.8 ± 5.5 mV and had a mean input resistance of 189.8 ± 94.9 MΩ ($n = 6$). The mean cell capacitance of this subset of neurons was 4.8 ± 2.7 pF and the mean current density was calculated as 297.9 ± 114.6 pA/pF. Single firing neurons expressed resting membrane potential of -49 ± 4.1 mV and had a mean input resistance of 190.2 ± 53.4 MΩ ($n = 5$). This subset of neurons had a cell capacitance of 4.4 ± 2.7 pF and a current density of 344.5 ± 96.8 pA/pF. In contrast to what had been expected, based on the different firing properties of neurons, there was no statistical difference between the two populations for any membrane property.

Current clamp

Whole-cell patch clamp recordings were carried out under current-clamp conditions on all DCN neurons, to observe their firing patterns and action potential parameters. Neurons were held at a holding potential (approximately −60 mV) using injection of DC current, and depolarizing pulses in steps of 20 pA (600 ms unless stated) were applied to the neurons from 0 to 180 pA. Neurons were found to have two distinct firing patterns: tonic firing and single action potential firing (Fig. 1). At the maximum depolarizing pulse injection (180 pA), the tonic firing neurons were found to have a mean maximum firing frequency (F_{max}) of 18.1 ± 6.9 Hz and a steady firing frequency (F_{steady}) of 14.2 ± 4.8 Hz ($n = 6$). Further, as the depolarizing pulse injection was increased, firing frequencies of the neurons also increased (Fig. 2). As single firing neurons only exhibited one action potential, regardless of the depolarizing pulse injection, no data on firing frequency could be obtained.

To further investigate action potential properties, single depolarizing pulse injections (300 ms) were applied to neurons at the stimulus strength that evoked a single action potential. In contrast to what had been expected, there was no statistical difference between tonic firing neurons and single firing neurons for any action potential parameter recorded. Therefore, the data were grouped together as one population ($n = 14$), whereby the neurons had a mean threshold of -42.2 ± 6.5 mV and mean action potential amplitude of 48.7 ± 9.5 mV. The mean half-width duration of the action potentials evoked was 2.9 ± 1 ms. Further, the neurons had a mean AHP amplitude of 19.6 ± 7.5 mV ($n = 13$) and a mean AHP duration of 61.2 ± 43.2 ms ($n = 13$).

As I_h currents have been previously observed in DCN neurons,[7, 33] hyperpolarizing pulse steps of 25 pA (600 ms) were applied to the neurons from 0 to −350 pA. Almost all neurons analysed exhibited a voltage-dependent depolarizing sag in response to a hyperpolarizing pulse step injection ($n = 11$), which had a mean maximum amplitude of 14.9 ± 10.1 mV (Fig. 3). The majority, but not all, of these neurons also exhibited RD ($n = 9$; 82%), of which the mean latency to the evoked action potential was 88.7 ± 40.8 ms (Fig. 3). The properties of the depolarizing sag or the RD were not examined further in this study.

Voltage clamp

To investigate the properties of potassium channels, in particular, Kv3 channels within DCN neurons, voltage-clamp experiments were carried out in the presence of TTX (1 μM), a voltage-sensitive sodium channel blocker.[34] This, therefore, eliminated voltage-sensitive sodium currents responsible for depolarization in DCN neurons, leaving voltage-sensitive potassium currents for investigation. Neurons were held at −90 mV, and the application of depolarizing stimuli in increments of 10 mV from the holding potential (−90 mV) to +40 mV revealed a voltage-dependent slowly inactivating outward current (Fig. 4). At +40 mV, the outward current had a mean peak amplitude

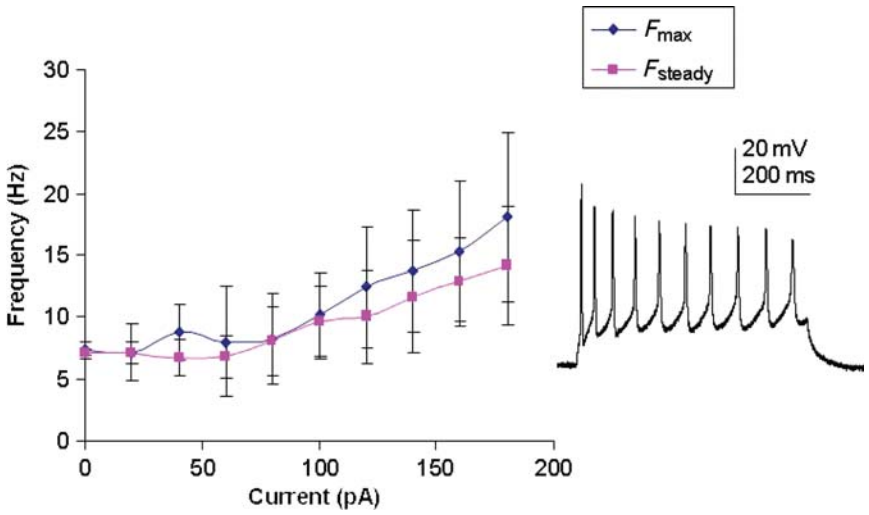

Figure 2. Mean maximum firing frequency (F_{max}) and steady firing frequency (F_{steady}) from a tonic firing neuron. Both F_{max} and F_{steady} increased in firing frequency with the increase in depolarizing stimuli. Inset shows a representative trace of a tonic firing neuron during 180 pA depolarizing pulse injection (600 ms).

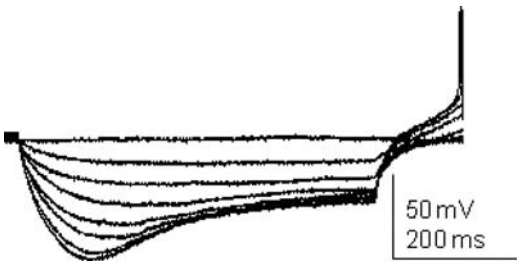

Figure 3. A representative trace of an I_h current, followed by a RD large enough to elicit an action potential, exhibited in neurons of the DCN. Traces show current elicited from hyperpolarizing steps of 50 pA from 0 to −350 pA.

Figure 4. A representative of the slowly inactivating outward current evoked during application of depolarizing voltage steps (increments of 10 mV from −90 to +40 mV). The initial peak current is where the outward current is at its highest, which transiently becomes a smaller steady current at all depolarizing steps.

of 1538.5 ± 1096.6 pA ($n = 10$). This mean peak amplitude was then reduced to 1192.7 ± 797.9 pA in the presence of TEA (100 μM), a statistically significant reduction of 19.3 ± 12.5% ($P < 0.05$; paired t-test). A 2 (treatment: control and drug) × 5 (voltage: 40, 30, 20, 10 and 0) repeated measures ANOVA was also conducted. Results showed a significant effect of treatment ($F(1, 14) = 12.469$, $P < 0.01$). *Post hoc* analyses showed the peak current amplitude to be significantly reduced as a result of the application of TEA ($P < 0.01$). This effect was reversible (Fig. 5; insets). Conductance–voltage curves of the potassium currents were fitted with a single, first-order Boltzmann function, yielding a mean peak $V_{1/2}$ of −12.9 ± 6.9 mV and a slope factor of 17.9 ± 3.5 mV (Fig. 5A).

The peak outward current transiently becomes steady outward current (Fig. 4) and at +40 mV the mean steady amplitude was 1463.6 ± 1148.4 pA ($n = 10$). Like the peak amplitude, the steady amplitude was reduced in the presence of TEA (100 μM) to 1132.6 ± 822.3 pA, which was also a statistically significant reduction of 19.4 ± 13.4% ($P < 0.05$; paired t-test). A 2 (treatment: control and drug) × 5 (voltage: 40, 30, 20, 10 and 0) repeated measures ANOVA was also conducted. Results showed a significant effect of treatment ($F(1, 14) = 5.069$, $P < 0.05$). *Post hoc* analyses showed the steady current amplitude to be significantly reduced as a result of the application of TEA ($P < 0.05$). This effect was also reversible (Fig. 5; insets). Conductance–voltage curves of the potassium currents were fitted with a single, first-order Boltzmann function, yielding a mean steady $V_{1/2}$ of 0.6 ± 7.4 mV and a slope factor of 16.8 ± 5.2 mV (Fig. 5B).

The TEA-sensitive current, which accounts for ∼19.3 ± 12.5% of the peak current and 19.4 ± 13.4% of the steady current, was calculated by digitally subtracting the currents observed in the presence of TEA from those observed under control conditions (Fig. 6). Here, the peak

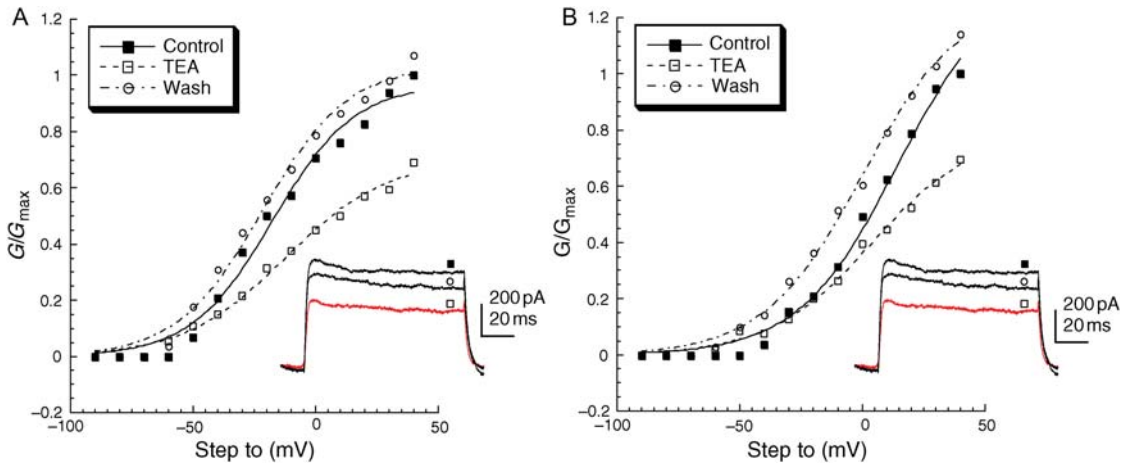

Figure 5. Representative of the conductance–voltage activation curves constructed for all cells. (**A**) The representative activation curve for the peak outward current. (**B**) The representative activation curve for the steady outward current. The Boltzmann function was fitted to the activation curves revealing a mean half-activation ($V_{1/2}$) and slope factor. The slope factors are quoted in the results section, along with the values for $V_{1/2}$. Insets show representative currents evoked at +40 mV depolarizing step for control (filled square), TEA (open square) and wash (open circle).

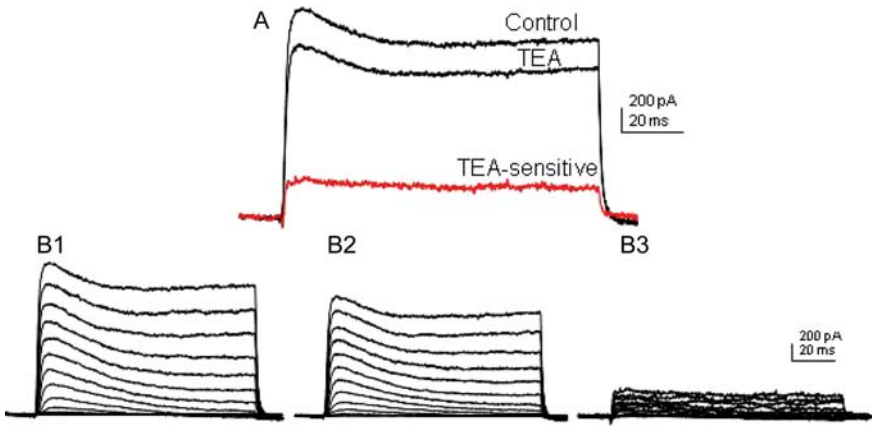

Figure 6. Outward potassium currents at control and in the presence of TEA combined with structured TEA-sensitive currents. (**A**) Control, TEA reduced and TEA-sensitive outward currents evoked at +40 mV depolarizing voltage step. (**B1–3**) Control (B1), TEA reduced (B2) and TEA-sensitive outward currents (B3) over all depolarizing voltage steps (increments of 10 mV from −90 to +40 mV). Scale bar in B3 applies to B1–B3.

TEA-sensitive current amplitude was 432.8 ± 461.7 pA (n = 7) and the steady TEA-sensitive current amplitude was 443.4 ± 509.3 pA (n = 7). Conductance–voltage curves of the TEA-sensitive current were then fitted with a single, first-order Boltzmann function, yielding a mean peak $V_{1/2}$ of 9.9 ± 10.1 mV and slope factor of 19.7 ± 10.1 mV and a mean steady $V_{1/2}$ of 21.5 ± 17.3 mV and slope factor of 16.1 ± 6.3 mV.

Discussion

On the basis of the fast spiking nature of DCN neurons,[7, 12] previous studies have speculated the presence of, and subsequently localized, Kv3 channels to DCN.[20, 23] This experiment aimed to investigate Kv3 channels in more detail, using electrophysiological analysis, under whole-cell voltage and current clamp. Here, low concentration TEA, a known selective blocker of Kv3 channels,[24] was applied to elucidate these channels, which were then investigated.

Current-clamp analysis of DCN neurons

Under current-clamp conditions, all neurons were held at −60 mV, which is ~20 mV below threshold,[7, 35] removing any spontaneous activity at rest. Using depolarizing stimuli, action potential firing was recovered and, based on the firing patterns exhibited during depolarizing stimuli (180 pA), two populations of neurons within the DCN were analysed. These consisted of tonic firing neurons, which fired action potentials repetitively, and single firing neurons, which fired a single action potential (Fig. 1).

Tonic firing neurons in the DCN are well documented, and were therefore expected,[7, 13] but single firing neurons appear to be uncharacterized. In contrast to what had been expected, there were no statistically significant differences between the two populations for any action potential parameter and these were therefore considered as one population that merely varied in firing frequency. Future experiments should collect more data from these single firing neurons to determine if they are tonic firing cells that have been 'silenced'[12] or a novel population of neurons within the DCN.

Increasing the depolarizing current injection was found to increase the maximum and steady firing frequencies observed in tonic firing neurons (Fig. 2). At the largest depolarizing current injection (180 pA), the firing frequencies observed were considerably slower than the rates observed *in vivo* (35–55 Hz[35]) and although relatively similar to the spontaneous firing frequencies of DCN neurons at rest, observed *in vitro* (18.3 ± 1.7[12] and 12.8 ± 6.1 Hz[7]), these too were found to increase upon depolarizing current injection. Interestingly, GABAergic DCN neurons have a spontaneous firing frequency of 9.9 ± 1 Hz at rest[35] which, although lower than the firing frequencies observed in this experiment, would increase upon depolarizing stimuli to potentially similar levels. It is, therefore, possible that analysed cells were GABAergic.

I_h and RD have been relatively well characterized in DCN[13, 33] and, like previous studies, the vast majority of cells exhibited the characteristic depolarizing 'sag' of the I_h current and RD, which led to evoked action potentials (Fig. 3). In agreement with the presence of an I_h 'sag', all four I_h channel subunits, HCN1–4, have been strongly immunolocalized to DCN[36] and when comparing the RD latency, the time to the first evoked action potential is consistent with the published data.[6]

Voltage-clamp analysis of DCN neurons

Under voltage-clamp conditions, the application of depolarizing stimuli elicited a rapid inward transient current (data not shown) followed by a slowly inactivating outward current in DCN neurons. In the presence of TTX (1 μM), the rapid inward current was abolished, revealing a voltage-dependent, slowly inactivating outward current that resembles a delayed rectifier current with a superimposed slowly inactivating transient current (Fig. 4), similar to the outward currents of DCN neurons observed elsewhere in TTX.[12] In the presence of low concentration TEA (100 μM), this outward current was reduced by ~20%, which was found to be statistically significant ($P < 0.05$; paired *t*-test) and reversible (Fig. 5; insets). This 20% reduction, therefore, represents the TEA-sensitive current and was extracted digitally (Fig. 6). As low concentration of TEA is a known selective blocker of Kv3 channels[24] and Kv3 subunits have been previously localized to DCN,[20, 23] it was speculated that any current reduction would be due to current mediated by Kv3 channels. The calculated $V_{1/2}$ for the TEA-sensitive current confirmed this speculation as it is in agreement with the published values for $V_{1/2}$ of Kv3 channels.[24]

Interestingly, the assumed Kv3-mediated TEA-sensitive current does not appear to have an obvious rapid transient component, suggesting the absence of A-type current mediating Kv3 subunits, Kv3.3 and Kv3.4.[25] In contrast with this speculation, Kv3.3 immunolocalization has been previously observed in DCN.[20, 23] This may be explained by the fact that Kv3.3 only appears to exhibit an A-type current when heterologously expressed in *Xenopus oocytes* and in mammalian cells, appears to express a delayed rectifier-like current.[24] It is still unknown what type of current Kv3.3 exhibits *in vivo*.

The fact that only 20% of the total current was found to be TEA-sensitive, and therefore mediated by Kv3 channels, may explain the slower firing frequency observed, as Kv3 channels contribute to the rate of firing frequency.[37] Neurons from published data may, therefore, have expressed more Kv3 channels, resulting in a higher-firing frequency. This can further lead to the speculation that different neurons in DCN express different amounts of Kv3 channels and, therefore, have different firing frequencies. This may also imply functional differences between cells that fire at different rates and in fact, each cerebellar nucleus is thought to have a different function.[38] Further, if the neurons recorded are GABAergic DCN neurons, it can be speculated that these fire at slower rates[35] due to a lower expression of Kv3 channels.

Kv3.1 and Kv3.3 channels in the cerebellum are as implicated in the control and modulation of motor skills as the cerebellum itself.[23] In fact, Kv3.1 and Kv3.3 double knockout mice exhibited impaired motor task execution and severe ataxia,[39, 40] suggesting that Kv3 channel activity in the cerebellum is crucial for its function. Interestingly in individuals with Filipino adult-onset ataxia and French child-onset ataxia, the causative gene maps to the gene encoding Kv3.3 channels.[41]

As only ~20% of the total current was sensitive to TEA, only 20% of the total current can be speculated to be mediated by Kv3 channels. The channels that mediate the remaining outward current, the TEA-resistant current, remain uncharacterized. Due to the voltage-dependence of the outward current, it can be speculated that the TEA-resistant current is mediated by a contribution of the three other main functional voltage-gated potassium channels Kv1, Kv2 and Kv4, of which Kv1 and Kv4 channels have been localized in DCN neurons.[22, 42]

It is thought that the main function of Kv4 channels is to facilitate regular spiking with low frequency, contrary to that of Kv3 channels.[43] The presence of these channels in DCN may, therefore, negatively modulate the firing frequencies,

whereas Kv3 channels would positively modulate firing frequencies. Interestingly, both Kv4 and Kv3 channels are dynamically regulated in neurons[24, 44] and, *in vivo*, this potential regulation may result in firing frequency plasticity occurring in response to functional requirements.

The applied Boltzmann function for the outward currents at control, in the presence of TEA and after wash (Fig. 5), calculates the $V_{1/2}$ for a mixture of outward currents. Interestingly, after calculating the mean $V_{1/2}$ value for all channels thought to be involved in the outward current (Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv3.1, Kv3.3, Kv4.2 and Kv4.3), this value (10.67 mV; calculated from values published[24, 45]) is consistent with the observed value in the experiment ($V_{1/2}$ value 12.9 ± 6.9).

Conclusions

In conclusion, DCN neurons are spontaneously active cells where the spontaneous activity is, at least in part, likely to be driven by Kv3 channels and this contribution is crucial to cerebellar function. Calculated $V_{1/2}$ values suggest that the TEA-sensitive current observed is mediated by Kv3 channels, therefore suggesting that Kv3 channels mediate ~20% of the total outward current, which is thought to vary between cells. Further, by comparing the $V_{1/2}$ value of the total outward potassium current with the mean calculated $V_{1/2}$ value for all Kv channels assumed to be involved, it can be speculated that Kv1, Kv3 and Kv4 potassium channels mediate the majority of the observed outward potassium current in DCN neurons, whereby Kv3 and Kv4 have implications in controlling the firing frequency exhibited.

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References

1. Robinson FR (1995) Role of the cerebellum in movement control and adaptation. *Curr Opin Neurobiol* 5: 755–762.
2. Purves D, Augustine GJ, Fitzpatrick D *et al.* (2001) Modulation of movement by the cerebellum. In *Neuroscience*. 2nd ed. MA, USA: Sinauer Associates, Inc., pp 409–426.
3. Crossman AR, Neary D (2000) Cerebellum. In *Neuroanatomy*. 2nd ed. Philadelphia, USA: Churchill Livingstone, pp 117–124.
4. Chambers WW, Sprague JM (1955) Functional localization in the cerebellum. II. Somatotopic organization in cortex and nuclei. *AMA Arch Neurol Psychiatry* 74: 653–680.
5. Sastry BR, Morishita W, Yip S *et al.* (1997) GABAergic transmission in deep cerebellar nuclei. *Prog Neurobiol* 53: 259–271.
6. Aizenman CD, Huang EJ, Linden DJ (2003) Morphological correlates of intrinsic electrical excitability in neurons of the deep cerebellar nuclei. *J Neurophysiol* 89: 1738–1747.
7. Czubayko U, Sultan F, Thier P *et al.* (2001) Two types of neurons in the rat cerebellar nuclei as distinguished by membrane potentials and intracellular fillings. *J Neurophysiol* 85: 2017–2029.
8. Sultan F, Czubayko U, Thier P (2003) Morphological classification of the rat lateral cerebellar nuclear neurons by principal component analysis. *J Comp Neurol* 455: 139–155.
9. Llinas R, Muhlethaler M (1988) Electrophysiology of guinea-pig cerebellar nuclear cells in the *in vitro* brain stem-cerebellar preparation. *J Physiol* 404: 241–258.
10. Watanabe M (2008) Molecular mechanisms governing competitive synaptic wiring in cerebellar Purkinje cells. *Tohoko J Exp Med* 214: 175–190.
11. Teune TM, van der Burg J, de Zeeuw CI *et al.* (1998) Single Purkinje cell can innervate multiple classes of projection neurons in the cerebellar nuclei of the rat: a light microscopic and ultrastructural triple-tracer study in the rat. *J Comp Neurol* 392: 164–178.
12. Ramen IM, Gustafson AE, Padgett D (2000) Ionic currents and spontaneous firing in neurons isolated from the cerebellar nuclei. *J Neurosci* 20: 9004–9016.
13. Aizenman CD, Linden DJ (1999) Regulation of the rebound depolarization and spontaneous firing patterns of deep nuclear neurons in slices of rat cerebellum. *J Neurophysiol* 82: 1697–1709.
14. Jahnsen H (1986) Electrophysiological characteristics of neurones in the guinea-pig deep cerebellar nuclei *in vitro*. *J Physiol* 372: 129–147.
15. Auman TD, Fetz EE (2004) Oscillatory activity in forelimb muscles of behaving monkeys evoked by microstimulation in the cerebellar nuclei. *Neurosci Lett* 361: 106–110.
16. Martin JH, Cooper SE, Hacking A *et al.* (2000) Differential effects of deep cerebellar nuclei inactivation on reaching and adaptive control. *J Neuropysiol* 83: 1886–1899.
17. Zhang W, Shin JH, Linden DJ (2004) Persistent changes in the intrinsic excitability of rat deep cerebellar nuclear neurones induced by EPSP or IPSP bursts. *J Physiol* 561(Pt 3): 703–719.
18. Hausser M, Raman IM, Otis T *et al.* (2004) The beat goes on: spontaneous firing in mammalian neuronal microcircuits. *J Neurosci* 24: 9215–9219.
19. Kanemasa T, Gan L, Perney TM *et al.* (1995) Electrophysiological and pharmacological characterization of a mammalian Shaw channel expressed in NIH 3T3 fibroblasts. *J Neurophysiol* 74: 207–217.
20. Weiser M, Vega-Saenz de Miera E, Kentros C *et al.* (1994) Differential expression of Shaw-related K^+ channels in the rat central nervous system. *J Neurosci* 14(Pt 1): 949–972.
21. Sah P (1996) Ca(2 +)-activated K^+ currents in neurones: types, physiological roles and modulation. *Trends Neurosci* 19: 150–154.
22. Chung YH, Joo KM, Nam RH *et al.* (2005) Immunohistochemical study on the distribution of the voltage-gated potassium channels in the gerbil cerebellum. *Neurosci Lett* 374: 58–62.
23. Erisir A, Lau D, Rudy B *et al.* (1999) Function of specific K(+) channels in sustained high-frequency firing of fast-spiking neocortical interneurons. *J Neurophysiol* 82: 2476–2489.
24. McMahon A, Fowler SC, Perney TM *et al.* (2004) Allele-dependent changes of olivocerebellar circuit properties in the absence of the voltage-gated potassium channels Kv3.1 and Kv3.3. *Eur J Neurosci* 19: 3317–3327.

25. Rudy B, Chow A, Lau D *et al*. (1999) Contributions of Kv3 channels to neuronal excitability. *Ann N Y Acad Sci* 868: 304–343.
26. Rudy B, McBain CJ (2001) Kv3 channels: voltage-gated K^+ channels designed for high-frequency repetitive firing. *Trends Neurosci* 24: 517–526.
27. Chang SY, Zagha E, Kwon ES *et al*. (2007) Distribution of Kv3.3 Potassium channel subunits in distinct neuronal populations of mouse brain. *J Comp Neurol* 502: 953–972.
28. Sung MJ, Ahn HS, Hahn SJ *et al*. (2008) Open channel block of Kv3.1 currents by fluoxetine. *J Pharmacol Sci* 106: 38–45.
29. Bean P (2007) The action potential in mammalian central neurons. *Nat Rev Neurosci* 8: 451–465.
30. Weiser M, Bueno E, Sekirnjak C *et al*. (1995) The potassium channel subunit KV3.1b is localized to somatic and axonal membranes of specific populations of CNS neurons. *J Neurosci* 15: 4298–4314.
31. Baranauskas G, Tkatch T, Nagata K *et al*. (2003) Kv3.4 subunits enhance the repolarising efficiency of Kv3.1 channels in fast-spiking neurons. *Nat Neurosci* 6: 258–266.
32. Pellegrino LJ, Pellegrino AS, Cushman AJ (1979) *A Stereotaxic Atlas of the Rat Brain*. New York, USA: Plenum Publishing Corporation.
33. Molineux ML, McRory JE, McKay BE *et al*. (2006) Specific T-type calcium channel isoforms are associated with distinct burst phenotypes in deep cerebellar nuclear neurons. *Proc Natl Acad Sci USA* 103: 5555–5560.
34. Catterall WA (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 20: 15–43.
35. Uusisaari M, Obata K, Knopfel T (2007) Morphological and electrophysiological properties of GABAergic and non-GABAergic cells in the deep cerebellar nuclei. *J Neurophysiol* 97: 901–911.
36. Notomi T, Shigemoto R (2004) Immunohistochemical localization of I_h channel subunits, HCN1-4, in the rat brain. *J Comp Neurol* 471: 241–276.
37. Lien CC, Jonas P (2003) Kv3 potassium conductance is necessary and kinetically optimised for high-frequency action potential generation in hippocampal interneurons. *J Neurosci* 23: 2058–2068.
38. Thach WT, Goodkin HP, Keating JG (1992) The cerebellum and the adaptive coordination of movement. *Annu Rev Neurosci* 15: 403–442.
39. Espinosa F, McMahon A, Chan E *et al*. (2001) Alcohol hypersensitivity, increased locomotion, and spontaneous myoclonus in mice lacking potassium channels Kv3.1 and Kv3.3. *J Neurosci* 21: 6657–6665.
40. Joho RH, Street C, Matsushita S *et al*. (2006) Behavioural motor dysfunction in Kv3-type potassium channel-deficient mice. *Genes Brain Behav* 5: 472–482.
41. Waters MF, Minassian NA, Stevanin G *et al*. (2006) Mutations in voltage-gated potassium channel KCNC3 cause degenerative and developmental central nervous system phenotypes. *Nat Genet* 38: 447–451.
42. Serodio P, Rudy B (1998) Differential expression of Kv4 K^+ channel subunits mediating subthreshold transient K^+ (A-type) currents in rat brain. *J Neurophysiol* 79: 1081–1091.
43. Martina M, Schultz JH, Ehmke H *et al*. (1998) Functional and molecular differences between voltage-gated K^+ channels of fast-spiking interneurons and pyramidal neurons of rat hippocampus. *J Neurosci* 18: 8111–8125.
44. Birnbaum SG, Varga AW, Yaun LL *et al*. (2004) Structure and function of Kv4-family transient potassium channels. *Physiol Rev* 84: 803–833.
45. Coetzee WA, Amarillo Y, Chiu J *et al*. (1999) Molecular diversity of K^+ channels. *Ann N Y Acad Sci* 868: 233–285.

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