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Altered Functional Expression of Purkinje Cell Calcium Channels Precedes Motor Dysfunction in *Tottering* Mice

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Abstract

In *tottering* mice, a point mutation in the gene encoding P-type ($\text{Ca}_v2.1$) voltage-gated calcium channels results in ataxia, absence epilepsy, and motor dystonia that appear 3–4 weeks postnatal. The aberrant motor behaviors have been linked to cerebellar dysfunction, and adult Purkinje cells (PCs) of *tottering* mice exhibit calcium-dependent changes in gene transcription suggestive of altered calcium homeostasis. In an attempt to identify early postnatal events important for the development of the behavioral phenotype, we examined calcium channel expression in cerebellar PCs from postnatal days 6 to 15 (P6–15). Whole cell recording was combined with selective calcium channel antagonists to allow discrimination of the various voltage-activated calcium channels types; early age-dependent differences between *tottering* and wild-type PCs were found. Wild-type PCs experienced a steady increase in P current density over this period, resulting in a two-fold change by P15. In *tottering*, by contrast, P current density remained unchanged from postnatal days 6 to 8 and was only 25% of the wild-type level by P8. A developmental delay in functional expression was implicated in this early deficit, since ensuing gains over the subsequent week brought *tottering* P current density close to the wild-type level by P15. At this age, *tottering* PCs also exhibited a 2.2-fold higher L-type calcium current density than that expressed by wild-type PCs. Increases in N current were apparent at some ages, most strikingly within a subset of *tottering* PCs at P15. Functional Rand T-type calcium current densities were equivalent to wild-type levels at all ages. We conclude that the *tottering* mutation brings about selective changes in functional calcium channel expression one to two weeks prior to the appearance of the behavioral deficits, raising the possibility that they represent an early, primary event along the path to motor dysfunction in *tottering*.

Keywords

voltage; gated Ca^{2+} channel; channelopathy; neural development

Voltage-gated calcium channels participate in cellular differentiation, neurotransmitter release, synaptic plasticity, and membrane excitability. In humans, mutations in these channels produce abnormalities that include ataxia, familial hemiplegic migraine, absence epilepsy, and/or cerebellar degeneration (Lorenzon and Beam, 2000). In *tottering* mice, a spontaneous point mutation in the gene encoding the P-type calcium channel ($\text{Ca}_v2.1$) changes proline to leucine near the putative outer vestibule of the channel's pore (Fletcher et al., 1996). The impact of

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this mutation on the biophysical properties of the channel—i.e., gating and permeation—are few and subtle (Wakamori et al., 1998), whereas the behavioral consequences are not—ataxia, absence epilepsy, and paroxysmal dystonia begin ~3–4 weeks postnatal (Green and Sidman, 1962; Noebels and Sidman, 1979).

Evidence suggests the involvement of diverse regions of the central nervous system in these varied neurological abnormalities (Noebels, 1984; Helekar and Noebels, 1991; Campbell and Hess, 1998; Caddick et al., 1999). In the cortex, abnormal spike wave discharges are thought to be an essential trigger for absence seizures (Noebels and Sidman, 1979), as both are eliminated following chemically induced ablation of the locus coeruleus (Noebels, 1984). In contrast, the prominent ataxia and dystonia exhibited by *tottering* animals are both considered of cerebellar origin (Campbell and Hess, 1998; Campbell et al., 1999), albeit through distinct underlying mechanisms, since surgical lesions of the cerebellar vermis eliminate stress-induced dystonia without altering ataxia (Abbott et al., 2000).

The involvement of the cerebellum in motor dysfunction in *tottering* is not unexpected, since P-type channels contribute ~85% of the total voltage-activated calcium current in Purkinje cells (Mintz et al., 1992), the sole output neuron of the cerebellum. Indeed aberrant PC function in *tottering* has been reported during optokinetic behavior as evidenced by irregular floccular PC spiking relative to wild-type cells (Hoebeek et al., 2005). PCs of adult *tottering* mice have also been shown to exhibit a selective 40% reduction in P current density (Wakamori, et al. 1998; but see Dove et al. 1998). In addition, the L-type ($\text{Ca}_v1.2$) channel mRNA is elevated in adult *tottering* PCs (Campbell and Hess, 1999), suggesting that changes in calcium channel expression play a role in the *tottering* phenotype.

Alterations in a number of other proteins have also been reported, some of which may contribute to the motor dysfunction in the mutant (Hess and Wilson, 1991; Austin et al., 1992; Fureman et al., 1999; Sawada and Fukui, 2001; Cicale et al., 2002; Frank-Cannon et al., 2007). In the case of tyrosine hydroxylase (TH), aberrations in calcium channel influx in *tottering* appear to precipitate abnormal developmental control of expression. In wild-type PCs, TH turns on by P21 and off by P35. By contrast, TH expression persists through adulthood in *tottering* PCs (Hess and Wilson, 1991; Austin et al., 1992); such aberrant TH expression can be blocked *in vivo* by administration of L channel antagonists (Fureman et al., 1999). Consistent with this finding, Sawada and Fukui (2001) demonstrated that enhanced Ca^{2+} influx into non-catecholaminergic neurons *in vitro* was able to trigger aberrant TH expression. Taken together, such expression studies link Ca^{2+} influx to control of expression in PCs and raise the possibility that, if alterations in calcium channel complement occur sufficiently early in *tottering*, they could interfere with normal PC development. We report here results of studies to explore the functional expression of all voltage-activated calcium channel types in PCs from *tottering* and wild-type mice during the first two weeks of postnatal development, prior to the onset of the behavioral phenotype. Our results provide strong support for the idea that changes in calcium influx may be an early factor precipitating the motor dysfunctions in *tottering*.

Experimental Procedures

Mice

All procedures with mice were approved by the Tufts Animal Case and Use Committee. Breeding pairs heterozygous for the *tottering* mutation on the C57BL/6J background (strain name B6.D2-*Cacna1atg/J*) were purchased from the Jackson Laboratories (Bar Harbor, ME) to establish our colony. Pregnant mice were checked at roughly the same time daily to establish the date of birth to within 24 hours. For simplicity all mice born within this 24-hour period of time were designated as postnatal day 1 (P1). For genotyping, the distal tips of the tails were removed from pups as early as P4, and the animals were tagged using the AIMS_{TM} pup tattoo

identification system. The mice used for controls consisted of wild-type offspring of heterozygous parents or offspring of wild type C57BL/6J.

Genotyping

Genotyping was performed following the procedure developed by Wakamori et al. (1998). Briefly, DNA was extracted from tail snips using the DNeasy kit from Qiagen (Valencia, CA) and polymerase chain reaction was used to amplify a region encompassing the T-to-G *tottering* mutation (Hot-Start kit, Qiagen). Because this point mutation removes an *Acil* restriction enzyme recognition site present in the wild-type gene, unambiguous assignment of genotypes was possible by *Acil* digestion and electrophoretic separation of the PCR reaction products on agarose gels.

Cell dissociation and acute culture

A single animal was used to generate PCs for each day's recording; averages of data reported for each time point were generated from multiple animals and dissociations. Pups were decapitated and the brains rapidly removed and placed into oxygenated ice-cold HEPES buffer (containing 82 mM Na₂SO₄, 30 mM K₂SO₄, 5 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4). The procedure for dissociation was modified from that of Mintz and Bean (1993). The cerebellar vermis was isolated and gently minced into pieces about 2 mm³ using featherweight razor blades. The tissue was transferred into protease solution (3 mg/ml protease XXIII in HEPES buffer) at 37 °C and digested for 6–7 minutes. The tissue was then rinsed twice with the HEPES buffer and transferred into 2 ml of Minimum Essential Medium with Earle's salts containing 10 mM glucose, 44 mM NaHCO₃, 1 mg/ml trypsin inhibitor, 1 mg/ml bovine serum albumin, and 0.1 mg/ml DNase. The tissue was minimally triturated—5 times through a fire polished glass Pasteur pipette with a ~2.0 mm diameter opening, then 10 times through a ~1.25 mm opening. Cells were plated onto tissue culture dishes (Becton-Dickinson) and placed at 37 °C in an O₂/CO₂ incubator. After an hour, dishes were supplemented with the medium described above after the cells were adequately attached for recording. Immediately prior to recording, cells were removed from the incubator and extracellular recording solution added (5 mM BaCl₂, 160 mM tetraethylammonium chloride, 10 HEPES, 1 μM tetrodotoxin, pH 7.4). Cells treated in this way typically remained viable for recording for at least 6 hours after dissociation.

Electrophysiology

Dissociated PCs were visually identified at 400× using a Zeiss inverted microscope with phase contrast, and were clearly distinguishable from granule or other neurons based on shape and size, ranging from ~15–25 μm in diameter. From P6–P9, PCs typically were oval or round with large eccentric visible nuclei (cf. images in Liljelund et al. 2000); by P15, PCs were typically larger and opaque, with a uniformly smooth appearance. Starting around P9, cells exhibited a combination of these features indicating a transition from the immature to the more mature morphology. P15 cells from *tottering* mice exhibiting low P-type current were from 3 out of 4 preparations of dissociated cells, and identified independently by 3 electrophysiologists.

Electrodes were pulled from Kimble capillary soda lime glass (Fisher Scientific) and had a typical resistance of 2 MΩ (range 1.5–4) when filled with an internal solution containing 150 mM CsCl, 10 mM HEPES, 5 mM tetracesium BAPTA, and 5 mM MgATP at pH 7.4. A List EPC-7 patch clamp amplifier was used for recording somatic currents in the whole cell mode. Capacitive transients were compensated and total cell capacitance noted from the C_{slow} dial. Series resistance was compensated, typically to ~30%. Currents were filtered at 3 kHz via a low pass, 8 pole Bessel filter (LPF8, Warner Instruments).

Data acquisition and analysis

Cell stimulation and data acquisition were controlled using Pulse software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Data were acquired at 10 or 40 kHz and a p/4 or p/6 protocol, respectively, was employed for subtracting leak currents. Data analysis was carried out off-line using IgorPro (Wavemetrics, Lake Oswego, OR); spreadsheets and statistical calculations were performed with Excel (Microsoft, Redmond, WA). Data are expressed as means $\pm$ standard errors of the mean (with statistical significance assessed by a Student's two-tailed t test).

Ba²⁺ replaced Ca²⁺ as the charge carrier in order to minimize calcium-dependent inactivation of the channels, thereby avoiding underestimations of current density (particularly important for L-type channels, which exhibit significant calcium-dependent inactivation, Liang et al., 2003). Since the peak current of PC from P15 mice occasionally exceeded 5 nA when using 5 mM Ba²⁺, 2.5 mM Ba²⁺ (and 164 mM tetraethylammonium chloride) was used in some cases to minimize access resistance errors. In this concentration range, Ba²⁺ current is a linear function of external Ba²⁺ (Cota and Stefani, 1984); thus, currents measured in 2.5 mM Ba²⁺ solution were scaled up by a factor of 2 during analysis to allow comparison with other data.

Somatic currents in Purkinje cells slowly increased during the first few minutes following whole cell access (Regan, 1991), and an additional increase accompanied the onset of perfusion of the control solution (i.e., no antagonists). Application of solutions containing Ca²⁺ channel blockers was initiated only after the voltage-activated current of a cell had reached a stable level in the control solution. Solutions were applied by gravity flow from tubes positioned directly in front of the cell under investigation. As nimodipine-induced inhibition of L current is voltage-dependent, we monitored the progress of inhibition during a test pulse to 0 mV preceded by a strong depolarizing conditioning prepulse (to 80 mV). After reaching a steady-state, full inhibition was measured at the end of a long (500 ms) depolarizing pulse without the conditioning pulse.

Chemical Reagents

All reagents, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in deionized water, stored as aliquot samples frozen at ca. -60° C unless otherwise indicated, and diluted into the final solution immediately prior to use. Stock concentrations were made for bovine serum albumin, trypsin inhibitor and protease XXIII at 100 mg/ml, DNase I (Roche Applied Science, Indianapolis, IN) at 10 mg/ml, tetrodotoxin (EMD Biosciences, San Diego, CA) at 3 mM (stored at -20° C), ω -agatoxin IVA and ω -conotoxin GVIA (Peptides International, Louisville, KY) at 0.1 mM, nimodipine at 10 mM in dimethylsulfoxide. BAPTA was obtained from Invitrogen (Carlsbad, CA). In all experiments employing ω -agatoxin IVA and ω -conotoxin GVIA, bovine serum albumin was added at 0.2 mg/ml to the final working solutions to minimize non-specific binding and, therefore, was also used in the controls.

Results

Functional calcium channel expression in early postnatal PCs

Somatic currents through voltage-activated calcium channels in PCs were recorded using tight seal, whole cell recordings from cells acutely dissociated from wild-type or *tottering* mouse cerebellar vermis. On average, peak Ba²⁺ current was significantly lower in *tottering* than in wild-type PCs at P6 (-1.83 $\pm$ 0.26 nA vs. -2.78 $\pm$ 0.33 nA, n=12 and 11, respectively, p<0.03). The smaller current in *tottering* PCs cannot be attributed to a reduction in cell size, because the average membrane capacitance (an indicator of cell membrane surface area) was actually larger in *tottering* than in wild-type PCs at this age (34.0 $\pm$ 4.0 pF vs. 24.7 $\pm$ 5.9 pF, respectively,

n=11, Fig. 1). This disparity in capacitance persisted until P15. Thus, the average current density in *tottering* PCs at P6 was ~50% lower than in their wild-type counterparts at P6 (-54 ± 4.6 pA/pF vs. -113.0 ± 9.7 pA/pF, respectively). This indicates that significant functional differences are apparent between PCs of the two genotypes as much as two weeks before the behavioral dysfunctions appear (Green and Sidman, 1962).

To identify which types of voltage-activated calcium channels were altered in these cells, we measured Ba^{2+} currents before and after exposure to saturating concentrations of selective Ca^{2+} channel antagonists. As shown in Fig. 1A and C, the P-type channel antagonist, ω -agatoxin IVA (300 nM), inhibited an average $77 \pm 9\%$ (n=11) of voltage-activated current in wild-type PCs at P6. In contrast, the *tottering* P-type current averaged only $46 \pm 3\%$ of the total at P6 (n=11), significantly different from wild-type ($p < 0.001$). The remaining “non-P type” current was not significantly different in the two groups of cells at this age (-28 ± 3 pA/pF for wild-type and -29 ± 2 pA/pF for *tottering*, n=11 for both). This result indicated that the reduced current density of immature *tottering* PCs can be attributed to a selective reduction in the functional expression of P-type channels.

In the continued presence of ω -agatoxin IVA to block P channels, N-type currents were isolated by applying the selective N channel antagonist, ω -conotoxin GVIA (at 1 μM). Overall, N-type current densities were equivalent for wild-type and *tottering* PCs at P6, -5 ± 2 pA/pF (n=4, wild-type) and -7 ± 1 pA/pF (n=5, *tottering*) (Fig. 1C). Thus, unlike P channels, functional expression of N channels in PCs was unaltered at this early postnatal age.

L-type current was then isolated by the addition of the selective L channel antagonist, nimodipine (1 μM), applied in the continuous presence of the P- and N-type channel antagonists. Because the efficacy of blockade by dihydropyridine antagonists, such as nimodipine, is enhanced at depolarized potentials (Sanguinetti and Kass, 1984), we measured the inhibitory effect at the end of a prolonged (500 ms) depolarizing test pulse (Fig. 1A, lower panels). The average density of nimodipine-sensitive current at P6 was also not significantly different in the *tottering* and wild-type PCs, being -7.7 ± 1.6 pA/pF in wild-type and -10.8 ± 0.9 pA/pF in *tottering* (n=8 for both).

Although the T- and R-type calcium channels are less amenable to pharmacological isolation than the other Ca^{2+} channel types, they can be distinguished by taking advantage of their different inactivation kinetics. T-type channels undergo rapid and complete inactivation during prolonged depolarization (Perez-Reyes, 2006) whereas R-type channels inactivate more slowly (Stotz and Zamponi, 2001). Thus, by applying a sufficiently long depolarizing voltage step (500 ms), the T-type current fully inactivates, appearing as a transient (Fig. 1A lower panels). After inactivation of the T-type current, with P-, N- and L-type channels blocked, the sustained current remaining was R-type current. At P6, wild-type and *tottering* PCs exhibited similar levels of R- and T-type current densities. R-type current densities were -4.2 ± 0.6 pA/pF (n=4) in wild-type and -3.2 ± 0.5 pA/pF (n=5) in *tottering*, and T-type current densities were -15.5 ± 1.8 pA/pF (n=4) in wild-type and -14.6 ± 2 pA/pF (n=5) in *tottering*. Thus, P-type channels were the only calcium channels whose functional expression was significantly affected in *tottering* PCs at P6.

Alterations in functional calcium channel expression in PCs with postnatal age

To compare the developmental changes in PC calcium channels from wild-type and *tottering* mice, voltage-activated current densities were measured over time between P6 to P15. Wild-type PCs exhibited a steady increase in total current density from P6-P9 and by P15 had increased overall by 1.7 fold (Fig. 2). Current densities of PCs from *tottering* exhibited a very different developmental progression; between P6 and P9, the total current density remained constant (at ca. -55 pA/pF). By P8-9, the total current density in *tottering* represented only

~40% of that in wild-type PCs. Between P8 and P15, however, a 2.7-fold increase in average current density occurred in *tottering* PCs. This delayed increase was so pronounced that, by the end of the second postnatal week, the total current density in *tottering* PCs reached levels not significantly different from those in wild-type cells (Fig. 2).

To determine which Ca^{2+} channel types contributed to the changes in current density, we used the pharmacological dissection of currents described above and in Figure 1. The steady rise in wild-type current density observed throughout the time period examined was due largely to an increase in P channels (Figs. 3A, 4A,C). Similarly, in *tottering*, the P current underlay the majority of the changes in total current density (Figs. 3A, 4A,C), increasing 3.2-fold between P8–P15.

We next tested whether concomitant changes in density occurred in other channel types as well, producing a change in channel complement as previously reported for adult *tottering* cerebella (Zhou et al., 2003). For both wild-type and *tottering* PCs between P6 and P9, the current through L-type channels was small and unchanging (at ca. -8 pA/pF). Over the ensuing week, however, L current density increased almost two-fold in *tottering* PCs, while it was unchanged in wild-type PCs (-16.3 ± 2.4 pA/pF in *tottering* and -8.7 ± 1.8 in wild-type, $n=9$, $p=0.002$) (Figs. 3C 4A,C). Thus, *tottering* PCs exhibit aberrant functional expression of L-type Ca^{2+} channels nearly a week before the appearance of motor deficits.

Functional expression of N-type channels in wild-type PCs dropped more than 3.5-fold from P6 to P15, from -5.3 ± 1.2 pA/pF ($n=4$) to -1.5 ± 0.4 pA/pF ($n=14$), $p=0.001$ (Figs. 3B, 4A,C). In *tottering* PCs, a deviation from wild-type was seen for the N-type current at P7 and P9 (also see below). Levels of R- and T-type channels were equivalent for wild-type and *tottering* PCs over this entire developmental window (Fig. 3D and 3E). Taken together, these results indicate that the mutation in the P-type channel in *tottering* is selectively accompanied by functional increases in L-type channels (and N-type at some ages), while R-, and T-type channels remained relatively unchanged.

Qualitative differences in the variance of the wild-type and *tottering* PCs became apparent by P15. Wild-type PCs were relatively homogeneous with regard to their Ca^{2+} channel complement, with high levels of P current, prominent T current, low levels of L current, and virtually no N current. *Tottering* PCs as a group showed more variation, particularly in regard to the levels of P current density as indicated by the non-normal distribution of values (Fig. 4B, right). Two groups of *tottering* PCs were apparent, one of which exhibited values for mean P current density remarkably close to the wild-type value of -157 ± 20 pA/pF ($n=16$). This group of cells was in the majority (7/12) with a mean P current density of -132 ± 19 pA/pF, whereas the remaining 5 cells exhibited a significantly lower density, -31 ± 3 pA/pF ($p<0.001$). These two groups of *tottering* PCs were otherwise indistinguishable based on morphological criteria, cell capacitance, and other physiological characteristics of PCs (see Experimental Procedures). Furthermore, two of these 5 cells with a low P-type current also exhibited a mean N-type current density that was significantly higher than that calculated from the entire sample of P15 *tottering* PCs (-73 ± 3 pA/pF vs. -5.4 ± 4.8 pA/pF, $n=2$ and 10 respectively, $p<0.001$). The current densities of all other Ca^{2+} channel types were equivalent in the two groups. These data indicate that the *tottering* mutation results in anomalous patterns of expression in a subset of PCs.

Discussion

Results reported here demonstrate that the P-type calcium channel mutation in *tottering* gives rise to significant changes in PC calcium channel complement over the first two weeks of postnatal development. Early in this window (by P8), the only manifestation of the mutation

was a ~75% reduction in P current density, while later in development (by P15) a two-fold enhancement of L current density was apparent in *tottering* PCs as compared to their wild-type counterparts. Given that the ataxia and seizures characteristic of the mutant do not manifest before 3 weeks of age, these results suggest the possibility that changes in calcium channel complement may be an early precipitating factor for the behavioral phenotype.

The reductions in macroscopic P current in postnatal *tottering* PCs reported here are most simply explained by a reduction in the surface expression of P channels, since the mutation has little effect on the gating and permeation properties of the channel itself (Wakamori et al., 1998; Pietrobon, 2002). However, a differential expression of alternative splice variants in wild-type and *tottering* PCs, for example, (Chaudhuri et al., 2005) or alterations in channel modulation (Mintz and Bean, 1993; Zhou et al., 2003) might also contribute to the changes in current density reported here. Although such a possibility cannot be ruled out, our results suggest that P-type calcium channels in PCs from *tottering* appear on the surface membrane later in development, reach a lower density, and exhibit more heterogeneity than their wild-type counterparts.

Given the variety of calcium-dependent processes known to contribute to normal PC function—gene expression, intracellular Ca^{2+} homeostasis, spontaneous firing properties and synaptic plasticity—the aberrations in calcium channel complement reported here are likely to have multiple functional consequences.

Consequences of postnatal deficits in P-type current

Voltage-gated calcium channels are key regulators of calcium-dependent gene transcription (Finkbeiner and Greenberg, 1998). Although L channels may be the dominant transcriptional regulators, P channels also contribute both to calcium-dependent transcription (Fields et al., 2001, Sutton et al., 1999) and to nuclear Ca^{2+} levels in early postnatal PCs (Gruol et al., 2005). Thus, the results reported here showing early 75% reductions in P-type current density and later two-fold increases in L-type current density in PCs during these first two weeks after birth are likely to bring about significant changes in transcription in the mutant and produce multiple effects. Further, such effects would likely not be limited to PCs, as other cell types with dominant expression of P channels may be similarly affected.

The Ca^{2+} influx through calcium channels is also selectively coupled to a number of other effector responses in PCs that might contribute to motor dysfunction in *tottering*. Of particular note are both large and small conductance calcium-activated potassium channels (BK and SK), which are key negative regulators of the spontaneous firing in PCs (Womack and Khodakhah, 2002). BK and SK channels are themselves selectively activated by Ca^{2+} influx through P-type Ca^{2+} channels in PCs (Womack et al., 2004), making it likely that changes in P channel expression directly affect PC excitability. In fact, blockade of P-type Ca^{2+} channels in wild-type PCs dramatically affects PC firing rates, as does direct blockade of the potassium channels (Womack and Khodakhah, 2002). Furthermore, the selective blockade of SK channels *in vivo*, through application of the channel antagonist apamin, promotes ataxia (Lallement et al., 1995). In support of this proposed mechanism for motor dysfunction in *tottering*, potassium channel blockers have been shown to inhibit stress-induced dystonia (Weisz et al., 2005). Another calcium channel mutant, *ducky*—which also displays reduced P-type current—exhibits altered frequency and regularity of spontaneous firing as well (Donato et al., 2006). Thus, the reductions in P-type Ca^{2+} channels that we report here for *tottering* would be expected to significantly alter PC firing rates and, thereby, promote motor abnormalities.

Consequences of late postnatal variability in Ca^{2+} channel complement

It is unclear whether the P15 *tottering* PCs characterized by unusually low P current density represent a functionally distinct population of PCs that persist into adulthood or, alternatively, disappear still later in postnatal development. The finding of cell-to-cell variability in PCs is not unprecedented. Considerable variation has been reported for calcium-dependent inactivation of calcium current in P10 wild-type PCs (Chaudhuri et al., 2005); furthermore, only subpopulations of PCs from *tottering* and *leaner* (another P channel mutant) exhibit elevated tyrosine hydroxylase expression (Austin et al., 1992). The two PC populations reported here would likely have significantly different firing properties based on their different calcium channel complements. Furthermore, such inhomogeneity (if found in the flocculus as well as the vermis) may underlie, in part, the reported decrease in the regularity of spiking recorded from PCs in the flocculus of *tottering* animals—the activity responsible for controlling eye movement; such circuit “noise” may help to explain *tottering*’s motor deficits (Hoebeek et al., 2005).

The differences in Ca^{2+} channel complement between the majority and minority samples, may serve to resolve a long-standing discrepancy between the results of Wakamori et al. (1998, in which low adult *tottering* current density was reported) and Dove et al. (1998, in which no significant differences were found). Our results indicate that the ratio of the two populations in a sample would be one key determinant of whether significant reductions are found on average.

Consequences of late postnatal increase in L-type current

As with P channels, changes in L channel expression are also likely to have acute physiological consequences. The increased expression of L channels that we report in two-week-old postnatal PCs is likely to be maintained in adult *tottering* animals, as increased L-type mRNA has been reported in adult PCs (Doyle et al., 1997; Campbell and Hess, 1999). Abundant pharmacological evidence also supports this conclusion. L channel antagonists (including nimodipine and nifedipine) administered to adult *tottering* animals, for example, prevent stress-induced dystonia, but are without effect in wild-type animals (Campbell and Hess, 1999). This finding, together with evidence that PCs in the cerebellar vermis are a focal point for such stress-induced seizures (Campbell and Hess, 1998; Abbott et al., 2000), raises the possibility that early elevations in Ca^{2+} influx through L channels in PCs bring about at least some of the motor dysfunction in *tottering*. Further support for this idea comes from experiments on wild-type animals in which the L channel agonist, BayK 8644, was applied *in vivo* and produced dystonia at concentrations well below those that induce seizures (Campbell and Hess, 1999). Finally, dantrolene-induced inhibition of intracellular Ca^{2+} release via ryanodine receptors in PCs (which are, themselves, activated by Ca^{2+} influx through L channels) also eliminates dystonia (Raïke et al., 2006). Interestingly, the ataxia (likely also of cerebellar origin) was unaltered by dihydropyridine administration (Campbell and Hess, 1999), indicating that reversible changes in L channel current do not directly underlie the ataxia in *tottering*.

The changes in L channel expression in PCs are interesting because Ca^{2+} influx through L channels is known to be a particularly important physiological regulator of these neurons. For example, L channel current underlies spontaneous, rhythmic oscillations in intracellular Ca^{2+} concentration in young (P4–P7) PCs (Liljelund, et al., 2000). Similar spontaneous Ca^{2+} oscillations, reported in other neurons, are known to alter several developmental processes, including neurite outgrowth and neurotransmitter phenotype (Spitzer et al., 1994; Gu and Spitzer, 1995; Gomez and Spitzer, 1999). In wild-type PCs, Ca^{2+} oscillations normally subside after P7 (Liljelund et al., 2000), at a time when P-type current is rising rapidly. Thus, we might anticipate that, in P15 *tottering* PCs, the unusually high L channel density and reduced P channel density might help to sustain Ca^{2+} oscillations (and the burst firing that drives them)

well beyond their appropriate developmental window, thereby maintaining the cells in a relatively immature state.

A two-fold elevation of the L current in postnatal PCs was also reported for cells from the P channel knockout mouse (Jun et al., 1999); as with *tottering*, the knockout exhibits dystonia and absence seizures and delayed cerebellar development. These animals do not survive to adulthood. *Tottering* is a mild Ca^{2+} channelopathy, by contrast (Lorenzon and Beam, 2000), with significant levels of P-type current. The fact that the L current elevation is triggered in both mouse models suggests that L channels are tightly regulated and readily upregulated following changes in P channel expression. It is tempting to propose that other P-type calcium channelopathies will also exhibit such compensatory L channel upregulation, even following modest reductions in P current. In addition, it would be intriguing if early treatment with L-type channel antagonists could abrogate or delay the development of motor dysfunction (as discussed above for dystonia in adult *tottering*) and/or prevent progression to cell death and degeneration that is characteristic of the most severe calcium channelopathies.

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Abbreviations

PCs	Purkinje Cells
TH	Tyrosine Hydroxylase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
pA	picoampere
pF	picofarad

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Figure 1. Ca^{2+} channel complement in PCs at postnatal day 6

A) Representative current recordings from 6 day-old wild-type and *tottering* PCs (as marked) in response to depolarizing voltage steps from -80 to 0 mV for 20 ms (upper panels) or from -80 to -10 mV for 500 ms (lower panels). Superimposed currents were taken under control conditions (a), then during exposure to ω -agatoxin IVA (b), then ω -agatoxin IVA + ω -conotoxin GVIA (c), then ω -agatoxin IVA + ω -conotoxin GVIA + nimodipine (d), each at saturating concentration, as noted in the text. B) Amplitude histograms illustrating the distribution of peak Ba^{2+} current densities measured in the sample of P6 wild-type (WT) and *tottering* (TG) PCs. C) Histogram illustrating the peak Ba^{2+} current densities through the pharmacologically-isolated calcium channel types recorded from wild-type (white bars) and *tottering* (grey bars) PCs (*, $p < 0.001$).

Figure 2. Functional Ca^{2+} channel density changes during postnatal development
 Plot illustrating the mean current density over time for PCs dissociated from wild-type (white circles) or *tottering* (black circles) animals from the ages shown on the abscissa. Error bars represent standard errors of the mean ($n = 8-24$ cells/point; *, $p < 0.001$).

Figure 3. Developmental changes in Ca^{2+} channel complement

Plots illustrating the mean current densities through the different Ca^{2+} channel types (noted on the ordinates) measured in PCs dissociated from wild-type (white circles) or *tottering* (black circles) animals from the ages shown on the abscissae. Error bars represent standard errors of the mean (n = 4–21 cells/point; *, $p < 0.001$, §, $p < 0.01$, ¥, $p < 0.05$).

Figure 4. Ca^{2+} channel complement in PCs at postnatal day 15

A) Representative current recordings from 15 day-old wild-type and *tottering* PCs (as marked) in response to depolarizing voltage steps from -80 to 0 mV for 20 ms (upper panels) or from -80 to -10 mV for 500 ms (lower panels). Superimposed currents were taken under control conditions (a), then during exposure to ω -agatoxin IVA (b), then ω -agatoxin IVA + ω -conotoxin GVIA (c), then ω -agatoxin IVA + ω -conotoxin GVIA + nimodipine (d), each at saturating concentration, as noted in the text. B) Amplitude histograms illustrating the distribution of peak Ba^{2+} current densities measured in the samples of P15 wild-type (WT) and *tottering* (TG) PCs. C) Histogram illustrating the peak Ba^{2+} current densities through the pharmacologically-isolated calcium channel types recorded from P15 wild-type (white bars) and *tottering* (grey bars) PCs (§, p<0.01; ¥, p<0.05).