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# The Status of Voltage-Dependent Calcium Channels in $\alpha_{1E}$ Knock-Out Mice

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It has been hypothesized that R-type Ca currents result from the expression of the $\alpha_{1E}$ gene. To test this hypothesis we examined the properties of voltage-dependent Ca channels in mice in which the $\alpha_{1E}$ Ca channel subunit had been deleted. Application of $\omega$-conotoxin GVIA, $\omega$-agatoxin IVA, and nimodipine to cultured cerebellar granule neurons from wild-type mice inhibited components of the whole-cell Ba current, leaving a "residual" R current with an amplitude of ~30% of the total Ba current. A minor portion of this R current was inhibited by the $\alpha_{1E}$-selective toxin SNX-482, indicating that it resulted from the expression of $\alpha_{1E}$. However, the majority of the R current was not inhibited by SNX-482. The SNX-482-sensitive portion of the granule cell R current was absent from $\alpha_{1E}$ knock-out mice. We also identified a subpopulation of dorsal root ganglion (DRG) neurons from wild-type mice that expressed an SNX-482-sensitive component of the R current. However as with granule cells, most of the DRG R current was not blocked by SNX-482. We conclude that there exists a component of the R current that results from the expression of the $\alpha_{1E}$ Ca channel subunit but that the majority of R currents must result from the expression of other Ca channel $\alpha$ subunits.

*Key words: dorsal root ganglia; cerebellar granule cells; pain; synaptic transmission; voltage-dependent calcium channels; $\alpha_{1E}$ knock-out mice*

Voltage-sensitive Ca channels are of great importance in coupling excitability to Ca-dependent events within cells. It is clear that there are many different kinds of Ca channels. The properties of these channels make them suitable for performing different tasks in neurons and other cells (Catterall, 1998). Voltage-sensitive Ca channels are multisubunit structures consisting of a major pore-forming subunit (the $\alpha_1$ subunit) and several ancillary subunits (Hoffman et al., 1999). At this time 10 different $\alpha_1$ subunits are known to exist (Ertel et al., 2000). Seven of these ($\alpha_{1A}$–$\alpha_{1E}$, $\alpha_{1F}$, and $\alpha_{1S}$) code for high-threshold Ca channels (Catterall, 1998; Hoffman et al., 1999), whereas $\alpha_{1G}$–$\alpha_{1I}$ code for low-threshold Ca channels (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999). It has been important to try and establish which $\alpha_1$ subunits are responsible for the different types of Ca currents that can be recorded from particular types of cells. However, there are many splice variants of the various Ca channel subunits, and different combinations of these result in Ca currents with a wide range of biophysical properties when they are expressed in heterologous expression systems (Parent et al., 1997; Pereverzev et al., 1998; Hoffman et al., 1999). Thus, the potential diversity of Ca current types is very large. However, the situation has been facilitated by the availability of a number of drugs and toxins that selectively target different $\alpha_1$ subunits. Thus, $\omega$-conotoxin GVIA is diagnostic for $\alpha_{1B}$, dihydropyridines are diagnostic for $\alpha_{1C}$, $\alpha_{1D}$, and $\alpha_{1S}$, $\omega$-agatoxin IVA is diagnostic for $\alpha_{1A}$, etc. (Catterall, 1998; Hoffman et al., 1999). One exception to this has been the Ca channel subunit encoded by the $\alpha_{1E}$ gene. Until very recently no specific toxin or drug was known that targeted $\alpha_{1E}$ Ca currents when these were expressed in heterologous expression systems (Newcombe et al., 1998). Consequently, the neuronal currents resulting from the expression of $\alpha_{1E}$ were not known with certainty. There has been much speculation that neuronal Ca currents that are insensitive to other drugs and toxins represent $\alpha_{1E}$ currents (Hilaire et al., 1997; Piedras-Renteria and Tsien, 1998; Tottene et al., 2000). Indeed, in certain instances these "residual" or "R-type" Ca currents do possess biophysical properties that resemble those resulting from the expression of $\alpha_{1E}$ subunits (Randall and Tsien, 1995; Tottene et al., 1996; Hilaire et al., 1997). Nevertheless, there has been considerable controversy about the nature of $\alpha_{1E}$-based Ca currents in neurons, and very little is known about their functions (Bourinet et al., 1996; Meir and Dolphin, 1998). Recently, a toxin (SNX-482) has been described that does selectively block the Ca currents that result from the expression of the $\alpha_{1E}$ subunit in heterologous expression systems (Newcombe et al., 1998). Interestingly, this toxin was unable to block neuronal R currents in several instances. On the other hand, experiments using antisense knock-out of $\alpha_{1E}$ subunits in neurons have supported the idea that R currents do indeed result from the expression of $\alpha_{1E}$ subunits (Piedras-Renteria and Tsien, 1998; Tottene et al., 2000).

To answer questions further concerning the nature and functions of $\alpha_{1E}$ Ca currents, we have generated mice in which the $\alpha_{1E}$ gene has been deleted. We demonstrate that R currents are heterogeneous, consisting of elements that result from the expression of $\alpha_{1E}$ as well as other non-$\alpha_{1E}$-dependent components.

## MATERIALS AND METHODS

*Construction of a* Cacna1e *null mutation.* A bacterial artificial chromosome (BAC) containing the *Cacna1e* gene was isolated by screening the Research Genetics CITB 129/Sv library with a *Cacna1e* cDNA. By the use of a probe specific for sequences encoding domain II of the *Cacna1e* protein, an ~18 kb *Bam*HI fragment was identified and cloned in the yeast shuttle vector pRS314. This vector had been modified previously to contain the thymidine kinase expression cassette. The DNA sequence of this fragment was determined and revealed to contain 11 exons that encode all of domain II.

The plasmid containing the 18 kb *Cacna1e* fragment was transformed

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into the yeast strain H1515 by LiCl. Yeast containing the *Cacna1e* plasmid were transformed with a PCR fragment that had been generated using a template containing the Neo and URA3 expression cassettes. The PCR fragment was generated using chimeric primers that contain 50 bp of intron sequences of *Cacna1e* and 20 bp that correspond to the 5′ or 3′ ends of the selection cassette. The fragment produced encodes both selection cassettes and 50 bp arms, which are homologous to intron 4 and intron 8. After selection on growth plates lacking uracil, DNA was isolated from uracil prototrophs and transformed into bacterial strain DH10B. Plasmids were sequenced to confirm that the PCR fragment had integrated into the desired site.

The *Cacna1e* targeting vector was linearized using *Not*I and electroporated into CJ7 ES cells. After selection with G418/FIAU, DNA was prepared from resistant clones and analyzed by genomic blot hybridization. An external 3′ probe was used to screen the *Nde*I-digested DNA for the presence of a rearranged 6.6 kb fragment and 6 kb endogenous fragment. Clones that exhibited the correct rearrangement were then analyzed using a 5′ internal probe to screen *Eco*RI-digested DNA for the presence of a 12 kb rearranged fragment and 8.7 kb endogenous fragment. Two clones were selected for blastocyst injection and produced several highly chimeric founder mice. Founders were then bred to C57BL/6J females.

*Western blotting.* Wild-type and $\alpha_{1E}$-deficient mice were decapitated, and the brains were removed on ice. The cerebellum was separated from the rest of the brain, and the brain regions were processed as follows. Tissue was homogenized for 20 strokes in an ice-cold buffer containing 0.32 M sucrose, 5 mM Tris, 5 $\mu$g/ml aprotinin, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml leupeptin, and 1 mM 4-(2 aminoethyl)-benzenesulfonyl-fluoride, pH 7.4. Homogenates were centrifuged at $1000 \times g$ for 10 min. The resulting supernatant was diluted to 15 ml in sucrose buffer and centrifuged for 15 min at $20{,}000 \times g$. The supernatant was discarded, and the outer membrane portion of the pellet was gently washed and resuspended in 1 ml of sucrose buffer leaving the inner mitochondrial pellet intact. The suspended pellet was centrifuged in a bench top centrifuge at 14,000 rpm for 10 min at 4°C to remove the sucrose, and the resulting pellet was resuspended in 5 mM Tris and 2 mM EDTA, pH 7.4. Lysates were stored at −80°C until use.

Protein samples were loaded onto a 6% polyacrylamide gel and run at 120 V for ~2 hr. Thirty micrograms of protein were loaded for brain homogenates, whereas 15 $\mu$g of $\alpha_{1E}$-transfected and untransfected human embryonic kidney (HEK) cell membranes was used. After transfer onto nitrocellulose, blots were blocked with 5% skim milk in Tris-buffered saline for 2 hr at room temperature. Blots were incubated with polyclonal $\alpha_{1E}$ antibody at 1 $\mu$g/ml for 2 hr at room temperature.

Anti-human $\alpha_{1E}$ antibody was generated by collaborators at Eli Lilly and Company. Amino acid sequences specific for $\alpha_{1E}$ were prepared as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*. Antigens were derived from the IIS-6/IIIS-1 cytoplasmic loop region spanning amino acids 984–1099 of the human $\alpha_{1E}$ subunit. The antibody was purified by protein A chromatography and affinity chromatography [for a detailed account of antibody preparation and purification, refer to Beattie et al. (1997)]. Antibody specificity was confirmed by ELISA and immunocytochemistry using human embryonic kidney cells transfected with appropriate $\alpha_1$ subunits (Volsen et al., 1995; Beattie et al., 1997). The specific use of the $\alpha_{1E}$ antibody used in these studies has been reported previously (Volsen et al., 1995; Day et al., 1996; Beattie et al., 1997; Grabsch et al., 1999). After washing, blots were incubated with HRP-conjugated secondary antibody (1:20,000; Promega, Madison, WI) for 1 hr at room temperature. Bands were visualized by incubation in ECL chemiluminescent reagent and development on chemiluminescent film.

*Dorsal root ganglion neuron culture.* Dorsal root ganglia (DRGs) were rapidly removed from all spinal segments of 2- to 5-d-old neonatal knock-out (KO) and wild-type mice under aseptic conditions and digested sequentially in collagenase (Sigma, St. Louis, MO), collagenase and dispase (Boehringer Mannheim, Indianapolis, IN), and trypsin (Life Technologies, Gaithersburg, MD) in HBSS (Life Technologies) for 10 min, respectively, at 37°C. Ganglia were washed in DMEM three times and resuspended in F12 media (Life Technologies), supplemented with 10% FBS (Summit Biotechnology), $N_2$ supplement (Life Technologies), 50 ng/ml nerve growth factor (Collaborative Biomedical Products), and penicillin and streptomycin (100 $\mu$g/ml and 100 U/ml, respectively). Single neuronal cells were obtained by trituration through a flame-polished pipette ~10 times, and the cell suspension was centrifuged at 800 rpm for 5 min. The pellet was resuspended in F12 with the additives listed above, except that serum was reduced to 0.5%. Isolated DRG neurons were plated on poly-ornithine (Sigma)- and laminin (Collaborative Biomedical Products)-coated glass coverslips (25 mm in diameter) and incubated in the same medium that was replaced every 48 hr. Cultures were maintained at 37°C in a water-saturated atmosphere with 5% $CO_2$ for up to 2 weeks.

*Preparation of cultured cerebellar neurons.* Cultured cerebellar neurons were prepared in a coculture system as described previously (Brorson et al., 1991). Briefly, 17- to 19-d-old embryos were removed from pregnant mice anesthetized previously with ether and killed by cervical dislocation. The cerebella were dissected out and incubated in trypsin (Worthington, Freehold, NJ; 100 mg/10 ml; 0.5 ml of trypsin in 4.5 ml of HBSS containing 25 mM HEPES) for 20 min at 37°C. After washing, tissue was resuspended in DNase and triturated through a flame-narrowed pipette until no visible tissue fragments remained. Cells were plated at a density of $3 \times 10^5$ cell/ml onto 15 mm round glass poly-L-lysine-coated coverslips. Neurons were suspended over a feeder layer of cortical astrocytes and maintained in serum-free defined medium containing 15 mM HEPES. After 2 d in culture at 37°C, in a humidified 5% $CO_2$ atmosphere, 5-fluoro-2-deoxyuridine (5 $\mu$M final concentration) was added to maintain astrocyte numbers at near confluency. The cultures were fed every 3–4 d by replacement of half of the medium. This preparation generated both cerebellar Purkinje cells and granule cells, which could be distinguished by their differences in size. Granule cells were used for electrophysiological analysis between 10 and 20 d *in vitro*.

*Whole-cell patch clamp.* The tight-seal whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used to record Ba currents. Recordings were made at room temperature (21–24°C). Currents were recorded using the Axopatch 1D (Axon Instruments, Foster City, CA) or EPC-7 (List Electronics, Darmstadt, Germany) amplifier, filtered at 2 kHz by the built-in filter of the amplifier, and stored on the computer. Capacitative transients were cancelled at 10 MHz, and their values were obtained directly, together with the series resistance values from the settings of the Axopatch 1D or EPC-7 amplifiers. Leak corrections were performed using a P/N protocol. Command pulses were delivered at 30 sec intervals from −80 mV to +10 mV to elicit Ba current. Soft, soda-lime capillary glass was used to make patch pipettes. Before seal formation, the resistances of the recording electrodes were 4.5–6 ΩM for granule cells and 2.5–4 ΩM for DRG neurons. The extracellular buffer solution for whole-cell voltage-clamp experiments was composed of (in mM): 151 tetraethylammonium chloride, 5 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose; pH was adjusted to 7.4 with TEAOH. The standard internal solution consisted of (in mM): 100 CsCl, 37 CsOH, 1 $MgCl_2$, 10 BAPTA, 10 HEPES, 3.6 MgATP, 1 GTP, 14 Tris2CP, and 50 U/ml creatine phosphokinase. The pH was adjusted to 7.3 with CsOH. The osmolarity of the pipette solution was 300 mOsm/1, and the osmolarity of the extracellular solution was between 325 and 340 mOsm/1. Toxins were applied by bath perfusion (cerebellar granule cells) or locally by a puff pipette (DRG neurons), always in the presence of 0.2 mg/ml chicken egg ovalbumin (Sigma), to block the low-affinity binding sites.

## RESULTS

### Disruption of the *Cacna1e* gene does not affect viability

A null allele of the *Cacna1e* gene was created using an 18 kb *Bam*HI fragment that encodes all of domain II of the $\alpha_{1E}$ protein. By the use of the homologous recombination system of yeast, the S4–S6 regions of domain II were replaced with a neomycin/URA3 selection cassette to generate a targeting vector (Fig. 1). We believed that by removing the pore-lining domain and its neighboring transmembrane domains that a null allele of *Cacna1e* would be created. This construct was introduced into embryonic stem cells, and the cells were screened for disruption of the *Cacna1e* locus. Approximately 110 neomycin-resistant clones were recovered and analyzed by Southern analysis. Of these 110 ES cells, 5 showed the desired disruption at the *Cacna1e* locus. Chimeric animals produced from blastocyst injection were then tested for the presence of mutant alleles. Two lines were established from independent ES cell clones, and intercross mating was used to generate homozygous mutant mice. Viable homozygous mutant animals were generated from both lines. Both of the lines generated gave similar results. There were no observed phenotypic differences between wild-type and homozygous knock-out mice.

### Western blotting of $\alpha_{1E}$-deficient and wild-type mice

Western blot analysis was used to confirm deletion of the $\alpha_{1E}$ protein in $\alpha_{1E}$-deficient mice compared with wild-type mice (Fig. 2). Cerebellum and whole brain minus cerebellum extracts from both 129/SV and C57BL/6J wild-type mice displayed a clear band at 230 kDa, the predicted size for the $\alpha_{1E}$ subunit (Fig. 2). In contrast a corresponding band was not observed in either the cerebellum or whole brain minus cerebellum from $\alpha_{1E}$-deficient mice, indicating the absence of the $\alpha_{1E}$ subunit from these mice (Fig. 2). Northern blot analysis revealed no detectable $\alpha_{1E}$ transcript I in the homozygous knock-out mice using a probe located either within the deleted region or downstream from the deletion (+5387–5966).

### Electrophysiology of Ba currents

We examined the properties of the voltage-sensitive Ba currents in cerebellar granule and DRG neurons cultured from $\alpha_{1E}$ KO mice as well as their parental wild-type strains.

Ba currents in cerebellar granule and DRG neurons exhibited

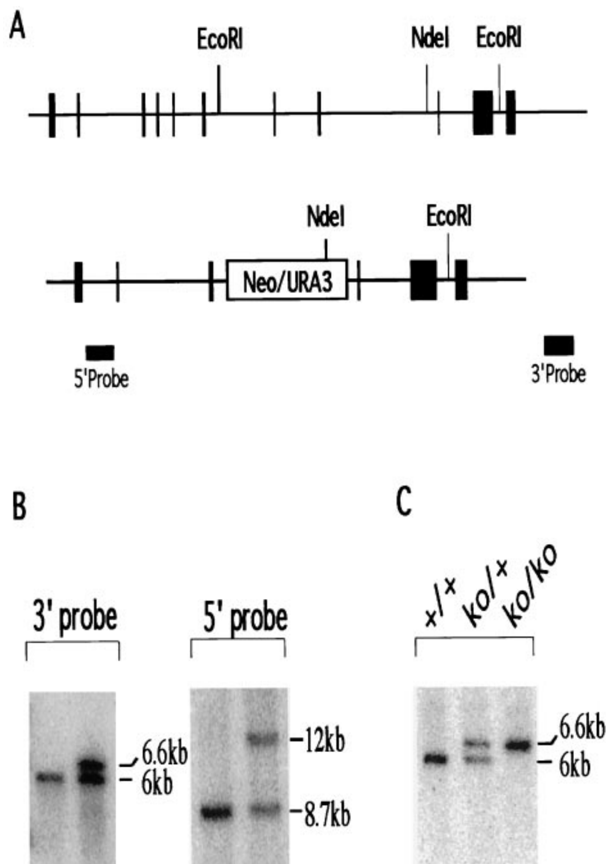


*Figure 1.* Generation of a mouse with a disrupted allele of the *Cacna1e* gene. *A,* Targeting of the *Cacna1e* gene is shown. *Top*, The genomic structure of the wild-type allele of the *Cacna1e* gene is shown with exons indicated by *black boxes*. Restriction sites used for determination of the disrupted allele are indicated. *Bottom*, A PGK *neo/URA3* expression cassette replaced the fourth through eighth exons of this fragment. The locations of probes used for genomic blot hybridization are shown *below* the disrupted allele. *B,* Genomic blot hybridization of DNA derived from neomycin-resistant ES cell clones is shown. ES cell DNA was probed with a 3′-flanking probe (*left*) and 5′ internal probe (*right*). Hybridization with the 3′ probe shows a 6 kb wild-type band and 6.6 kb mutant band, whereas the 5′ internal probe revealed an 8.7 kb wild-type band and 12 kb mutant band. *C,* Tail tip DNA from three intercross littermates was probed with the 3′-flanking probe and showed the expected size for wild-type, heterozygous, and homozygous mutant. *ko*, Knock-out.

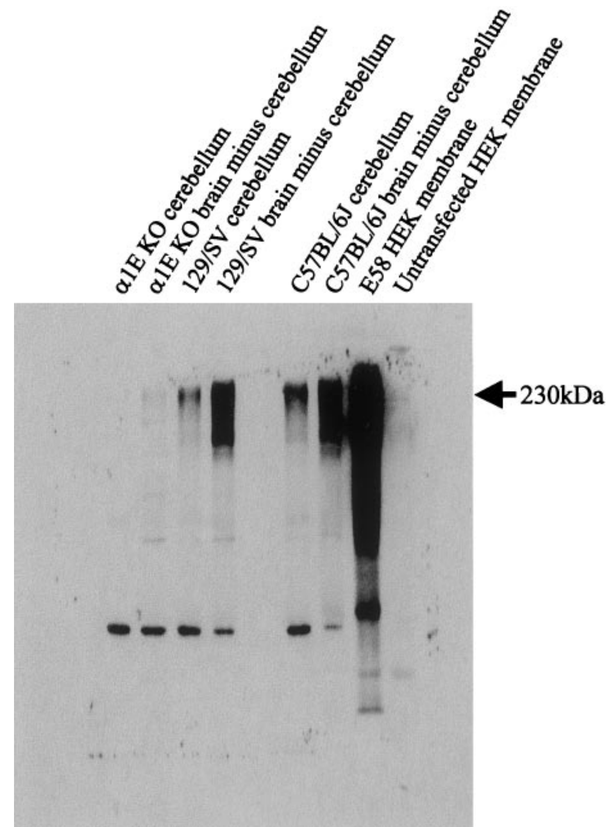


*Figure 2.* Western blot analysis of $\alpha_{1E}$ protein in wild-type and $\alpha_{1E}$-deficient mice. Brain lysates from 129/SV and C57BL/6J wild-type mice show the expression of the 230 kDa $\alpha_{1E}$ protein, whereas the protein is clearly absent from brain tissue of $\alpha_{1E}$ KO mice. Specificity of the antibody was confirmed by detection of a band in $\alpha_{1E}$-transfected HEK cell membranes and the lack of staining in untransfected HEK cell membranes.

differential sensitivity to a number of toxins and drugs that have been shown previously to target different types of voltage-sensitive Ca channels (Figs. 3, 4). In the case of cerebellar granule neurons (Fig. 3), components of the Ba current were blocked by the sequential addition of nimodipine (L-type blocker) ω-agatoxin VIA (P/Q-type blocker), and ω-conotoxin GVIA (N-type blocker), leaving a component of the current that we designated the R current on the basis of previous criteria in the literature (Fig. 3). We examined the effect of the $\alpha_{1E}$-selective toxin SNX-482 on the R current in these neurons. In both of the parental lines SNX-482 at a supramaximally effective concentration of 1 $\mu$M blocked ~30% of the granule cell R current. However, it was clear that the majority of the R current was not blocked by this toxin. Although the overall magnitude of the whole-cell Ca current in $\alpha_{1E}$ KO mice was the same as that in wild-type mice, the SNX-482-sensitive component of the Ba current was completely absent from $\alpha_{1E}$ mice. However, a large R current still remained in these KO mice after sequential application of all of the drugs and toxins. We also noted that the size of the N-current was slightly larger in the granule neurons from $\alpha_{1E}$ KO mice. The absolute magnitude of the currents recorded in these experiments can be seen in Table 1.

In DRG neurons sequential addition of drugs and toxins also inhibited different components of the Ba current, leaving a substantial R current (Fig. 4). Interestingly, we observed two populations of DRG neurons from wild-type mice. In the first type, addition of SNX-482 was ineffective in blocking the R current. In a second population of neurons, addition of SNX-482 produced a clear inhibition of the R current although, as in the case of cerebellar granule neurons, the majority of the R current was not blocked by SNX-482. Both types of DRG neurons (i.e., SNX-482 sensitive and insensitive) were observed in both wild-type parental strains. Interestingly, in neurons that exhibited SNX-482-sensitive R currents, we found that ω-agatoxin IVA was relatively ineffective (Fig. 4*E*). DRG neurons from $\alpha_{1E}$ KO mice exhibited Ba currents with the same magnitude as those from wild-type mice. The Ba currents in these neurons exhibited components that were blocked by nimodipine, ω-agatoxin IVA, and ω-conotoxin GVIA, leaving a substantial R current, but this was not blocked by SNX-482.

## DISCUSSION

Voltage-sensitive Ca channels are a diverse family of molecules (Ertel et al., 2000). Of the nine genes whose expression produces channels of this type, the properties and functions of the $\alpha_{1E}$ channel are the least well understood. Although $\alpha_{1E}$ subunits appear to be widely distributed throughout the CNS and peripheral nervous system (Volsen et al., 1995; Day et al., 1996), it is not clear which types of Ca currents result from their expression. One of the problems has been the lack of a drug or toxin that specifically targets these channels, although the recent identification of the $\alpha_{1E}$-selective toxin SNX-482 has helped to alleviate this problem (Newcombe et al., 1998). Furthermore, because $\alpha_{1E}$ subunits are structurally diverse (Pereverzev et al., 1998; Vajna et al., 1998; Spaetgens and Zamponi, 1999) and because their combination with different ancillary subunits results in a variety of Ca currents with differing biophysical characteristics (Parent et al., 1997; Nakashima

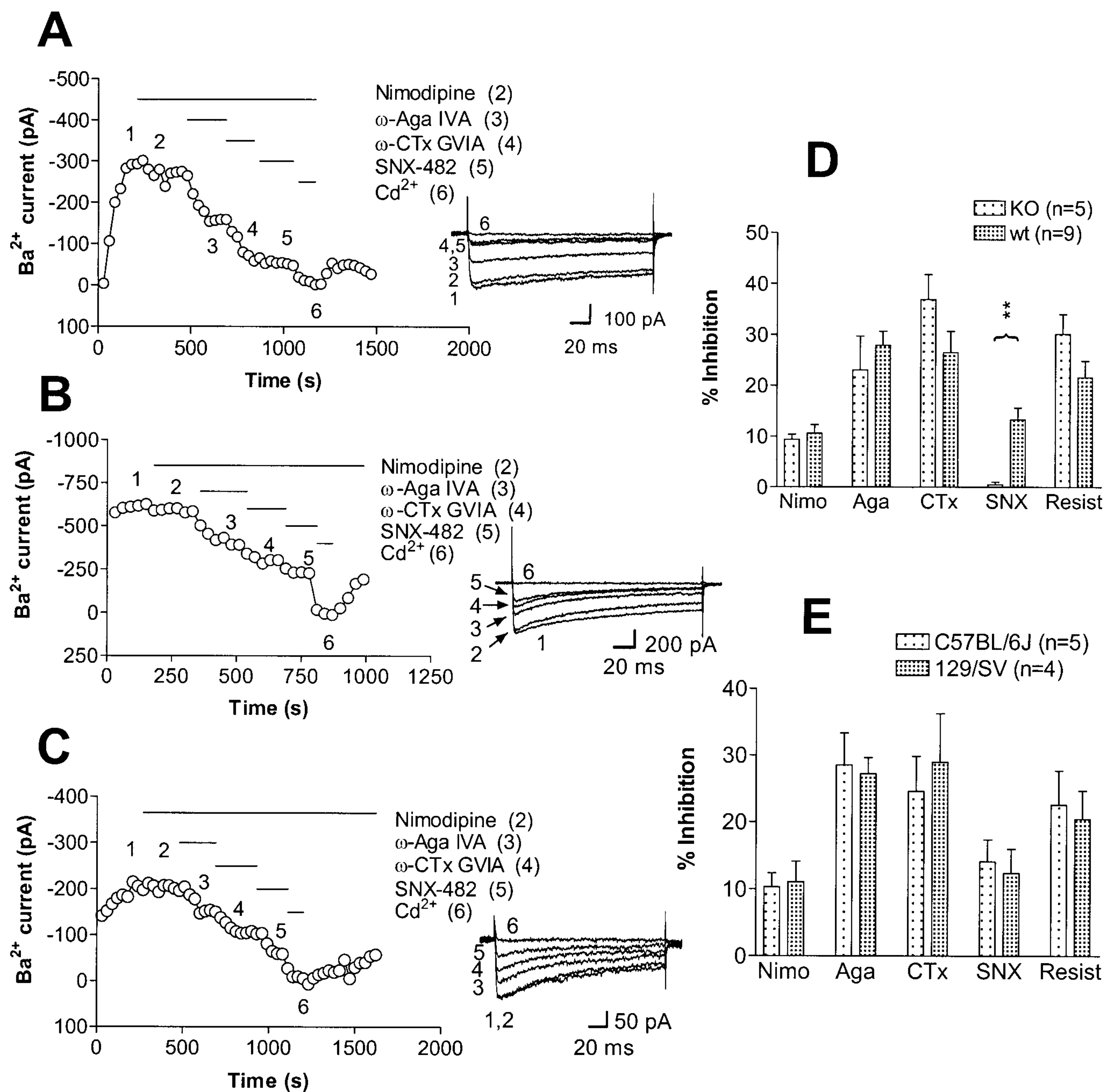


*Figure 3.* Effects of drugs and toxins on the cerebellar granule cell $I_{Ba}$. *A*, Plot of the peak $I_{Ba}$ versus time in an $\alpha_{1E}$ KO mouse shows a resistant component of the high voltage-activated $I_{Ba}$ remaining after consecutive bath application of specific drugs and toxins. SNX-482 had no effect on the toxin-resistant current. *Inset,* Representative *traces* at *numbered* points are shown. *B*, Plot of the peak $I_{Ba}$ versus time from a wild-type C57BL/6J mouse is shown. *Inset,* Representative *traces* are shown. SNX-482 inhibited a component of the R current remaining after nimodipine, ω-agatoxin IVA (*ω-Aga IVA*), and ω-conotoxin GVIA (*ω-CTx GVIA*) application. *C*, Plot of the peak $I_{Ba}$ versus time from a wild-type 129/SV mouse is shown. SNX-482 also inhibited part of the R current in this case. *Inset*, Representative *traces* are shown. *D*, Comparison of inhibitory effects of specific drugs and toxins on the granule cell $I_{Ba}$ in $\alpha_{1E}$ KO and control mice is shown. Drugs and toxins were used in the following concentrations: nimodipine (*Nimo*), 500 nM; ω-conotoxin GVIA (*CTx*), 500 nM; ω-Aga IVA (*Aga*), 200 nM; SNX-482 (*SNX*), 1 μM; $Cd^{2+}$, 100 μM. The data from the two parental control mouse strains were pooled. SNX-482 was only effective in inhibiting the $I_{Ba}$ from control mice (**$p < 0.01$). The *numbers* in *parentheses* represent the number of experiments. *E*, The effects of the specific drugs and toxins on the $I_{Ba}$ from the two control mouse strains did not differ significantly from each other. The *numbers* in *parentheses* represent the number of experiments. *Resist*, Resistant; *wt*, wild type.

et al., 1998), there is still considerable uncertainty about the types of Ca currents $\alpha_{1E}$ subunits produce in neurons and other cells.

To overcome this problem some investigators have resorted to antisense approaches to examine the relationship between Ca currents and the expression of $\alpha_{1E}$ subunits. For example Piedras-Renteria and Tsien (1998) observed that antisense oligonucleotides for $\alpha_{1E}$ partially reduced the size of the R current in rat cerebellar granule cells. They suggested either that more than one $\alpha_1$ subunit was responsible for the R current in these cells or that the apparent heterogeneity was caused by the expression of multiple forms of the $\alpha_{1E}$ subunit that were differentially sensitive to antisense treatment. Furthermore, Newcombe et al. (1998) reported that the R current in cerebellar granule neurons was insensitive to SNX-482, suggesting a dissociation between this current and $\alpha_{1E}$ expression. On the other hand, Tottene et al. (2000) reported that the R current in cerebellar granule neurons consisted of multiple components, some of which were sensitive to SNX-482 and some of which were resistant. However, these authors also demonstrated that antisense treatment was nearly completely effective in reducing both the toxin-sensitive and -insensitive components of the current. Thus, it

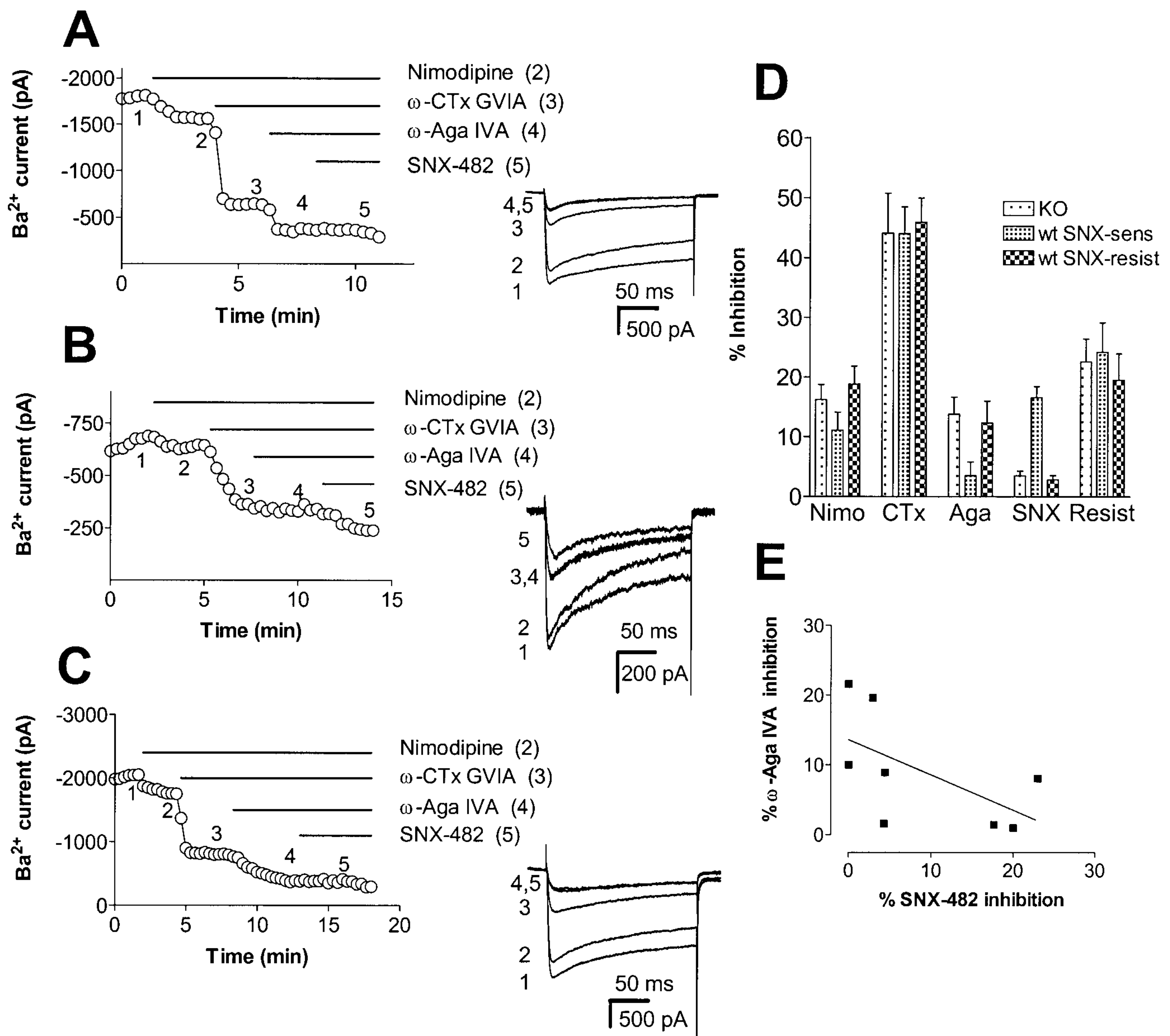


*Figure 4.* Effects of drugs and toxins on the $I_{Ba}$ in DRG neurons from wild-type and $\alpha_{1E}$ KO mice. *A,* Plot of the peak $I_{Ba}$ versus time in a DRG neuron from an $\alpha_{1E}$ KO mouse. An R current still remained after sequential application of the different drugs and toxins. The $I_{Ba}$ from $\alpha_{1E}$ KO mice was insensitive to SNX-482. *Inset,* Representative currents. *B*, Time course of the peak $I_{Ba}$ from a wild-type DRG neuron exhibiting sensitivity to SNX-482. *C*, Time course of the peak $I_{Ba}$ from a wild-type DRG neuron that was insensitive to SNX-482. *D*, Average inhibition (mean ± SEM) of the peak $I_{Ba}$ by specific drugs and toxins. Drugs and toxins were used in the following concentrations: nimodipine, 2 μM; ω-conotoxin GVIA, 1 μM; ω-Aga IVA, 200 nM; SNX-482, 1 μM. DRG neurons from $\alpha_{1E}$ KO mice ($n = 7$) and a population of DRG neurons from wild-type mice ($n = 8$) were insensitive to SNX-482 application. The $I_{Ba}$ inhibition by SNX-482 in sensitive DRG neurons ($n = 10$) was significant ($p < 0.01$). *E*, Interrelationship between sensitivity to ω-Aga IVA and SNX-482 in wild-type DRG neurons ($n = 8$). The correlation was significant ($p = 0.0265$) using the Spearman rank correlation test.

is still unclear whether the R current, at least in cerebellar granule neurons, is a homogeneous entity or not and what its relationship to $\alpha_{1E}$ expression might be.

To settle this issue, we have generated mice that completely lack the $\alpha_{1E}$ transcript and protein. It is therefore clear that any Ca currents recorded from these animals cannot result from the expression of $\alpha_{1E}$. It is important to note therefore that there was a substantial R current in all of the neurons from $\alpha_{1E}$ KO mice from which we made recordings. Therefore, we can conclude that the majority of R currents, at least in cerebellar granule and DRG neurons, do not result from the expression of $\alpha_{1E}$ subunits. Having said this, it is also true that there appears to be a component of the R current in some cell types that does result from the expression of $\alpha_{1E}$, although this component is relatively small. The situation in mouse cerebellar granule cells therefore seems to be similar to that reported by Piedras-Renteria and Tsien (1998). Although the magnitude of $\alpha_{1E}$-dependent R current in their studies was larger than

**Table 1. $Ba^{2+}$ currents in cultured mouse neurons**

| | KO | Wild type |
|---|---|---|
| Total current | 364.9 ± 50.4 | 375.8 ± 47.6 |
| Nimodipine | 36.1 ± 8.2 | 44.7 ± 8.4 |
| ω-Aga IVA | 75.0 ± 15.0 | 105.3 ± 15.6 |
| ω-CTx GVIA | 139.2 ± 30.7 | 95.4 ± 22.1 |
| SNX-482 | 1.8 ± 1.3 | 45.5 ± 6.9 |
| Resistant | 112.8 ± 26.8 | 84.9 ± 20.54 |

Comparison of the peak $I_{Ba}$ (pA) from cultured mouse cerebellar granule cells ($\alpha_{1E}$ knock-out, $n = 5$; wild type, $n = 9$). Data represent peak current amplitudes (pA) blocked by the specific toxin or drug indicated and the remaining resistant current. Data are given as mean ± SEM.

that in ours, their conclusion that the R current in granule neurons did not completely result from the expression of $\alpha_{1E}$ seems similar to ours. Furthermore, our results are also consistent with the work of Newcombe et al. (1998), who observed SNX-482-resistant R currents in several types of neurons.

The identity of R currents in sensory neurons also seems to be complex. It is interesting to note that two different patterns of Ca current sensitivity were observed in these neurons. We only identified an SNX-482-sensitive component in those wild-type neurons with a relatively small ω-agatoxin IVA-sensitive current. We do not yet know what this phenotype means in terms of the overall classification of DRG neurons. However, as in the case of granule neurons, the magnitude of the SNX-482-sensitive current, in those DRG cells in which it is observed, was relatively small.

If the expression of $\alpha_{1E}$ is not responsible for the majority of the R current in most neurons, then what is? It is interesting to note that Jun et al. (2000) have shown recently that there was a large decline in the magnitude of the cerebellar granule neuron R current in $\alpha_{1A}$ KO mice [although, interestingly, Piedras-Renteria and Tsien (1998) found that $\alpha_{1A}$ antisense did not reduce the R current in these neurons]; the decline in R current magnitude in these mice was much greater than that in the $\alpha_{1E}$ KO mice reported here. Jun et al. (2000) demonstrated that ~80% of the R current was absent in their $\alpha_{1A}$ KO mice. Thus, it is conceivable that this portion, together with the SNX-482-sensitive portion described here, accounts for virtually all of the granule neuron R current, although exactly what the relationship is between the non-$\alpha_{1E}$-dependent R current and $\alpha_{1A}$ is not clear at this time. Nevertheless, it is also interesting to note that in our studies on DRG neurons there was a negative correlation between the magnitude of the ω-agatoxin IVA-sensitive and the SNX-482-sensitive currents. It is also of interest that the SNX-482-sensitive current was restricted to a particular population of DRG neurons. Exactly what this population represents from the functional point of view awaits further identification but could indicate a role for $\alpha_{1E}$-based currents in the processing of a particular type of sensory information, such as pain (Saegusa et al., 2000).

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