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Alterations in Intracellular Calcium Ion Concentrations in Cerebellar Granule Cells of the CACNA1A Mutant Mouse, Leaner, During Postnatal Development

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Abstract

Maintaining calcium ion (Ca^{2+}) homeostasis is crucial for normal neuronal function. Altered Ca^{2+} homeostasis interferes with Ca^{2+} signaling processes and affects neuronal survival. In this study, we used homozygous leaner and tottering mutant mice, which carry autosomal recessive mutations in the gene coding for the α_{1A} pore forming subunit of $Ca_V2.1$ (P/Q-type) voltage-gated calcium channels (VGCC). Leaner mice show severe ataxia and epilepsy, while tottering mice are less severely affected. Leaner cerebellar granule cells (CGC) show extensive apoptotic cell death that peaks at postnatal (P) day 20 and continues into adulthood. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations in leaner and tottering mouse Purkinje cells have been described, but $[Ca^{2+}]_i$ concentrations have not been reported for granule cells, the largest neuronal population of the cerebellum. Using the ratiometric dye, Fura-2 AM, we investigated the role of Ca^{2+} homeostasis in CGC death during postnatal development by demonstrating basal $[Ca^{2+}]_i$, depolarization induced Ca^{2+} transients, and Ca^{2+} transients after completely blocking $Ca_V2.1$ VGCC. From P20 onward, basal $[Ca^{2+}]_i$ levels in leaner CGC were significantly lower compared to age-matched wild-type CGC. We also compared basal $[Ca^{2+}]_i$ levels in leaner and wild-type CGC to basal $[Ca^{2+}]_i$ in tottering CGC. Potassium chloride induced depolarization revealed no significant difference in Ca^{2+} transients between leaner and wild-type CGC, indicating that even though leaner CGC have dysfunctional P/Q-type VGCC, Ca^{2+} transients after depolarization are the same. This suggests that other VGCC are compensating for the dysfunctional P/Q channels. This finding was further confirmed by completely blocking $Ca_V2.1$ VGCC using ω-Agatoxin IV-A.

Keywords

Basal intracellular calcium; Cerebellum; Voltage-gated calcium ion channels; Calcium ion homeostasis; Leaner mutant mice

Calcium (Ca^{2+}) is a ubiquitous intracellular signaling messenger that is involved in all events taking place in the cells of the nervous system. Maintaining intracellular calcium ($[Ca^{2+}]_i$) homeostasis is crucial for normal neuronal function as alterations significantly affect neuronal survival. We are studying leaner mutant mice, which carry an autosomal recessive mutation in the CACNA1A gene, coding for the α1A pore forming subunit of $Ca_V2.1$ (P/Q-type) voltage-gated calcium channels (VGCC). Homozygous leaner mice

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exhibit cerebellar ataxia and absence epilepsy starting soon after postnatal (P) day 10-12. There is excessive loss of leaner cerebellar granule cells (CGC) starting at P15, which peaks at P20 and continues into adulthood (Lau et al. 2004; Bawa and Abbott 2008). Several autosomal dominant human neurological disorders are associated with mutations in the CACNA1A gene including; familial hemiplegic migraine, generalized epilepsy with ataxia, episodic ataxia type 2 and spinocerebellar ataxia type-6.

Although considerable work has been done to elucidate $[Ca^{2+}]_i$ concentrations in Purkinje cells of these mutant mice (Dove et al. 1998), no one has reported $[Ca^{2+}]_i$ concentrations in granule cells, which are the largest neuronal population of the cerebellum and peak granule cell death coincides with the onset of the ataxic phenotype. We measured basal $[Ca^{2+}]_i$ as an index of calcium homeostasis in CGC during postnatal development and compared the results with CGC from another mutant mouse, tottering. Tottering mice also carry an autosomal recessive mutation in the CACNA1A gene, but it is not in the same location as the leaner mutation, and these mice do not exhibit neuro-degeneration as juveniles or young adults (Fletcher et al. 1996).

According to Dove et al. (1998), there is a 60% decrease in Ca^{2+} currents in leaner Purkinje cells, and there is also three-fold reduced probability of channel opening. P-type calcium channels contribute approximately 90% of whole cell Purkinje cell Ca^{2+} current (Dove et al. 1998), whereas in granule cells P/Q-type constitutes 46% of whole cell Ca^{2+} current (Randall and Tsien 1995). In the second part of the experiment we looked at Ca^{2+} transient by depolarizing granule cells using potassium chloride and blocking P/Q-type currents using ω-Agatoxin IV-A.

Wild-type (+/+), homozygous leaner (tg^{la}/tg^{la}) and homozygous tottering (*tg/tg*) mice on an inbred C57BL/6 background were used. All mice were bred and housed at the Laboratory Animal Research and Resource facility at Texas A&M University. Details of leaner mouse management are described elsewhere (Bawa and Abbott 2008). Three male and three female wild-type and homozygous leaner mice at four postnatal age groups (P10, P20, P30, and P40) were used, whereas the comparison with homozygous tottering was done at P20 and P30. Procedures for animal use were approved by the Texas A&M University Laboratory Animal Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

Cerebellar granule cells were acutely isolated and the protocol was confirmed by different experiments as described in detail elsewhere (Bawa and Abbott 2008). Briefly, at the specified ages, mice were anesthetized using isoflurane and killed by decapitation. The cerebellum was removed, chopped into small pieces, and transferred to 50 ml chilled minimum essential medium with Earle's salts (MEM; Life Technologies inc., Rockville, MD), then transferred to MEM containing 1.5 U/ml protease (Sigma, St. Louis, MO, USA), and stirred. During stirring 0.2% DNase (Sigma) was added to digest genomic DNA released by damaged cells. The media containing cells was centrifuged at 1,000*g* for 10 min, and the cells were resuspended in MEM.

For basal $[Ca^{2+}]_i$ measurements, acutely isolated CGC were plated onto chambered slides (VWR, West Chester, PA, USA) and incubated at 37°C for 20 min using 95% O_2 and 5% CO_2. Ten microliter of 20% pluronic acid (Sigma) was added to 50 μg of Fura-2 AM and added to media to achieve a final concentration of 5 μM. Cells were incubated with the Fura-2AM at 37°C for 30 min. De-esterification of the dye was carried out by washing the cells twice with fresh MEM and incubating at 37°C for an additional 30 min. After de-esterification, the CGC were kept in phenol red free media (Sigma). Fura-2AM changes

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fluorescence intensity differentially at excitation wavelengths of 340 and 380 nm upon binding Ca^{2+}. The cells were exposed to dual excitation at 340 and 380 nm and fluorescence was monitored at 510 nm. Fluorescent images were acquired using a 40× UV objective on an Olympus 1X-70 microscope and a three-chip Hamamatsu ORCA-ER cooled charge-coupled device camera. Negative and positive controls were run to validate the experimental design as described in Bawa and Abbott (2008).

After measuring basal $[Ca^{2+}]_i$, we ascertained how $Ca_V2.1$ calcium channels in leaner mice behave when depolarized. Ca^{2+} transients were evoked by KCl-induced depolarization and imaged using Fura-2AM. Five wild-type and five leaner mice were used. After measuring basal $[Ca^{2+}]_i$ as mentioned-above, extracellular KCl was increased to 40 mM by superfusing granule cells with 20 μl of 1 mM KCl. K^+ induced Ca^{2+} transients were measured for 10 min and data points demonstrating peak $[Ca^{2+}]_i$ were used for comparison with the initial basal levels. Data are expressed as the peak $[Ca^{2+}]_i$ minus the initial basal $[Ca^{2+}]_i$ levels, which is referred as calcium transient. A minimum of 3–4 cells were analyzed from each animal for each age group and genotype.

To demonstrate the overall percentage of $Ca_V2.1$ current in leaner granule cells we used a specific $Ca_V2.1$ channel blocker, ω-Agatoxin IVA (Peptide International, Louisville, KY). Acutely isolated CGC from P20 wild-type and leaner mice were loaded with Fura-2AM. The cells were then incubated in 200 nM of ω-Agatoxin IVA for 20 min at room temperature, then depolarized with KCl. Peak $[Ca^{2+}]_i$ post depolarization was recorded, and data are expressed as the peak $[Ca^{2+}]_i$ minus the initial basal $[Ca^{2+}]_i$ and referred to as calcium transient.

Image capturing and *R*-values were calculated using Simple PCI Version 5.0.0.1503 Compix Inc. and Imaging System (Cranberry Township, PA). $[Ca^{2+}]_i$ was determined by the equation:

$$\left[Ca^{2+}\right]_i = K_D \ (F_{min}/F_{max}) \ [(R - R_{min}) / (R_{max} - R)] .$$

Background was subtracted from 340 and 380 fluorescence intensity before calculating the *R*-value. F_{min} and F_{max} indicate fluorescence intensity at 380 nm in the absence of Ca^{2+} and high Ca^{2+}, respectively. R_{min} and R_{max} represent the ratio of F340/380 in the absence of Ca^{2+} and high Ca^{2+}, respectively. The values used for F_{min}/F_{max}, R_{min}, and R_{max} were 8.36, 0.2, and 8.47, respectively, calculated using the calcium calibration kit (Molecular Probes, Carlsbad, CA, USA).

Data are presented as means ± standard error of mean. All data were analyzed by statistical software SPSS (Version 12.0.1 for windows) using General linear model (GLM)-univariate analysis of variance (ANOVA) at $\alpha = 0.05$. Significant differences among genotypes were interpreted using Tukey's HSD post-hoc test.

Mean basal $[Ca^{2+}]_i$ data in CGC during postnatal development are shown in Fig. 1. A GLM-univariate ANOVA indicated an overall significant difference between genotypes and postnatal days ($P < 0.001$). No significant difference in basal $[Ca^{2+}]_i$ was observed between wild-type and leaner CGC at P10, whereas basal $[Ca^{2+}]_i$ of wild-type CGC at P10 was significantly reduced compared to P20, P30, and P40 wild-type CGC. This could be due to the developmental expression of various VGCC and their subunits, since expression of α and β subunits of $Ca_V2.1$ Ca^{2+} channel is most evident after P10, and their co-expression is required for proper channel functionality (Tanaka et al. 1995). Starting from P20, there was reduced basal $[Ca^{2+}]_i$ in CGC of leaner mice compared to age-matched wild-type CGC ($P <$

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0.001). These findings are in contrast to leaner Purkinje cells, where basal $[Ca^{2+}]_i$ levels were not different from age-matched wild-type Purkinje cells (Dove et al. 1998). We speculate that this difference could be due to different type of Ca^{2+} channels present in CGC as compared to Purkinje cells in which 90% of calcium current is dependent on P/Q-type VGCC. Multiple VGCC are present in CGC that could be responding differently in leaner mice as a form of functional compensation and resulting in altered Ca^{2+} homeostasis and reduced basal $[Ca^{2+}]_i$.

Interestingly, the CGC of tottering mice also showed reduced basal $[Ca^{2+}]_i$ levels compared to wild-type CGC ($P < 0.001$) and the level was not different from leaner CGC ($P = 0.376$). Since tottering mouse CGC do not show excessive neuronal cell death, this suggests that reduced basal $[Ca^{2+}]_i$ alone is not directly responsible for neuronal cell death in leaner CGC. Therefore, we looked at depolarization induced Ca^{2+} transients in CGC during postnatal development.

KCl-induced depolarization is commonly used to observe Ca^{2+} transients in CGC. A control experiment was run to see if the observed changes in fluorescent intensity were due to extracellular Ca^{2+} influx. Isolated CGC were loaded with Fura-2AM and depolarized using KCl both in the presence and absence of extracellular Ca^{2+} in the media. A significant increase in $[Ca^{2+}]_i$ was observed in granule cells from both wild-type and leaner mice depolarized with KCl in the presence of extracellular Ca^{2+}, whereas no significant increase was observed in Ca^{2+}-free media (data not shown). The time course of change in $[Ca^{2+}]_i$ following depolarization of CGC with KCl exhibited two phases. The first phase was a transient peak, representing Ca^{2+} entry into the cells via Ca^{2+} channels and presumably calcium induced calcium release from intracellular stores. During the second phase (i.e., the plateau phase), $[Ca^{2+}]_i$ reaches an equilibrium as a result of continued depolarization and continuous buffering (Werth and Thayer 1994). Our experimental setup showed both phases of Ca^{2+} depolarization (data not shown). We were interested only in the first phase, the calcium transient, when there was peak $[Ca^{2+}]_i$ to understand the effect of the leaner mutation on whole cell Ca^{2+} currents in CGC.

General linear model-univariate ANOVA analysis showed no difference in Ca^{2+} transients between genotypes in all age groups. There was significant difference in Ca^{2+} transients in P10 wild-type CGC compared to CGC from P20, P30, and P40 wild-type mice (Fig. 2). The developmental changes observed in Ca^{2+} current are relevant to the processes of granule cell maturation and excitability. Our result is in line with large body of evidence suggesting Ca^{2+} currents are lower in immature granule cells (Randall and Tsien 1995). At P10, leaner CGC showed the same pattern as P10 wild-type CGC except, unlike wild-type CGC, leaner CGC showed no difference in Ca^{2+} transients between P10 and P20 (Fig. 2). This suggests either delayed maturation of CGC or developmental defects due to the $Ca_V2.1$-type channel mutation in leaner mice.

Our results suggest that even though leaner CGC have dysfunctional $Ca_V2.1$ calcium channels still there is no major change in overall Ca^{2+} transients between wild-type and leaner CGC post depolarization. This means that either there is a possible functional compensation by other VGCC for dysfunctional $Ca_V2.1$ channels or reduced Ca^{2+} buffering in CGC, such that the increased $[Ca^{2+}]_i$ occurring in leaner CGC is not buffered efficiently, leading to increased Ca^{2+} transients. Another possibility is the presence of both of the above mentioned mechanisms. We previously have observed decreased calretinin expression, the major Ca^{2+} binding protein, in leaner CGC compared to age-matched wild-type granule cells (Nahm et al. 2002). Reduced calretinin expression in leaner granule cells could exaggerate the response to K^+-induced $[Ca^{2+}]_i$ increase due to lower Ca^{2+} buffering capacity. Another interpretation could be that $Ca_V2.1$ Ca^{2+} channels are not contributing a

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major portion of the whole cell Ca^{2+} current in leaner CGC. To test this hypothesis, we depolarized CGC in the presence and absence of the specific $Ca_V2.1$ channel blocker, ω-Agatoxin IV-A, which is known to block both P- and Q-type Ca^{2+} currents on the basis of different inactivation kinetics and sensitivity (Randall and Tsien 1995).

Results using ω-Agatoxin IV-A showed a significant reduction of almost 40% in depolarization induced Ca^{2+} transients in CGC of P20 wild-type mice. Our data are in agreement with a previous report that approximately 45% of the Ca^{2+} current in CGCs can be accounted for by $Ca_V2.1$ VGCC (Randall and Tsien 1995). However, ω-Agatoxin IV-A was not able to significantly reduce depolarization induced Ca^{2+} transients in leaner CGC (Fig. 3). Although, there was 17% reduction in agatoxin-treated whole cell Ca^{2+} transients in CGC of leaner mice, the response was not significantly different from nonagatoxin treated Ca^{2+} transients. This suggests that there is a functional compensation for $Ca_V2.1$ calcium channels by other VGCC.

The types of channels that are compensating for dysfunctional $Ca_V2.1$ channels in leaner CGC are not known. CNS neurons typically have diverse subtypes of Ca^{2+} channels that overlap in function. Recently, Etheredge et al. (2007) reported functional upregulation of Ca_V1 (L-type) channels to compensate for reduced $Ca_V2.1$ channel function in basal forebrain neurons from leaner mice. Like CGC and unlike Purkinje cells, basal forebrain neurons possess the full complement of Ca^{2+} channel subtypes (Etheredge et al. 2007). Similarly, $Ca_V2.2$ (N-type) channels have been shown to compensate for reduced P-type function in tottering mice (Leenders et al. 2002). The T-type calcium channel α_{1G} subunit is shown to be differently regulated in leaner cerebellar Purkinje and granule cells (Nahm et al. 2005). While cerebellar Purkinje cells show increased α_{1G} subunit expression, CGCs show decreased α_{1G} subunit expression (Nahm et al. 2005). Thus, it seems that the genetic alteration in $Ca_V2.1$ Ca^{2+} channels causes different consequences in Ca^{2+} channel expression and functions of cerebellar Purkinje and granule cells.

In conclusion, we report for the first time basal and depolarization induced $[Ca^{2+}]_i$ in CGC of leaner mice using the ratiometric dye Fura-2AM. Our study showed that there is reduced basal $[Ca^{2+}]_i$ in leaner CGC; however, the depolarization induced Ca^{2+} transients in leaner CGC are similar to wild-type CGC that can be attributed, at least in part, to reduced Ca^{2+} buffering in CGC that has been reported previously (Nahm et al. 2002). However, blocking entire $Ca_V2.1$ Ca^{2+} channels did not reduce whole cell current, indicating functional compensation by other VGCC. Future research in this area would be to elucidate the VGCC that are compensating for dysfunctional $Ca_V2.1$ Ca^{2+} channels in CGC of leaner mice.

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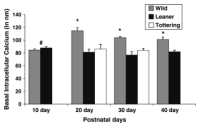

Fig. 1.

Mean basal $[Ca^{2+}]_i$ in cerebellar granule cells (CGC) during postnatal development. Graph shows mean basal $[Ca^{2+}]_i$ data from isolated CGC of wild-type (Wild), leaner (Leaner), and tottering (Tottering) mice ($n = 6$). The basal $[Ca^{2+}]_i$ level in wild-type CGC was significantly higher than observed in leaner and tottering CGC at P20 and P30 ($P < 0.001$). There also were significant differences between wild-type and leaner basal $[Ca^{2+}]_i$ at P40 ($P < 0.001$). However, no significant difference in basal $[Ca^{2+}]_i$ between wild-type and leaner CGC at P10 was observed. "*" indicates significant differences between wild-type and other genotypes and "#" indicates significant differences within the wild-type mouse groups. Data were analyzed using GLM-univariate analysis of variance at $\alpha = 0.05$

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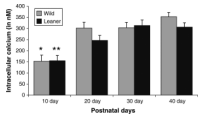

Fig. 2.

Mean calcium transients in cerebellar granule cells (CGC) of wild-type (Wild) and leaner (Leaner) mice during postnatal development. *Graph* shows mean Ca^{2+} transient data in leaner and wild-type CGC after they were depolarized using 40 mM KCl. A minimum of 3–4 cells from each mouse were analyzed for each age group and genotype. There were no significant differences between wild-type and leaner CGC for all age groups observed. However, Ca^{2+} transients in P10 wild-type CGC were different from the entire wild-type group ("*", $P < 0.05$) and Ca^{2+} transients in P10 leaner CGC were different from P30 and P40 ("**", $P < 0.05$). Data were analyzed using GLM-univariate analysis of variance at $\alpha = 0.05$

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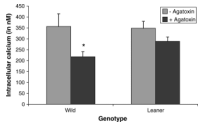

Fig. 3.

Mean Ca^{2+} transients data from leaner and wild-type cerebellar granule cells (CGC) after they were incubated in the presence or absence of ω-Agatoxin IV-A and depolarized with KCl. Reduced Ca^{2+} transients were observed only in wild-type (Wild) but not in leaner (Leaner) CGC after incubation with the $Ca_V2.1$ channel specific blocker ω-Agatoxin IV-A ($P < 0.05$; $n = 30$). Data were analyzed using GLM-univariate analysis of variance at $\alpha = 0.05$

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