Age-Dependent Contribution of P/Q- and R-Type Ca^{2+} Channels to Neuromuscular Transmission in *Lethargic* Mice

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ABSTRACT

β -Subunits of voltage-gated calcium channels (VGCCs) regulate assembly and membrane localization of the pore-forming α_1 -subunit and strongly influence channel function. β_4 -Subunits normally coassociate with α_{1A} -subunits which comprise P/Q-type ($\text{Ca}_v2.1$) VGCCs. These control acetylcholine (ACh) release at adult mammalian neuromuscular junctions (NMJs). The naturally occurring *lethargic* (*lh*) mutation of the β_4 -subunit in mice causes loss of the α_1 -binding site, possibly affecting P/Q-type channel expression or function, and thereby ACh release. End-plate potentials and miniature end-plate potentials were recorded at hemidiaphragm NMJs of 5–7-week and 3–5-month-old *lh* and wild-type (wt) mice. Sensitivity to antagonists of P/Q- [ω -agatoxin IVA (ω -Aga-IVA)], L- (nimodipine), N- (ω -conotoxin GVIA), and R-type [$\text{C}_{192}\text{H}_{274}\text{N}_{52}\text{O}_{60}\text{S}_7$ (SNX-482)] VGCCs was compared in

juvenile and adult *lh* and wt mice. Quantal content (*m*) of adult, but not juvenile, *lh* mice was reduced compared to wt. ω -Aga-IVA (~60%) and SNX-482 (~45%) significantly reduced *m* in adult *lh* mice. Only Aga-IVA affected wt adults. In juvenile *lh* mice, ω -Aga-IVA and SNX-482 decreased *m* by >75% and ~20%, respectively. Neither ω -conotoxin GVIA nor nimodipine affected ACh release in any group. Immunolabeling revealed α_{1E} and α_{1A} , β_1 , and β_3 staining at adult *lh*, but not wt NMJs. Therefore, in *lh* mice, when the β -subunit that normally coassociates with α_{1A} to form P/Q channels is missing, P/Q-type channels partner with other β -subunits. However, overall participation of P/Q-type channels is reduced and compensated for by R-type channels. R-type VGCC participation is age-dependent, but is less effective than P/Q-type at sustaining NMJ function.

Introduction

Voltage-gated calcium channels (VGCCs) of the high-voltage-activated (HVA) class are composed of α_1 -, β -, $\alpha_2\delta$ -, and sometimes γ -subunits (Tsien et al., 1991). The α_1 -subunits form the Ca^{2+} -selective pore and determine most of the subtype-specific attributes of VGCCs (Zhang et al., 1993; Catterall, 1995, 1998). Five α_1 -subunits exist for neuronal HVA VGCCs (Tsien et al., 1991; Catterall, 1995). The α_{1A} -, α_{1B} -, and α_{1E} -subunits comprise the P/Q- ($\text{Ca}_v2.1$), N- ($\text{Ca}_v2.2$), and R-type ($\text{Ca}_v2.3$) VGCCs, respectively. The α_{1C} - and α_{1D} -subunits are found in neuronal L-type VGCCs ($\text{Ca}_v1.2$ – 1.3) (Tsien et al., 1991; Catterall, 1995; Catterall et al., 2005).

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VGCC β -subunits regulate the assembly and membrane localization of the α_1 -subunits. They also modulate current amplitude, rate, and voltage dependence of activation and inactivation, as well as ligand-binding sites (Catterall, 1995; Chien et al., 1995; Walker and De Waard, 1998; Brice and Dolphin, 1999). Four β -subunits (β_{1-4}) exist; each is encoded by a separate gene (Buraei and Yang, 2010). Distinct pairings of β -subunits with given α_1 -subunits occur (Day et al., 1998). This pairing is essential for proper targeting, membrane insertion, channel density, kinetic parameters, and interactions of the channel with vesicular release site proteins (Wittmann et al., 2000; Murakami et al., 2003). In the absence of the normally associating β -subunit, alternate β -subunits can interact with α_1 -subunits to restore most of the VGCC's functions, albeit in an altered manner (Burgess et al., 1999; Qian and Noebels, 2000).

The β_4 -subunit is normally widely expressed in mammalian brain (Ludwig et al., 1997). It typically associates with the α_{1A} -subunit of P/Q-type VGCCs (Wittmann et al., 2000). Spontaneous mutations in the β_4 -subunit cause several neurological syndromes in mice (Burgess et al., 1997; Burgess and Noebels, 1999). The *lethargic* (*lh*) mutation is one such example. *Lethargic* mice exhibit ataxia, *lethargic* behavior, spike-wave epilepsy, and paroxysmal dyskinesia. The mutation includes loss of the α_1 -binding site (Burgess et al., 1997; Burgess and Noebels, 1999), disrupting the normal coupling found in functional

ABBREVIATIONS: ACh, acetylcholine; ω -Aga-IVA, ω -agatoxin IVA; ω -CTx-GVIA, ω -conotoxin GVIA; EDL, extensor digitorum longus; EPP, end-plate potential; HVA, high-voltage-activated; *lh*, *lethargic*; *m*, quantal content; MEPP, miniature end-plate potential; nACh, nicotinic acetylcholine; Nim, nimodipine; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; PND, postnatal day; SNX-482, $\text{C}_{192}\text{H}_{274}\text{N}_{52}\text{O}_{60}\text{S}_7$; *tg*, *tottering*; VGCC, voltage-gated calcium channel; wt, wild type.

P/Q-type channels, a principal regulator of neurotransmitter release. Loss of P/Q channels, their replacement by other VGCC subtypes, or even substitution of other β -subunits for β_4 could markedly alter neurotransmitter release.

Although mature mammalian motor nerve terminals contain almost exclusively P/Q-type VGCCs (Uchitel et al., 1992; Katz et al., 1995), under certain specific conditions, subtypes of VGCC that are not normally associated with acetylcholine (ACh) release at motor nerve terminals can assume control (Flink and Atchison, 2002; Urbano et al., 2003; Pagani et al., 2004; Pardo et al., 2006; Kaja et al., 2007a). Adaptation to loss of the α_{1A} -subunit obviously involves substitution of other α_1 -subunits; however, the impact of loss of a specific β -subunit, in this case β_4 , is less clear. Normally, β_4 should combine with α_{1A} to form functional P/Q channels to support ACh release. In its absence, other α_{1A} -containing VGCCs may populate adult mammalian neuromuscular junctions (NMJs). If they do, they must substitute other β -subunit(s) which may alter their function or expression. McEnery et al. (1998b) suggested that β_4 expression was required for VGCC maturation at the synapse. In the forebrain and cerebellum of *lh* mice, there is increased expression of the β_{1b} and coassociation with the α_{1B} -subunit. Additionally, in situ hybridization histochemistry demonstrated that *lh* brain exhibits increased expression of β_3 mRNA (Lin et al., 1999). The *lh* mutation did not compromise P/Q- or N-type VGCC function at hippocampal Schaffer collateral synapses, implicating rescue of presynaptic Ca^{2+} currents by other available β -subunits (Qian and Noebels, 2000). Therefore, when the β_4 -subunit is absent, compensatory mechanisms are activated, yet this compensation is not fixed, and seems to be age- and tissue-dependent. A prior study of neuromuscular transmission of 5–7-week-old *lh* mice reported no reduction in evoked release of ACh and almost exclusive control by P/Q-type channels (Kaja et al., 2007a). However, the period of development of VGCC phenotype extends beyond 6 weeks, and adult *lh* mice could have a pronounced reduction in neuromuscular transmission as development progresses due to substitution of other HVA VGCC subtypes for P/Q-type due to lack of the β_4 -subunit. Additionally, the ability of R-type VGCCs to contribute to ACh release in *lh* mice was not tested. In adult *tottering* (*tg*) mice, in which the α_{1A} -subunit is mutated, R-type channels play a major role in regulating ACh release (Pardo et al., 2006), whereas in juvenile *tg* mice, R-type contribution is minor (15%) (Kaja et al., 2006).

Consequently, the present study was designed to compare 1) which VGCC subtypes, as determined pharmacologically, control ACh release at the motor nerve terminals of adult and juvenile *lh* mice; 2) which β -subunit(s) substitute for the β_4 in the adult; and 3) whether R-type channels played a differential, age-dependent role. We hypothesized that P/Q-type channels would be replaced with other HVA VGCC subtypes, including L- ($\text{Ca}_v1.2$), N-, or R-type, as occurs when P/Q-type channels become dysfunctional (Qian and Noebels, 2000; Pardo et al., 2006; Kaja et al., 2007b) or decrease in number (Flink and Atchison, 2002; Nudler et al., 2003; Pagani et al., 2004), and that loss of β_4 would in turn be compensated by replacement with other β -subunits. The functional consequences of either α_1 - and/or β -subunit substitution could be responsible for the *lh* phenotype.

Materials and Methods

Mice. Breeding pairs of heterozygous *Cacnb4^{lh4J}* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently

maintained in a breeding colony at Michigan State University Laboratory Animal Resources (East Lansing, MI). Litters were genotyped at weaning, 3 weeks after birth. Homozygous mice (*lh*) were also identified by their characteristic phenotype consisting of a mild ataxia, wobbly gait behavior, and smaller body size. Adult mice (3–5 months of age) and juvenile mice (5–7 weeks of age) were used. All experiments were performed in accordance with the local university (Michigan State University Laboratory Animal Resources) and national guidelines (National Institutes of Health) and were approved by the University Animal Use and Care Committee.

Chemicals. Nimodipine was purchased from Sigma-Aldrich (St. Louis, MO), ω -agatoxin IVA (ω -Aga-IVA) from Alomone Labs (Jerusalem, Israel), ω -conotoxin GVIA (ω -CTx-GVIA) from Bachem (Torrance, CA), and SNX-482 ($\text{C}_{192}\text{H}_{274}\text{N}_{52}\text{O}_{60}\text{S}_7$; structure in Newcomb et al., 1998, 2000) from Ascent Scientific (Princeton, NJ). HEPES, EGTA, paraformaldehyde, Triton X-100, and tetramethylrhodamine α -bungarotoxin were all purchased from Sigma-Aldrich. μ -Conotoxin GIIIB and antibodies against the various α_1 -subunits were obtained from Alomone Labs. The presence of the various β -subunits was tested using antibodies for β_1 , β_2 , and β_4 (Neuromab, University of California, Davis, CA) and β_3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (heavy + light chains) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Pacific Blue goat anti-mouse IgG (heavy + light chains) was obtained from Invitrogen (Carlsbad, CA). All antibodies were used in a dilution of 1:200.

Electrophysiology. Animals were sacrificed by decapitation following anesthesia with 80% CO_2 and 20% O_2 . The diaphragm muscle with its attached phrenic nerves was then removed and the hemidiaphragm pinned out at resting tension in a Sylgard-coated chamber. For control recordings, the tissue was perfused continuously at a rate of 1–5 ml/min with oxygenated (100% O_2) physiological saline solution containing 137.5 mM NaCl, 5.0 mM KCl, 1 mM MgCl_2 , 11 mM D-glucose, 4 mM HEPES, and 2 mM CaCl_2 and maintained at room temperature (23–25°C). The pH was adjusted to 7.4 at room temperature using NaOH. Muscle action potentials were inhibited by pretreating the tissue with 2.5–4 μM μ -conotoxin GIIIB for 15 minutes. This toxin preferentially blocks muscle Na^+ channels (Cruz et al., 1985; Hong and Chang, 1989) and thus suppresses muscle contractility. Preparations were continuously perfused with physiological saline at a rate of 1–5 ml/min, and were retreated with 2.5–4 μM μ -conotoxin GIIIB for 15 minutes, after ~60–90 minutes, to maintain contractile block. This allowed end-plate potentials (EPPs) to be recorded from intact myofibers without depressing ACh release (high $[\text{Mg}^{2+}]$, low $[\text{Ca}^{2+}]$) or blocking postjunctional ACh receptors (*d*-tubocurarine) (Pardo et al., 2006). Separate preparations were used for each experiment, and a given hemidiaphragm received only one treatment.

Relative contributions of specific HVA VGCC subtypes were examined using antagonists with relative subtype specificity. Involvement of L-type channels in ACh release was examined using the L-type antagonist nimodipine (Nim) (Atchison, 1989). Paired comparisons were made for each preparation between the drug-free treatment (control) and following application of Nim. Values are expressed as the percentage of quantal content (*m*) from Nim-treated preparations to that of preparation before addition of the drug (control). Likewise, sensitivity to ω -CTx-GVIA, SNX-482, and ω -Aga-IVA was used to test for the contribution of N-, R-, and P/Q-type Ca^{2+} channels, respectively, to ACh release at *lh* motor nerve terminals. Cd^{2+} was used to block all Ca^{2+} channels nonspecifically. The P/Q-, N-, and R-type antagonists are all essentially irreversible, so only one toxin or drug was applied to any preparation. The hemidiaphragm was preincubated in 5 ml of solution containing ω -Aga-IVA, SNX-482, or ω -CTx-GVIA for 1 hour before commencing electrophysiological recordings. Toxin experiments involved recording at 5–10 end plates for at least 5 minutes each, as a control prior to toxin administration. Subsequently, 5–10 end plates were again sampled for 5 minutes each following toxin exposure. The last end plate sampled, prior to treatment, was the first one sampled after treatment. The solution was constantly aerated with

100% O₂ during the exposure. For experiments involving Nim and Cd²⁺, the hemidiaphragm was superfused with the constantly oxygenated (100% O₂) solution in which the compound was diluted. ω -Aga-IVA, ω -CTx-GVIA, and SNX-482 were used at 100 (adult) to 200 nM (young), 3 μ M, and 1 μ M, respectively, and diluted in 5 ml of physiological saline solution. Nim and Cd²⁺ were used in concentrations of 10 μ M and diluted in 20 ml of physiological saline solution. These concentrations were chosen based on literature determining their effectiveness at murine neuromuscular junctions (Atchison, 1989; Xu et al., 1998; Santafé et al., 2000; Urbano et al., 2001, 2003; Flink and Atchison, 2002; Kaja et al., 2006).

Miniature end-plate potentials (MEPPs) and EPPs were recorded using intracellular glass microelectrodes (1.0 mm o.d.; WPI, Sarasota, FL) having a resistance of 5–15 M Ω when filled with 3 M KCl. Constant-current stimuli (0.5 Hz, 50 microseconds) were provided by a suction electrode coupled to a Grass SIU stimulus isolation unit (Grass Instruments, Quincy, MA) and Grass S48 stimulator. Signals were amplified using a WPI 721 amplifier, digitized using a PC-type computer and Axoscope 9.0 software (Axon Instruments, Foster City, CA), and analyzed using MiniAnalysis 6.0 software (Synaptosoft, Decatur, GA). Amplitudes of MEPPs and EPPs were normalized to a membrane potential of –75 mV, and recordings were rejected if the 10–90% rise time was greater than 1.5 milliseconds or if the membrane potential was more depolarized than –55 mV. Normalized EPPs were corrected for nonlinear summation (McLachlan and Martin, 1981). The *m* value was calculated using the ratio of the mean amplitudes of corrected EPPs and MEPPs (see Flink and Atchison 2003; Pardo et al., 2006).

The number of animals used in any given experiment is indicated in the respective figure legend. Measurements are expressed as the mean $\pm$ S.E.M. for *n* $\geq$ 5. Statistical significance between the various treatment groups was analyzed using a one-way analysis of variance (Prism; GraphPad Software Inc., San Diego, CA). Post-hoc differences among sample means were analyzed using Tukey's test. For all experiments, statistical significance was set at *P* < 0.05.

Protein Isolation and Western Blot Analysis. Western blots were not sufficiently sensitive to detect the scarcity of VGCC subunits in the presynaptic area of the diaphragm muscle (data not shown). Therefore, the protein levels of VGCC α_{1A} - and β -subunits of the cerebellum, which express Ca_v2.1 abundantly, were compared in adult *lh* and wild-type (wt) mice. The tissue sample was ground in a mortar containing 1 ml of 2 $\times$ lysis buffer with 50 μ l of each protease (20 $\times$ stock solution of pepstatin, leupeptin, ethylenediaminetetraacetic acid, EGTA, and protease inhibitors; Roche, Indianapolis, IN). The ground lysate was centrifuged for 10 minutes at 22,000g. The supernatant was stored at –80 $^{\circ}$ C. The protein concentration was determined using the bicinchoninic acid assay, and quantified using a Beckman DU 640 spectrophotometer (Beckman, Brea, CA). The proteins were loaded onto a 10% SDS (w/v) polyacrylamide gel and migrated at a constant current of 40 mA. They were then transferred to a nitrocellulose membrane at 4 $^{\circ}$ C and a constant voltage of 90 mV. The membrane was probed against β -actin (dilution 1:20,000) as a loading control, and against the α_{1A} - and β_{1-4} -subunits. The molecular weight markers used in the Western blots (Precision Plus Protein WesternC standards) are from Bio-Rad (Hercules, CA) and were used at 1:10 dilution.

Immunohistochemistry. Localization of the different Ca²⁺ channel α_1 -subunits at *lh* and wt mouse motor nerve terminals was compared using fluorescence immunohistochemistry in the extensor digitorum longus (EDL) muscles from animals whose diaphragms were used for pharmacological studies. The EDL is a homogeneous fast twitch type muscle; thus, concerns associated with myofiber type-dependent differences in structure or function of the neuromuscular junctions were minimized (Gertler and Robbins, 1978; Prakash et al., 1996). EDL muscles from adult wt and *lh* mice (*n* $\geq$ 5) were pinned out and fixed for 30 minutes in 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; composition –137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH₂PO₄, and 4.3 mM Na₂HPO₄, pH 7.4). Tissues were washed in

PBS for 1 minute and treated with 0.1% (w/v) Triton X-100 in PBS for 30 minutes, after which they were washed for 15 minutes with PBS and cryoprotected in 20% and 30% (w/v) sucrose, each for 24 hours. Tissue was then placed in optimal cutting temperature compound (Tissue Tek, Tokyo, Japan) in a plastic mold and stored at –20 $^{\circ}$ C until used. Longitudinal sections (20- μ m thick) were cut (Cryostat model Microm HM 525; Thermo Shandon Inc., Pittsburgh, PA) and mounted onto gelatin-coated slides. The tissue was subsequently stained with specific antibodies. α -Bungarotoxin was used as a marker for the muscle ACh receptors. The motor nerve ending was stained with antibodies against α_1 - and β_{1-4} -subunits. Preparations were viewed using a Nikon Eclipse TE 2000-U Diaphot-TMD microscope (Nikon, Melville, NY) with a Hamamatsu Orca 285 charge-coupled device camera (Bridgewater, NJ). Images were acquired using Metamorph software (Molecular Devices, Sunnyvale, CA).

Averages of the mean values of fluorescence obtained from all of the individual nerve terminals sampled were calculated for each specific subunit studied. Averaged values of fluorescence were compared between the *lh* and the wt preparations using values obtained from wt preparations as control. Subsequently, the percentage of juxtaposition of the green and the red dye was calculated by dividing the surface of each picture taken into an area of 5 $\times$ 5 squares for a total of 25 inner squares. Each inner square in which the green and the red dyes were juxtaposed was taken as 4% juxtaposition (Pardo et al., 2006).

Results

β_4 Is Replaced by Other β -Subunits in Cerebellum of Adult *lh* Mice. As *lh* mice lack the β_4 -subunit, we first wanted to determine whether this mutation affected the protein levels of the remaining β -subunits of adult *lh* mice. We initially tried to assay for protein level in the diaphragm and phrenic nerve. This technique has been used in the past for abundant motor nerve terminal proteins (Kalandakanond and Coffield, 2001). However, due to the scarcity of VGCC proteins present in this tissue and the poor sensitivity of the antibodies in Western blots, we were unable to detect any subunit (no bands were revealed in these blots; results not shown). We subsequently probed for the different VGCC subunits in the adult cerebellum. The β_4 -subunit is normally extensively expressed in the cerebellum (Ludwig et al., 1997), and this region of the brain is responsible for motor coordination. *lh* Mice exhibit poor motor coordination and balance (Dickie, 1964; Sidman et al., 1965), indicating that cerebellar dysfunction likely occurs. Because the β_4 -subunit normally coassociates with α_{1A} , we assayed for α_{1A} levels to determine if the absence of β_4 affects the levels of α_{1A} . Figure 1 shows a representative Western blot and composite results for VGCC protein levels in the cerebellum of *lh* mice. As reported by McEnery et al. (1998a), no β_4 immunoreactivity was detectable in *lh* mice. Interestingly, α_{1A} levels were not affected in the cerebellum, whereas β_1 and β_3 were significantly increased. Western blot assays showed that even though *lh* mice lack the β_4 -subunit, the protein levels of the α_{1A} -subunit are comparable for *lh* and wt animals (Fig. 1, A and B).

Nerve-Evoked ACh Release Is Decreased in Adult but Not in Juvenile *lh* Mice. When inducing evoked release of ACh, the amplitude of the EPPs and *m* in juvenile mice did not differ significantly between genotypes. The mean amplitude of EPPs was 14.3 $\pm$ 0.5 mV in wt and 15.6 $\pm$ 0.6 mV in *lh*, and the *m* values were 23.1 $\pm$ 0.6 in wt and 20.9 $\pm$ 1.1 in *lh*. However, in adult *lh* mice, there was a significant decrease in both EPP amplitude (18.8 $\pm$ 2.0 mV in wt and 7.9 $\pm$ 1.4 mV in *lh*) as well as *m* (18.8 $\pm$ 2.0 in wt and 5.3 $\pm$ 1.1 in *lh*) (Fig. 2).

Fig. 1. Effect of *lh* mutation on VGCC subunit protein levels. (A) Representative Western blots are depicted of VGCC subunits present in cerebellar proteins (40 μ g). Protein levels from adult *lh* and wt cerebellar preparations were probed with antibodies for α_{1A} and β_{1-4} . Six separate blots are shown: one each for the α_1 - and β -subunits and one for β -actin. The bands at 88 and 55 kDa both are attributable to β_1 as per the antibody manufacturer. Levels of α_{1A} are approximately equal in wt and *lh*, but there is no β_4 in *lh*. There is prominent expression of β_4 in wt cerebellum but not in *lh*. β -Actin was used as a loading control. (B) Relative levels of VGCC subunits in adult *lh* cerebellum. Protein levels were quantified using the program ImageJ (NIH, Bethesda, MD). Values are expressed as the percentage of the wt value. Each value represents the mean $\pm$ S.E.M. of seven animals. *Significant difference between the two genotypes ($P < 0.05$).

These results indicate that as *lh* mice age, defects in neuromuscular transmission become evident.

Evoked ACh Release Is Controlled by P/Q- and R-Type VGCCs in Adult *lh* Mice. Western blot results suggested that the *lh* mutation did not affect the α_{1A} protein levels as compared with their wt littermates. However, these mice are apparently compensating for the absence of β_4 with increased levels of β_1 and β_3 . This could lead to a change in sensitivity to VGCC antagonists in adult *lh* mice. We wanted to determine if atypical association of β_1 - and β_3 -subunits affected the pattern of VGCC expression at NMJs of adult

Fig. 2. Comparative effects of evoked release in adult and juvenile *lh* and wt mice. The *m* value was calculated for each NMJ preparation using the ratio of the average EPP amplitude to the average MEPP amplitude in juvenile and adult wt and *lh* mice. Each value represents the mean $\pm$ S.E.M. ($n \geq 4$). *Significant difference between the two genotypes ($P < 0.05$).

lh mice. Consequently, we tested the sensitivity of neuromuscular transmission to different VGCC antagonists.

Figure 3 compares the pharmacological contribution of VGCC control of motor nerve ACh release in *lh* and wt adult preparations. ω -Aga-IVA significantly decreased the *m* to $40.8 \pm 6.1\%$ and $24.3 \pm 2.3\%$ of toxin-free control in *lh* and wt animals, respectively. The difference between the two genotypes was not statistically significant ($P > 0.05$). SNX-482 significantly decreased the *m* to $54.3 \pm 8.5\%$ in *lh* mice, but not wt mice ($91.7 \pm 9.9\%$). Neither ω -CTx-GVIA nor Nim significantly altered the *m* of either genotype ($P > 0.05$). CdCl₂ (10 μ M) completely blocked EPPs in both genotypes (results not shown).

Coapplication of ω -Aga-IVA and SNX-482 preparations in adult *lh* reduced ACh release to a level similar to that observed with ω -Aga-IVA alone in wt mice. Taken together, these results indicate that, in adult *lh* animals, ACh release is controlled by P/Q- and R-type VGCCs, and that both types contribute to a similar extent. The contribution of L- and N-type VGCCs to control of ACh release was negligible in both wt and *lh* mice.

Effects of ω -Agatoxin IVA and SNX-482 on ACh Release of Young wt and *lh* Mice. In adult *lh* mice, R-type VGCCs play a significant role in ACh release at motor nerve terminals. A prior study (Kaja et al., 2007a) reported that P/Q-type VGCCs controlled ACh release in young *lh* mice, but did not test specifically for the presence of R-type VGCCs. As such, we sought to determine if R-type VGCCs contributed to ACh release at NMJs of 5–6-week-old *lh* mice. We first verified the sensitivity of ACh release in young *lh* mice to ω -Aga-IVA. To maintain consistency with the results of the Kaja et al. (2007a) study, we used a higher ω -Aga-IVA concentration (200 nM) for these experiments. The SNX-482 concentrations remained the same as in our experiments with adults. Unlike the situation in adult *lh* mice, EPP amplitudes of 5–7-week-old *lh* mice did not differ from wt. Moreover, both wt and *lh* mice had similar responses of ACh release to

Fig. 3. ACh release is controlled by P/Q- and R-type VGCCs in adult *lh* mice. Effect of VGCC antagonists on nerve-evoked ACh release from motor nerve terminals of adult *lh* and wt mice. The tissue of wt or *lh* mice was treated with VGCC antagonists: ω -Aga-IVA (100 nM), ω -Ctx-GVIA (3 μ M), Nim (10 μ M), or SNX-482 (1 μ M). ω -Aga-IVA reduced the *m* in *lh* animals to 40%, whereas the same concentration reduced the *m* in wt mice to 24%. This difference was not statistically significant ($P > 0.05$). Application of ω -Ctx-GVIA and Nim did not significantly change the *m* of either wt or *lh* mice. SNX-482 significantly reduced the *m* in *lh* animals, but not in wt mice. SNX-482 and ω -Aga-IVA were applied simultaneously to measure the combined effect of the two antagonists. Application of both toxins in *lh* animals had an additive effect of reducing *m* to the level caused in wt by ω -Aga-IVA alone. Conversely, SNX-482 had no additive effect over that of ω -Aga-IVA in wt. *Significant difference from the control pretreated preparation; #significant difference between genotypes. Data represent the mean $\pm$ S.E.M. ($n \geq 5$). Statistical significance was set as $P < 0.05$.

ω -Aga-IVA (Fig. 4); *m* was reduced to $19.1 \pm 4.1\%$ of control in wt and $25.4 \pm 3.1\%$ in *lh*. SNX-482 pretreatment for 1 hour did not alter *m* in wt, but reduced it to $80.2 \pm 5.9\%$ of control in *lh* mice (Fig. 4). Thus, whereas in juvenile *lh* mice P/Q-type VGCCs are mainly responsible for ACh release, R-type VGCCs are already beginning to contribute. This developmental switch is increased as *lh* mice mature to adulthood. The increase in R-type VGCC compensation in adult *lh* mice might be a form of synaptic plasticity to balance the reduced ACh release with increased age.

Spontaneous Release of ACh Is Altered in Juvenile, but Not Adult *lh* Mice. Table 1 compares MEPP amplitude and frequency for juvenile and adult *lh* NMJs. Whereas in adult *lh* mice, neither MEPP amplitude nor frequency was significantly different from wt, in juvenile *lh* mice, MEPP amplitude was significantly increased ($P < 0.05$). Although MEPP frequency also tended to be higher in *lh* juvenile mice, the effect was not significant ($P > 0.05$).

Immunohistochemical Localization and Identity of VGCC Subunits in Adult *lh* and wt Mice. Western blot results showed an increased level of β_1 - and β_3 -subunits in adult *lh* mice, whereas electrophysiological results indicated that P/Q- and R-type VGCCs control ACh release at adult *lh* NMJs. Moreover, work done by Pagani et al. (2004) provided evidence that the β_4 -subunit as well as β_{1b} and β_{2a} are present at the NMJs of adult wt mice (Pagani et al., 2004). Consequently, we wanted to determine to what extent the various α_1 - and β -subunits occurred at NMJs of adult *lh* and wt mice. As demonstrated by the representative immunostaining

Fig. 4. Effects of ω -Aga-IVA and SNX-482 on ACh release of young wt and *lh* mice. Treatment with ω -Aga-IVA significantly decreased *m* in both genotypes; however, SNX-482 only decreased *m* in *lh* but not wt. *Significant difference from the control pretreated preparations ($P < 0.05$). Data represent the mean $\pm$ S.E.M. ($n \geq 4$).

images in Fig. 5, wt animals have staining of α_{1A} (green), β_4 (blue), and α -bungarotoxin (red); adult *lh* mice have no immunostaining of β_4 , but do have α_{1A} . Moreover, the α_{1A} and postsynaptic staining clearly overlap even in the absence of β_4 . In wt, the three proteins also overlap completely. Figure 5 demonstrates extensive immunostaining for α_{1E} in *lh*, but not in wt. The α_{1E} staining again superimposes with that of α -bungarotoxin. Similarly at *lh* endplates, there is immunostaining of β_3 , and β_1 , which again overlaps with that of the α_1 (green) and α -bungarotoxin (red) (Figs. 6 and 7). In wt, β_1 was not present, and β_3 , although present, did not exhibit the abundance that it did in *lh* (Fig. 5).

Figure 8 compares the extent of juxtaposition of the VGCC subunits against the nicotinic acetylcholine (nACh) receptor present in both genotypes. In *lh* mice, there is a $56.2 \pm 6.5\%$ and $48.8 \pm 9.6\%$ juxtaposition of α_{1A} and α_{1E} , respectively, whereas in wt, there was $80.3 \pm 8.1\%$ juxtaposition of α_{1A} with α -bungarotoxin. With regards to the β -subunits, in *lh* mice, β_1 and β_3 juxtaposed with the nACh receptor at $73.9 \pm 21.3\%$ and $58.2 \pm 7.4\%$, respectively. In wt, the only VGCC subunits that exhibited significant overlap with α -bungarotoxin were α_{1A} and β_4 .

TABLE 1

Effects of age on MEPP amplitude and frequency at *lh* and wt mouse neuromuscular junctions

Genotype	MEPP Frequency	MEPP Amplitude
	Hz	mV
Juvenile		
wt	0.95 ± 0.06^a	0.66 ± 0.03
<i>lh</i>	1.05 ± 0.08	0.79 ± 0.04^b
Adult		
wt	1.11 ± 0.04	1.14 ± 0.02
<i>lh</i>	1.14 ± 0.04	1.10 ± 0.01

^aMean $\pm$ S.E.M. of $n > 7$ mice.^bSignificantly greater than wt ($P < 0.05$).

Discussion

Mutations in VGCC subunits are associated with several human neurological disorders (Catterall et al., 2008; Pietrobon, 2010). Studying these channelopathies has yielded important information regarding Ca^{2+} -dependent neurotransmission. The *lh* mutation is one such example. It disrupts transmitter release, but is not lethal. Moreover, it is directed not at the fundamental pore-forming α_1 -subunit, whose identity defines the basic functions of a VGCC, but rather at an auxiliary subunit which nonetheless plays critical roles in the targeting and modulation of the channels. The questions addressed in this study were as follows: 1) Given that the normal partner of the α_{1A} -subunit, the β_4 -subunit, is absent in adult *lh* mice, then which VGCCs are involved in the control of ACh release at NMJ of these in adulthood? 2) Was the change age dependent?

Our results demonstrate the following: 1) even though *lh* animals lack the β_4 -subunit, levels of the α_{1A} -subunit in the

cerebellum are similar for adult *lh* and wt animals; this is likely due to the increased level of β_1 and β_3 to compensate for a lack of the β_4 -subunit; 2) in adult, but not juvenile, *lh* mice, quantal content of ACh is diminished; 3) ω -Aga-IVA decreases *m* in both genotypes and in young and adult *lh* mice, but its contribution decreases with age; 4) in both young and adult *lh* mice, R-type VGCCs contribute to ACh release, but the extent of this contribution increases with age; conversely, R-type VGCCs do not contribute to ACh release in wt mice of either age group, and this result is consistent in both young and adult mice, which indicates the essential role in compensating for loss of NMJ function; 5) In adult *lh* mice, β_1 - and β_3 -subunits apparently associate with α_{1A} and α_{1E} , whereas for wt, β_4 appears to be solely responsible for coupling with α_{1A} at NMJs; and 6) in each adult group, there is a small percentage of *m* not accounted for by P/Q- or R-type channels at the concentration of antagonists tested, but which is sensitive to Cd^{2+} .

In normal adult mammals, ACh release at the NMJ is almost exclusively controlled by P/Q-type VGCCs (Uchitel et al., 1992; Protti and Uchitel, 1993; Katz et al., 1995). Disrupting this relationship should have pronounced consequences for neuromuscular transmission. That disruption could result from either 1) reducing expression of α_{1A} or 2) disrupting the normal relationship between α_{1A} and its normally obligatory coexpressing β_4 -subunit. In some respects, it does have severe consequences. For example, on the one hand, α_{1A} knockout mice do not survive more than 15–21 days after birth (Jun et al., 1999). On the other hand, several naturally occurring mutants in which the P/Q-type channel is altered remain viable. In the *tg* mutant, there is a point mutation in the α_{1A} -subunit leading to its being dysfunctional (Fletcher et al.,

Fig. 5. Immunostaining of wt and *lh* NMJ with α_{1A}/α_{1E} and β_4 . EDL muscles from wt and *lh* mice were stained with specific antibodies against VGCC α_1 - (green) (α_{1A} , A; α_{1E} , B) and β_4 - (blue) subunits as well as α -bungarotoxin (red). Epifluorescence was used to determine the superimposition of the various subunits. The bar scale is 10 μm . An identical protocol was used in Figs. 6 and 7. AChR, ACh receptor.

Fig. 6. Immunostaining of wt and *lh* NMJ with α_{1A}/α_{1E} and β_1 . EDL muscles from wt and *lh* mice were stained with specific antibodies against VGCC α_1 - (green) (α_{1A} , A; α_{1E} , B) and β_1 - (blue) subunits as well as α -bungarotoxin (red), and epifluorescence determined the superimposition of the various subunits. The bar scale is 10 μ m. The protocol used was identical to that of Fig. 5. AChR, ACh receptor.

1996). However, despite central nervous system and neuromuscular impairment, *tg* mice live to adulthood. *lh* Mice, in which the β_4 -subunit that normally partners with α_{1A} is lost, also live to adulthood, and are in fact fertile. Thus, when the α_{1A} -subunit expression is present, even if it is ultimately afunctional to critical functions, such as neuromuscular transmission, compensatory changes occur in VGCC expression that permit multiple subtypes to participate. However, the conditions that determine which VGCCs contribute are unclear and appear to be complex. Understanding this could have important implications in therapeutics for patients with channelopathy-related neurological impairment, as well as for basic neurophysiology.

When the α_{1A} -subunit is mutated, such as in *tg* mice (Kaja et al., 2006; Pardo et al., 2006), or in the passive transfer model of Lambert-Eaton myasthenic syndrome when P/Q function is impeded (Flink and Atchison, 2002), subtypes of VGCCs that normally do not mediate ACh release assumed this role. The identity of the VGCC subtype that serves in the compensatory role varies, with respect to both the source of the impairment as well as the synapses involved. For example, in the mouse passive transfer model of Lambert-Eaton myasthenic syndrome, P/Q-type VGCC contribution at the NMJ is reduced by ~40%, but partial compensation occurs through L-type channels (Flink and Atchison, 2002). Conversely, when the α_{1A} -subunit is impaired in *tg* mice, compensation involves not L-type but rather R- and to a slight extent N-type VGCCs at the NMJ (Kaja et al., 2006; Pardo et al., 2006), and primarily N-type VGCC in hippocampal Schaffer collaterals (Burgess and Noebels, 1999). Yet L-type channel upregulation apparently does occur in *tg* mice and contributes to the dystonia seen in these mice (Campbell and Hess, 1999). Thus, the channel type which compensates for

impairment of P/Q-type function is definitely not immutable, and appears to be synapse specific. Moreover, as demonstrated in this paper, the compensation is certainly age dependent.

The situation becomes more complicated in the case of abnormalities in the β -subunit. The role that the β -subunit has in this compensatory process is unclear. It normally regulates the assembly and membrane localization of the α_1 pore-forming subunit of VGCCs (Berrow et al., 1995; Walker and De Waard, 1998). Thus, absence of a particular β -subunit could profoundly affect synaptic function if it was an obligatory partner of an α_1 -subunit that normally regulated release, as α_{1A} -containing P/Q-type VGCCs do at the adult mammalian NMJ. The normal coassociation of β_4 -subunits with α_{1A} -subunits is lost in *lh* mice (Burgess et al., 1997). However, *lh* mice are viable, so neuromuscular function is more or less retained. As such, we hypothesized that *lh* mice compensated for absence of the β_4 -subunit at the motor nerve terminal with other types of VGCC. In this case, two possibilities existed: 1) α_{1A} would be retained at motor nerve terminals and would partner with other β -subunits, or 2) α_{1A} would be lost completely and another α_1 -subunit (α_{1E}) would replace it.

In *lh*, both of these possibilities occur. P/Q-type channels, as defined by their sensitivity to ω -Aga-IVA, clearly contribute to synaptic transmission at *lh* NMJs. While the extent of ω -Aga-IVA block of EPPs was reduced in *lh*, the effect was not significant. Nevertheless, the *m* of EPPs at *lh* NMJs was approximately half that of wt.

No significant difference was observed in α_{1A} abundance in adult *lh* mice using either Western blotting of a tissue with a high level of expression of P/Q-type channels (cerebellum) or immunohistochemistry at the NMJ. Neither Western blotting

Fig. 7. Immunostaining of wt and *lh* NMJ with α_{1A}/α_{1E} and β_3 . EDL muscles from wt and *lh* mice were stained with specific antibodies against VGCC α_1 - (green) (α_{1A} , A; α_{1E} , B) and β_3 - (blue) subunits as well as α -bungarotoxin (red), and epifluorescence determined the superimposition of the various subunits. The bar scale is 10 μ m. The protocol used was identical to that of Fig. 5. AChR, ACh receptor.

of the cerebellum nor immunohistochemistry of motor nerve terminals detected the presence of β_4 . Thus, in *lh* mice, association of β_4 with α_{1A} is apparently not obligatory for functional expression of P/Q-type channels at motor endplates. Alternate β -subunits substitute for the lack of β_4 . However, interestingly, even though cerebellar expression of α_{1A} is apparently normal, *lh* mice still express ataxia.

There is an increase in β_3 - and β_1 -subunit immunofluorescence at NMJs in *lh* mice. The immunostaining for α_{1A} juxtaposed staining with both β_1 or β_3 , as well as with α -bungarotoxin, so the α_{1A} present at the terminal includes channels with multiple phenotypes based on the contributory β -subunit. Neither β_1 nor β_3 appreciably stains end plate regions in wt, although they were present, albeit with lower staining intensity, in the cerebellum of wt mice. Shuffling of β -subunits for α_{1A} and α_{1B} has been reported for the cerebellum of *lh* mice (Burgess et al., 1999).

Despite the presence of functional P/Q-type channels in *lh* mice, R-type channels now intrude the motor nerve terminal and contribute measurably to ACh release. This is demonstrated by the differential effect of SNX-482 on wt and *lh* mice (Newcomb et al., 1998). The immunohistochemistry data confirmed the electrophysiology data; α_{1E} staining overlaps that of nACh receptors at the NMJ of *lh* mice, and is especially intense. Further, it overlaps with both β_1 - and β_3 -subunits not found at wt motor endplates. However, this compensation is not "seamless," as it comes at a price of reduced quantal release.

The contributions of the R-type channel are age dependent. At 5–7 weeks in *lh* mice, we found a measurable contribution of SNX-482-sensitive channels. The prior study of neuromuscular transmission in juvenile *lh* mice (Kaja et al., 2007a) did not include sensitivity to SNX-482, and therefore missed this

contribution. NMJ transmission is reduced in adult but not juvenile *lh* mice, so perhaps the substitution of P/Q-type with R-type reduces overall efficiency of transmission.

The marked overall reduction in EPP amplitude despite the presence of α_{1A} -containing channels has interesting and important implications for VGCC composition at *lh* mice NMJs. The "hybrid" channels produced by the combination of α_{1A} with subunits other than β_4 had a reduced capacity to support release. If a similar number of α_{1A} -containing channels were present, and yet each was less effective in inducing ACh release, then a relatively similar effect of ω -Aga-IVA implies that their function was impaired by loss of $\alpha_{1A}\beta_4$ pairing. Alternatively, there may have been α_{1A} -containing channels that were not ω -Aga-IVA-sensitive and which did not convey Ca^{2+} adequately to support ACh release at a normal level. Novel splice variants of the α_{1A} subunit in rats have been described (Rajapaksha et al., 2008). These lack the "synprint" site, but still conduct current, albeit with significant differences in amplitude and kinetics compared with the full-length variant. Whether the reduced quantal release in *lh* motor axon terminals involves the relative localization of the R-type channels, fundamental differences in biophysical properties between α_{1A} and α_{1E} , or differences imparted by the β -subunit, is unknown.

In both genotypes, a component of release is not sensitive to the combined presence of the various presumed subtype-specific toxins, yet does retain sensitivity to Cd^{2+} . The basis for this is unknown and commonly reported. Perhaps it reflects a use of inadequate concentrations of toxins, although a more interesting possibility is the presence of splice variants of the relative channel genotypes which exhibit reduced sensitivity to

Fig. 8. Percent juxtaposition of α_1 - and β -subunits of wt and *lh* NMJ with ACh receptors (AChR). (A) Percent juxtaposition of α -bungarotoxin and various α_1 -subunits of VGCC in *lh* and wt NMJ. (B) Percent juxtaposition of α -bungarotoxin and various β -subunits of VGCC in *lh* and wt NMJ. *Significant difference ($P < 0.05$) from wt preparations ($n = 8$).

the various toxins. This represents yet one more interesting enigma in the VGCC field.

The only other report of NMJ function in *lh* mice is by Kaja et al. (2007a). They reported no change in EPP amplitude or MEPP frequency for 5–7-week-old mice, but noted an increase in MEPP amplitude and almost 90% block of *m* by ω -Aga-IVA in *lh* mice. When we repeated the work by Kaja et al. (2007a) using animals of the same age, we saw that there was a significant decrease in ACh release in young *lh* mice when we applied ω -Aga-IVA, as well as when we applied SNX-482 (ACh was reduced to $25.4 \pm 3.1\%$ and $80.2 \pm 5.9\%$, respectively). Thus, there is a contribution of SNX-482-sensitive channels in juvenile *lh* mice; however, it is smaller, albeit significant, than in adult *lh* mice. These results imply that, in young *lh* mice, ACh release is controlled by both P/Q- and R-type VGCCs. It seems that the contribution of R-type VGCC increases as *lh* mice age. VGCC subunit expression is age dependent (Tanaka et al., 1995; McEnery et al., 1998b). Moreover, expression of splice variants of the α_{1A} -subunit is also age dependent (Vigues et al., 1998; Chang et al., 2007). Some of these splice variants have dramatically different sensitivities of ω -Aga-IVA (Kanumilli et al., 2006) as well as biophysical properties (Soong et al., 2002). Burgess et al. (1997) reported that the period between postnatal day (PND) 15 and PND 60 was critical for development of the complete *lh* phenotype, and after 2 months, these mice have recovered from deficits in other organ systems, and could have a normal life span. Thus, when using mice at PND ≤ 49 , it is possible that the final developmental changes associated with

VGCCs had not occurred. There might have been a greater dependence on P/Q-type and a lesser contribution of R-type channels than 3-month-old animals, or age-dependent splice variants in α_{1A} could have altered sensitivity to ω -Aga-IVA. Additionally, there are slight differences in the methodology used between us. These differences could possibly contribute to differences in our results from those of Kaja et al. (2007a).

In conclusion, the type of VGCC channel that can control ACh release at the mammalian NMJ is not fixed (Flink and Atchison, 2002; Urbano et al., 2003; Pagani et al., 2004; Pardo et al., 2006; Kaja et al., 2007a). Recruitment of alternative types of VGCCs to compensate for a deficit is possible and occurs in *lh* mice. This form of VGCC plasticity appears to be an important contribution to synaptic function when deficits in function or number of P/Q-type VGCCs are present. This type of compensatory “shuffling” has been reported by others for the central nervous system (Burgess et al., 1999). Understanding the basis for this form of synaptic plasticity could help in understanding how Ca²⁺ dependence of transmitter release occurs in normal synaptic function. The compensatory response produced appears to be very much individualized depending on the brain region and specific channel deficit involved.

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Authorship Contributions

Participated in research design: Molina-Campos, Xu, Atchison.

Conducted experiments: Molina-Campos, Xu.

Contributed new reagents or analytic tools: Molina-Campos, Xu, Atchison.

Performed data analysis: Molina-Campos, Xu, Atchison.

Wrote or contributed to the writing of the manuscript: Molina-Campos, Xu, Atchison.

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