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Impaired Long-term Potentiation and Enhanced Neuronal Excitability in the Amygdala of $Ca_V1.3$ Knockout Mice

Brandon C. McKinney[1,2], **Wilson Sze**[4], **Benjamin Lee**[4], and **Geoffrey G. Murphy**[2,3,4]

[1] Medical Scientist Training Program, University of Michigan, Ann Arbor, MI 48109-0069

[2] Neuroscience Program, University of Michigan, Ann Arbor, MI 48109-0069

[3] Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI 48109-0069

[4] Molecular & Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI 48109-0069

Abstract

Previously, we demonstrated that mice in which the gene for the L-type voltage-gated calcium channel $Ca_V1.3$ is deleted ($Ca_V1.3$ knockout mice) exhibit an impaired ability to consolidate contextually-conditioned fear. Given that this form of Pavlovian fear conditioning is critically dependent on the basolateral complex of the amygdala (BLA), we were interested in the mechanisms by which $Ca_V1.3$ contributes to BLA neurophysiology. In the present study, we used *in vitro* amygdala slices prepared from $Ca_V1.3$ knockout mice and wild-type littermates to explore the role of $Ca_V1.3$ in long-term potentiation (LTP) and intrinsic neuronal excitability in the BLA. We found that LTP in the lateral nucleus (LA) of the BLA, induced by high-frequency stimulation of the external capsule, was significantly reduced in $Ca_V1.3$ knockout mice. Additionally, we found that BLA principal neurons from $Ca_V1.3$ knockout mice were hyperexcitable, exhibiting significant increases in firing rates and decreased interspike intervals in response to prolonged somatic depolarization. This aberrant increase in neuronal excitability appears to be at least in part due to a concomitant reduction in the slow component of the post-burst afterhyperpolarization. Together, these results demonstrate altered neuronal function in the BLA of $Ca_V1.3$ knockout mice which may account for the impaired ability of these mice to consolidate contextually-conditioned fear.

Keywords

L-type voltage-gated calcium channels; afterhyperpolarization; long-term potentiation; conditioned fear; memory mechanisms; spike accommodation

Introduction

We have previously demonstrated that deletion of the L-type voltage-gated calcium channel $Ca_V1.3$ disrupts Pavlovian fear conditioning in $Ca_V1.3$ knockout mice. Specifically, $Ca_V1.3$ knockout mice exhibited normal consolidation of contextually-conditioned fear memories at

Send all correspondence to: Geoffrey G. Murphy, Ph.D., University of Michigan, 5037 Biomedical Sciences Research Building, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, Office (734) 936-8926, Lab (734) 936-8934, Fax (734) 936-2690, Email: murphyg@umich.edu.

1 and 6 hours after training but were unable to retain these memories long-term exhibiting significant impairments 24 hours after conditioning (McKinney and Murphy, 2006). While contextually-conditioned fear is thought to be dependent on both the amygdala as well as the hippocampus (Blanchard, Blanchard, and Fial, 1970; Kim and Fanselow, 1992; Maren, Aharonov, and Fanselow, 1997; Phillips and LeDoux, 1992) the consolidation deficits observed in the $Ca_V1.3$ knockout mice likely reflects a disruption of neuronal function within the amygdala proper, as spatial learning in tasks that require intact hippocampal function (e.g. the Morris water maze) are unaltered in these mice (McKinney and Murphy, 2006).

Currently there is general agreement that the basolateral complex of the amygdala (BLA), which includes the basal (BA) and lateral (LA) nuclei, is a critical structure for the processing of fear memories (reviewed by Kim and Jung, 2006; Sah, Westbrook, and Lüthi, 2008; Sigurdsson, Doyère, Cain, and LeDoux, 2007) and thus the neurophysiological function of $Ca_V1.3$ in this structure is of interest. Voltage-gated calcium channels (VGCC) and the L- type voltage gated calcium channels (L-VGCCs) in particular, have been implicated in several putative neurophysiological correlates of learning and memory. However, at present the relative contribution of the specific L-VGCC subtype(s) to these neurophysiological processes remains unknown. Of the presumed cellular mechanisms thought to subserve learning and memory, long-term potentiation (LTP) has been perhaps the most intensively studied.

Long-term potentiation can be induced *in vitro* in amygdala slice preparations using a variety of induction protocols and has been demonstrated at synapses within the BLA formed by afferent projections from the thalamus (the thalamic pathway) or the cortex (the cortical pathway). L-type VGCCs have been implicated in the induction of LTP at synapses in the thalamic pathway (Bauer, Schafe, and LeDoux, 2002; Weisskopf, Bauer, and LeDoux, 1999) as well as the cortical pathway (Drephal, Schubert, and Albrecht, 2006; Schroeder and Shinnick-Gallagher, 2004; Tsvetkov, Carlezon Jr, Benes, Kandel, and Bolshakov, 2002).

In addition to LTP, L-VGCCs have been demonstrated to play a role in regulating neuronal excitability. In the hippocampus, blockade of L-VGCCs with nimodipine decreases spike accommodation in a concentration dependent manner (Moyer, Thompson, Black, and Disterhoft, 1992). Although similar experiments specifically targeting L-VGCCs have not been performed in the amygdala, non-specific blockade of voltage-gated calcium channels with cadmium has been demonstrated to significantly increase repetitive firing rates in principle neurons of the BLA (Faber, Callister, and Sah, 2001; Faber and Sah, 2002; 2003; Washburn and Moises, 1992). The increased excitability that results from blockade of VGCCs is thought to be a direct result of a reduction in the post-burst afterhyperpolarization or AHP. The AHP is a hyperpolarizing voltage deflection that follows a burst of action potentials and serves to limit firing to a sustained depolarizing input (Alger and Nicoll, 1980; Hotson and Prince, 1980; Madison and Nicoll, 1984). The AHP is often described as having 3 components: a fast, medium and a slow AHP. The fast AHP (fAHP) occurs immediately after individual action potentials and lasts only 1–5 ms. The medium AHP (mAHP) is typically observed after a burst of action potentials and has a decay constant of approximately 100 ms. The slow AHP (sAHP) has a time constant of 1–5 seconds and is voltage-independent (Faber and Sah, 2007). Several studies using L-VGCC antagonists have shown that blockade of these channels in hippocampal pyramidal neurons leads to a significant reduction in the currents underlying the AHP, suggesting that the AHP is generated by calcium influx via L-VGCCs (Lima and Marrion, 2007; Marrion and Tavalin, 1998; Power, Wu, Sametsky, Oh, and Disterhoft, 2002; Shah and Haylett, 2000). While it has been previously demonstrated that blockade of voltage-gated calcium channels in the amygdala can significantly reduce the AHP (Faber et al., 2001; Faber and Sah, 2002; 2003; Washburn and Moises, 1992) to what extent that L-type voltage-gated calcium channels contributed to the generation of the AHP is unknown as is the relative contribution of the specific subtypes.

In the present study, we sought to determine to what extent the L-type calcium channel subtype $Ca_V1.3$ contributed to amygdala LTP and neuronal excitability. We found that $Ca_V1.3$ is critical for LTP induced in an *in vitro* horizontal slice preparation using a HFS induction protocol. In addition, we found that neurons in the BLA of the $Ca_V1.3$ knockout mice were hyperexcitable, exhibiting substantial increases in repetitive firing rates and decreased accommodation which were accompanied by a substantial decrease in the sAHP. Together, these results suggest that neuronal function is impaired in the BLA of $Ca_V1.3$ knockout mice and this may account for the impaired ability of these mice to consolidate contextually-conditioned fear.

Materials and Methods

Mice

The $Ca_v1.3$ knockout mice were generated by introducing a neomycin cassette into exon 2 of the gene for the pore-forming subunit of the $Ca_v1.3$ calcium channel (Platzer, Engel, Schrott-Fischer, Stephan, Bova, Chen, Zheng, and Striessnig, 2000). The null allele was maintained on a C57BL/6NTac background by successively crossing (> 12 generations) heterozygous null offspring with C57BL/6NTac wild-type mice purchased from Taconic Farms (Hudson, NY). Experimental mice were bred onto a 129S6B6F2/Tac hybrid genetic background. To obtain experimental mice, 129S6B6F1/Tac hybrid mice were generated first by crossing heterozygous null C57BL/6NTac mice from the maintenance cross with wild-type 129S6/SvEvTac mice purchased from Taconic Farms (Hudson, NY). The $Ca_v1.3$ heterozygous null 129S6B6F1/Tac mice were subsequently intercrossed to obtain experimental mice. For all experiments approximately equal numbers of young (2–6 mos) male and female mice were used. All comparisons were made between $Ca_v1.3$ knockout mice and wild-type littermates and the experimenter was kept blind as to the genotype throughout the experiment. All experiments were conducted in accordance with the guidelines set forth by the University of Michigan Committee on Use and Care of Animals.

Slice Preparation

The “horizontal slice preparation” was utilized for all of the *in vitro* slice experiments. We elected to use this particular preparation because the external capsule (EC) of the horizontal slice preparation contains intact afferents from brain regions including the hippocampus, perirhinal as well as entorhinal cortices (von Bohlen und Halbach and Albrecht, 1998; 2002) and it has been previously demonstrated that in the horizontal slice preparation, LTP in the lateral amygdala that results from stimulation of the EC can be induced in the absence of GABA antagonists such as picrotoxin (Drephal et al., 2006). Mice were anesthetized with isoflurane and decapitated. The brain was removed and horizontal slices were prepared as described in Drephal et al. (2006) and Stoop and Pralong (2000). Briefly, the brain was divided at the midline and each hemisphere was positioned flat on its medial surface. The olfactory bulb, the cerebellum, and the brain stem were subsequently removed and the dorsal side of the brain was cut along a plane which was tilted at a 10° posterosuperior-anteroinferior angle. The exposed dorsal side of the brain was glued onto a cutting block, the brain was covered with ice-cold (<1°C) oxygenated sucrose-based cutting solution containing the following (in mM): 2.8 KCl, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 1 $CaCl_2$, 206 sucrose, 26 $NaHCO_3$, 10 D-glucose, 0.40 ascorbic acid and horizontal slices (400 μm) were prepared with a vibrating blade microtome (VT1000S, Leica Microsystems). These slices contained the ventral hippocampus, the medial and lateral divisions of the entorhinal cortex, the perirhinal cortex, and the amygdaloid complex (see Figure 1A). Slices were transferred to a holding chamber filled with oxygenated artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 2.8 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 26 $NaHCO_3$, 10 D-glucose, 0.40 ascorbic acid at room temperature and remained there for at least 1 hour before being individually transferred to a submersion chamber and continuously perfused (~1.5 ml/min) with oxygenated ACSF heated to 31°C.

Electrophysiology

All recordings were made using a Dagan BVC-700A amplifier in bridge mode and filtered at 1–3 kHz. Recordings were digitized using an Axon Instruments 1440 DigiData A/D converter at a rate of 10 kHz and acquired to a Dell Optipex GX 620 desktop computer with pClamp 10 (Axon Instruments). Field potential recordings were made using glass pipettes made from Clark Borosilicate Standard Wall glass (Warner Instruments), pulled with a P-97 Flaming-Brown pipette puller (Sutter Instruments), filled with ACSF (tip resistances ~ 1 MΩ), and placed in the lateral nucleus of the BLA. Bipolar (Pt/Ir) stimulation electrodes were used to stimulate the EC. Stimulation of the EC with square wave pulses (100 μs) resulted in a compound waveform that contained the summation of both EPSPs and synchronized action potentials (Doyere, Schafe, Sigurdsson, and LeDoux, 2003; Watanabe, Saito, and Abe, 1995). Because the slope measure of this waveform in the LA is more sensitive to variability and noise (Doyere et al., 2003), we analyzed the amplitude of field potentials in the present study.

Basal synaptic transmission was assessed by examining the input-output relationship within the LA. Input-output response curves were constructed by varying the stimulation intensity and averaging 5 responses for each stimulation intensity. For experiments that examined LTP, the stimulus intensity that evoked a field potential amplitude equal to ~50% of the maximal response was then used for HFS as well as single-pulse stimulations that preceded and followed the HFS. A stable baseline of responses was obtained for at least 5 minutes and then a HFS consisting of two trains at 100 Hz (1 s duration; 30 s apart) was administered. Subsequent responses to single stimuli were recorded for 60 min, and their amplitude quantified as change in percentage with respect to baseline. Stimulus frequency pre- and post-HFS was 0.067 Hz.

To investigate the impact of $Ca_V1.3$ deletion on intrinsic neuronal properties, whole-cell recordings were made from principal neurons in the amygdala horizontal slice preparation using the “blind” patch method (Blanton, Lo Turco, and Kriegstein, 1989). While the initial recording electrode position for these experiments was within the lateral nucleus of the BLA it is possible that as the recording electrode was advanced through the horizontal slice, the final recording location may have been in the ventral portion of the basal nucleus and therefore, hereafter we refer to these as principal neurons of the BLA without distinguishing the subnuclei.

Patch-pipettes were made from Clark Borosilicate Standard Wall glass (Warner Instruments), pulled using a P-97 Flaming-Brown pipette puller (Sutter Instruments) with resistances of 3–6 MΩ, and filled with the following internal solution (in mM): 120 potassium methylsulfate, 20 KCl, 10 HEPES, 4 Na_2-ATP, 2 $MgCl_2$, 0.3 Tris-GTP, 0.2 EGTA, 7 phosphocreatine. Seal resistances of >1 GΩ were achieved prior to rupturing into whole-cell mode. Principal neurons within the BLA were distinguished from interneurons based on 1) spike width, 2) frequency accommodation, 3) the presence of a post burst afterhyperpolarization (Mahanty and Sah, 1998), and were considered healthy if they had a resting membrane potential of −58 mV or below and exhibited action potentials that overshot 0 mV. Single action potentials were evoked by a depolarizing square current step (5 ms) of increasing amplitude delivered via the head stage. Single action potentials were measured from rest and analyzed for spike threshold, amplitude and width (measured at ½ of the action potential amplitude). Repetitive spiking was examined at rest in these neurons using a series of 500 ms current steps of increasing intensity (−0.05 nA to 0.35 nA, .05 nA steps). Spike accommodation was assessed at the highest level of depolarization in each group (0.35 nA). To record the post burst AHP neurons were held at 5 mV below action potential threshold and the AHP was evoked using a 100 ms current step sufficient to elicit 5 action potentials. AHP measurements were made from the average of 10 successive traces from each neuron.

All recordings were analyzed off-line using Clampfit 10.0 (Axon Instruments). Sample sizes refer to number of slices in the synaptic physiology experiments and the number of cells in the

whole-cell experiment and all values are expressed as mean ± S.E.M. Statistical analysis was performed using student t-tests or repeated measures ANOVA using post hoc comparisons (Fisher's PLSD) where appropriate.

Results

$Ca_V1.3$ knockout mice exhibit intact synaptic transmission, but impaired LTP

The in vitro horizontal slice preparation of the amygdala is pictured in Figure 1A. An input-output response curve was constructed to assess basal synaptic transmission in $Ca_V1.3$ knockout mice (Figure 1B). A repeated measures ANOVA revealed that field potential amplitudes increased with EC stimulus intensity ($F_{(6, 150)} = 90.3$, $p < 0.05$), but there was no significant difference ($F_{(1, 25)} = 0.16$, $p > 0.05$) between wild-type (15 slices from 8 mice) and $Ca_V1.3$ knockouts (12 slices from 7 mice).

Having established that basal EC-LA synaptic transmission was unaltered in $Ca_V1.3$ knockout mice, we sought to investigate the ability of $Ca_V1.3$ knockout mice to exhibit LTP following HFS delivered via the EC. Figure 2 shows the effect of HFS on field potential amplitude in the LA. Paired t-tests reveal that average field potential 55–60 minutes post-HFS was significantly potentiated over the average field potential during the five-minute baseline in wild-type (12 slices from 9 mice; $t_{(1, 11)} = 5.2$, $p < 0.05$) and $Ca_V1.3$ knockout mice (10 slices from 7 mice; $t_{(1, 9)} = 2.8$, $p < 0.05$). However, field potential amplitudes were significantly less potentiated ($t_{(1, 20)} = 2.8$, $p < 0.05$; unpaired t-test) in $Ca_V1.3$ knockout mice (112.7 ± 4.6 %) than wild-type mice (144.3 ± 8.6 %). These results demonstrate that deletion of $Ca_V1.3$ results in a significant decrease in long-term potentiation of EC-LA synapses.

BLA neurons from $Ca_V1.3$ knockout mice exhibit enhanced excitability and decreased post-burst AHP

To investigate the role of $Ca_V1.3$ in regulating intrinsic excitability, whole-cell current clamp recordings were made from BLA principal neurons in slices prepared from $Ca_V1.3$ knockout mice (14 cells from 6 mice) and wild-type mice (16 cells from 8 mice). There was no significant difference in resting membrane potential between $Ca_V1.3$ knockout mice and wild- type mice (−63.6 ± 1.2 & −65.4 ± 1.5 mV, respectively; unpaired t-tests, $p > 0.05$). Similarly, there was no significant difference in input resistance between $Ca_V1.3$ knockout mice and wild-type mice (147.1 ± 13.9 & 173.3 ± 10.3 MΩ, respectively; unpaired t-tests, $p > 0.05$). In addition, BLA principal neurons from $Ca_V1.3$ knockout mice were not significantly different from wild-type BLA principal neurons when membrane properties measured during a single evoked spike were examined (Figure 3). We did not observe any significant differences between $Ca_V1.3$ knockout mice and wild-type mice with regard to action potential threshold, width, or peak amplitude (Figure 3A, B & C respectively; unpaired t-tests for all measures, $p > 0.05$).

While deletion of $Ca_V1.3$ did not significantly alter membrane properties at rest or during a single action potential, loss of $Ca_V1.3$ resulted in substantial changes in firing properties in response to prolonged depolarization (Figure 4). As the representative traces presented in Figure 4A indicate, prolonged current steps (0.35 nA; 500 ms) consistently generated more spikes in BLA principal neurons from $Ca_V1.3$ knockout mice when compared with wild-type mice. A repeated measures ANOVA of the group data for the average number of spikes for the various current levels (Figure 4B) revealed a significant main effect of genotype ($F_{(1, 28)} = 6.5$; $p < 0.05$) as well as a significant interaction between genotype and average spike number ($F_{(6, 168)} = 5.5$; $p < 0.05$). Post-hoc comparisons of the average number of spikes at the individual current levels indicated that the BLA neurons from $Ca_V1.3$ knockout mice fire more action potentials than BLA neurons from wild-type mice at all levels of depolarization except the two lower depolarizing steps (0.05 & 0.10 nA; Figure 4B).

In addition to firing more action potentials, neurons from $Ca_V1.3$ knockout mice exhibited a significant reduction in spike accommodation (Figure 4C). To assess spike accommodation, the duration of the interspike interval (ISI) for the first six spikes was measured during repetitive spiking evoked by a 500 ms, 0.35 nA current step in BLA principal neurons from $Ca_V1.3$ knockout mice (14 cells from 6 mice) and wild-type mice (15 cells from 8 mice; one neuron could not be driven to fire six spikes). While neurons from both groups of mice exhibited spike accommodation, the average duration of the ISI was significantly less for $Ca_V1.3$ knockout mice when compared to wild-type mice ($F_{(1,27)} = 6.1$; $p < 0.05$; main effect of genotype). From the group data it appears that deletion of $Ca_V1.3$ has a greater impact upon spiking that occurs later in the train of action potentials; an observation that is confirmed by post-hoc comparisons that were only significant for ISI 3–5. Taken collectively these data indicate that deletion of $Ca_V1.3$ leads to an aberrant increase in neuronal excitability within BLA principal neurons.

The firing frequency of principal neurons in the BLA is modulated in part by calcium- activated potassium currents that underlie the post burst AHP (Faber and Sah, 2002; 2005; Power and Sah, 2008; Washburn and Moises, 1992). Therefore, we hypothesized that increased neuronal excitability observed in the BLA of $Ca_V1.3$ knockout mice was due to a decrease in the AHP. Indeed, the AHP that resulted after 5 action potentials was significantly smaller in the $Ca_V1.3$ knockout mice in overall area (5552.8 ± 762.2 mV • ms) when compared with the AHP recorded in wild-type mice (10003.8 ± 1249.6.2 mV • ms; $t_{(1, 28)} = 2.9$, $p < 0.05$; Figure 5). A change in the overall area of the AHP could be the result of alterations in either the mAHP or sAHP. To determine whether the mAHP was affected in $Ca_V1.3$ knockout mice, we measured the amplitude of the AHP at its peak (~ 60 ms after current stimulus offset) and 200 ms after current stimulus offset. The peak amplitude of the AHP did not differ between wild-type (−9.9 ± 0.6 mV) and $Ca_V1.3$ knockout mice (−9.6 ± 0.5 mV; $t_{(1, 28)} = 0.6$, $p > 0.05$; Figure 6A). There was a strong, but statistically non-significant, trend for a smaller AHP amplitude at 200 ms after current stimulus offset in $Ca_V1.3$ knockout mice (−4.8 ± 0.4 mV) compared with wild-type mice (−6.0 ± 0.5 mV; $t_{(1, 28)} = 2.0$ $p = 0.06$; Figure 6B). To assess if the sAHP was reduced in $Ca_V1.3$ knockout mice, we measured the amplitude of the AHP at 1000 ms after current stimulus offset. The AHP amplitude at 1000 ms was significantly smaller in $Ca_V1.3$ knockout mice (−1.7 ± 0.3 mV) than in wild-type mice (−2.9 ± 0.3 mV; $t_{(1, 28)} = 2.4$, $p < 0.05$; Figure 6C) suggesting that deletion of $Ca_V1.3$ selectively impacts the generation of the sAHP. Additionally, AHP duration is significantly shorter in $Ca_V1.3$ knockout mice (4.4 ± 0.7 seconds) than in wild-type mice (6.7 ± 0.6 seconds, $t_{(1, 28)} = 2.5$; $p < 0.05$, Figure 6D).

Discussion

The primary finding of the current study is that deletion of the voltage-gated calcium channel $Ca_V1.3$ results in altered neuronal function within the amygdala. Specifically, we have demonstrated that LTP is significantly reduced in the amygdala of $Ca_V1.3$ knockout mice. In addition, we report that principal neurons in the BLA of $Ca_V1.3$ knockout mice exhibit enhanced excitability as measured by an increase in repetitive firing and a decrease in the post-burst AHP.

Our experiments using the horizontal slice preparation demonstrate that LTP induced by HFS of the afferent fibers of the EC is significantly reduced in mice that lack the $Ca_V1.3$ calcium channel. Importantly, the deficit in HFS LTP was observed in the absence of any alteration in basal synaptic transmission. Long-term potentiation in the amygdala is dependent upon activation of either NMDA receptors, L-type voltage gated calcium channels or both. The relative contribution of L-VGCCs to the induction of LTP in the amygdala appears dependent on the type of slice preparation, the specific stimulation protocol used to induce LTP and the afferents that are stimulated (Sah et al., 2008). Specifically, LTP induced in horizontal slices using 2 bouts of 100Hz stimulation (1 sec duration; 30 sec apart) of the external capsule can

be blocked by either NMDA receptor antagonists or calcium channel blockers such as nifedipine (Drephal et al., 2006). Thus our results are consistent with the notion that the bulk of the LTP that is blocked by nifedipine is mediated by the L-type calcium channel $Ca_V1.3$. To limit our assumptions regarding which neuronal subpopulations (i.e. excitatory or inhibitory) were or were not impacted by the deletion of $Ca_V1.3$ we elected to use the horizontal slice preparation because LTP in this preparation can be induced without blocking inhibitory transmission (Drephal et al., 2006). Thus, while it seems likely that the observed deficit in LTP reflects an alteration in calcium signaling postsynaptically, it is possible that deletion of $Ca_V1.3$ disrupts plasticity in inhibitory interneurons such that there is an increase in inhibition after LTP induction in the $Ca_V1.3$ knockout mice. In addition to permitting LTP induction in the absence of GABA antagonists, the EC of the horizontal slice preparation contains intact afferents from the hippocampus, perirhinal and entorhinal cortices (von Bohlen und Halbach and Albrecht, 1998; 2002), brain regions critical for encoding contextual information (Burwell, Saddoris, Bucci, and Wiig, 2004; Kim and Fanselow, 1992) of the type with which $Ca_V1.3$ knockout mice are impaired in forming associations (McKinney and Murphy, 2006).

Deletion of $Ca_V1.3$ did not significantly alter the passive membrane properties of the principle neurons in the BLA. We did not observe any statistically significant changes in resting membrane potential or input resistance in the $Ca_V1.3$ knockout mice. Similarly, there was no change in the spike threshold, height or half width of single action potentials recorded in $Ca_V1.3$ knockout BLA principle neurons. Neuronal excitability in the amygdala has been reported to be regulated by voltage-gated calcium channels. Repetitive firing in response to a prolonged depolarizing step is significantly increased in the presence of cadmium (Faber et al., 2001; Faber and Sah, 2002; Gean and Shinnick-Gallagher, 1989; Washburn and Moises, 1992). Consistent with these reports, we observed an increase in neuronal excitability as measured by a significant increase in the firing frequency and a decrease in the interspike interval in response to prolonged current steps in the $Ca_V1.3$ knockout mice. It is worth noting that although the difference in input resistance between wild-types and knockouts was not statistically significant, there was a trend toward a decrease in the $Ca_V1.3$ knockout mice. This modest decrease in input resistance would be predicted to result in less membrane depolarization in response to somatic current injection making our finding of increased excitability even more striking. At present we do not know the cellular mechanism that underlies this putative change in input resistance but it is not unreasonable to hypothesize that this may be a compensatory mechanism which would functionally oppose the increased neuronal excitability observed in the $Ca_V1.3$ knockout mice.

Previous reports have suggested that VGCC modulation of neuronal excitability is regulated at least in part by the AHP (Faber et al., 2001; Faber and Sah, 2002; Gean and Shinnick-Gallagher, 1989; Washburn and Moises, 1992). Indeed, the $Ca_V1.3$ knockout mice exhibited significant reductions in the overall area of the AHP as compared to wild-type littermates. Deletion of $Ca_V1.3$ significantly reduced the overall area of the AHP as well as the AHP amplitude measured at 1000 ms after current stimulus offset. We did not observe a significant change in the peak amplitude of the AHP and although there was a trend towards a difference, the AHP amplitude measured at 200 ms was not significantly different in $Ca_V1.3$ knockout mice. In addition, the AHP duration is significantly shorter in $Ca_V1.3$ knockout mice than in wild-type mice. Taken collectively we conclude that $Ca_V1.3$ is selectively involved in the generation of the sAHP.

Though significantly reduced, a residual AHP (measured by overall area and amplitude at 1000 ms) is present in the principal BLA neurons of $Ca_V1.3$ knockout mice. At present the calcium source for the residual AHP is not known. In the hippocampus, where the putative role that L-VGCCs play in sAHP generation has been studied in more detail, it appears that blockade of L-VGCCs with dihydropyridines does not completely abolish the underlying calcium-activated

potassium current that underlies the sAHP (Power et al., 2002; Shah and Haylett, 2000; Tanabe, Gahwiler, and Gerber, 1998). In these studies the residual calcium-activated potassium current might reflect the relatively poor blockade of L-VGCCs by dihydropyridines (Helton, Xu, and Lipscombe, 2005; Xu and Lipscombe, 2001). Clearly, in the present study this is not the case. One possibility is that the calcium source for the residual AHP is calcium influx through the other brain-expressed L-VGCC, $Ca_V1.2$. Although we have not examined the role of $Ca_V1.2$ in the generation of the sAHP in BLA principle neurons, we have recently demonstrated that deletion of $Ca_V1.2$ in CA1 pyramidal neurons within the hippocampus does not significantly reduce the sAHP while deletion of $Ca_V1.3$ reduced the sAHP by approximately 50% (Kuo, McKinney, White, and Murphy, 2006).

Alternatively one might imagine that calcium from intracellular calcium stores may be responsible for the residual sAHP. In hippocampal pyramidal neurons, disruption of calcium-induced calcium release (CICR) from intracellular calcium stores has been shown to reduce sI_{AHP}—the current that underlies the AHP (Borde, Bonansco, de Sevilla, Le Ray, and Buno, 2000; Shah and Haylett, 2000; Tanabe et al., 1998; Torres, Arfken, and Andrade, 1996; van de Vrede, Fossier, Baux, Joels, and Chameau, 2007). While similar experiments have not been carried out in projection neurons in the amygdala it has recently been reported that calcium released from intracellular stores in response to cholinergic stimulation does not activate calcium-activated potassium channels that give rise to the sI_{AHP} suggesting that CICR likely does not significantly contribute to the sAHP (Power and Sah, 2008). In addition, it has recently been demonstrated that calcium loading of the endoplasmic reticulum in BLA projection neurons can be achieved with subthreshold depolarization and it has been suggested that this calcium influx is gated by $Ca_V1.3$ (Power and Sah, 2005). Therefore if CICR is responsible for the residual sAHP, our data would suggest that the source of calcium that triggers the calcium release from internal stores does not require $Ca_V1.3$ and that the internal calcium stores can be replenished in the absence of $Ca_V1.3$.

A final explanation for the residual sAHP is the potential contribution of other voltage-gated calcium channels. While there has not been a systematic examination of which voltage-gated calcium channels mediate the sAHP in the amygdala, it is clear that voltage-gated calcium channels play a critical role in sAHP. The sAHP (or the underlying sI_{AHP}) is almost completely abolished by cadmium (Faber et al., 2001; Faber and Sah, 2002; 2003; Washburn and Moises, 1992) and high concentrations of nickel (Faber and Sah, 2002; 2003). In support of the suggestion that $Ca_V1.3$ operates in concert with other VGCCs is the observation that the N-type calcium channel blocker ω-conotoxin-GVIA significantly reduces sI_{AHP} in cultured rat hippocampal pyramidal neurons (Shah and Haylett, 2000). Whatever the other source of calcium may be, the data presented here suggest that $Ca_V1.3$ plays a key role in the generation of the sAHP in amygdala principle neurons.

The fact that BLA-LTP and neuronal excitability, two hypothesized neurobiological substrates for learning and memory, are altered in $Ca_v1.3$ knockout mice makes it tempting to speculate that one or both of the abnormalities leads to the impaired ability of $Ca_v1.3$ knockout mice to consolidate contextual fear learning. Within the BLA, a strong case can be made that LTP is necessary for fear learning. Sensory information about many potential conditioned and unconditioned stimuli, including auditory, contextual, and somatosensory information, converge on the BLA (Romanski, Clugnet, Bordi, and LeDoux, 1993; von Bohlen und Halbach and Albrecht, 1998; 2002) and LTP has been demonstrated in each of these afferent pathways (Bauer et al., 2002; Chapman, Kairiss, Keenan, and Brown, 1990; Drephal et al., 2006; Huang, Martin, and Kandel, 2000; Rogan, Staubli, and LeDoux, 1997; Weisskopf et al., 1999). Additionally, fear learning has been shown to modify synaptic strength of afferents on to BLA neurons in a way similar to experimentally-induced LTP (McKernan and Shinnick-Gallagher, 1997). Further, fear learning and BLA-LTP share similar stimulus contingencies (Bauer,

LeDoux, and Nader, 2001) and molecular mechanisms (Rodrigues, Schafe, and LeDoux, 2004). Together, these observations suggest that an LTP-like mechanism underlies fear learning in the BLA. An example of the shared molecular mechanisms of fear learning and BLA-LTP which is consistent with our findings comes from a study in which Bauer et al (2002) demonstrated that the L-VGCC antagonist blocks BLA-LTP as well as long-term fear memory when a L-VGCC antagonist was infused into the BLA. Based on these studies it would be logically consistent to hypothesize that the deficits in contextual fear conditioning consolidation observed in the $Ca_v1.3$ knockout mice is due to a reduction in BLA-LTP, however this hypothesis may be overly simplistic.

There is a significant disparity in time course between the BLA-LTP experiments and the deficits observed in the $Ca_v1.3$ knockout mice with regard to the consolidation of contextual fear conditioning (see McKinney and Murphy 2006, Figure 2). This disparity has been reported frequently in the literature. Many manipulations (genetic and pharmacological) that impair long-term but not short-term memory block LTP within minutes after induction. The work of Bauer et al. (2002) provides a relevant example. Application of the L-VGCC antagonist verapamil impaired BLA-LTP induced by paired stimulation of the EC immediately, whereas intra-amygdala infusion of the same L-VGCC antagonist before fear learning resulted in impairment performance 24 hours later, but not at earlier time points. Some have argued that this disparity in time course may reflect a difference in the way in which LTP is engaged naturally *in vivo* and the way it is studied *in vitro.* These differences include patterns of stimulation and modulation by other areas of the brain (Schafe, Nader, Blair, and LeDoux, 2001). Alternatively, the LTP underlying fear memories may not be induced during training, but rather at a later time point, for example, during sleep. There is evidence that consolidation of memories may occur via the replaying of newly acquired patterns of activity during sleep (Axmacher, Mormann, Fernández, Elger, and Fell, 2006) and thus replaying of specific patterns of neuronal activity during sleep may induce LTP.

Like LTP, changes in neuronal excitability have been proposed as a neurobiological substrate of learning. It has been suggested that enhanced excitability is not part of the encoding of learning per se, but rather serves a permissive function for synaptic modification (Zhang and Linden, 2003). It is proposed that when a neuron is more excitable, the threshold for LTP is lower, thus information encoding is facilitated. Following this line of reasoning, one would expect animals with more excitable neurons to be better capable at encoding information and thus more efficient learners. Our results suggest this is not the case in $Ca_v1.3$ knockout mice. That is, though $Ca_v1.3$ knockout mice exhibit enhanced excitability in principal neurons of the BLA (the present study) and in CA1 neurons of the hippocampus (Kuo et al., 2006), neither hippocampus-dependent learning (Morris water maze) nor BLA-dependent learning (contextual fear conditioning) is enhanced (McKinney and Murphy, 2006). It could be that enhanced excitability does facilitate learning, but $Ca_v1.3$ knockout mice have other deficits that interfere with learning (e.g., impaired BLA-LTP). Further behavioral characterization of $Ca_v1.3$ knockout mice may reveal that there are some learning tasks on which they perform better than wild-type mice. Our data, however, are consistent with other data in which manipulations that enhance excitability rescue learning and memory deficits in aged animals (Disterhoft, Thompson, Moyer, and Mogul, 1996; Murphy, Fedorov, Giese, Ohno, Friedman, Chen, and Silva, 2004), but lead to impairments in young animals (Giese, Storm, Reuter, Fedorov, Shao, Leicher, Pongs, and Silva, 1998).

Voltage-gated calcium channels have previously been implicated in amygdala neurophysiology, including LTP and neuronal excitability, but these data are the first to implicate the $Ca_v1.3$ subtype specifically. While it seems likely that the disruption of BLA-LTP or the aberrant increase in neuronal excitability could account for the impaired ability of $Ca_v1.3$ knockout mice to consolidate conditioned fear, a thorough understanding of the relative

contributions of these two neurophysiological changes to the memory deficits observed in the $Ca_v1.3$ knockout mice awaits further study.

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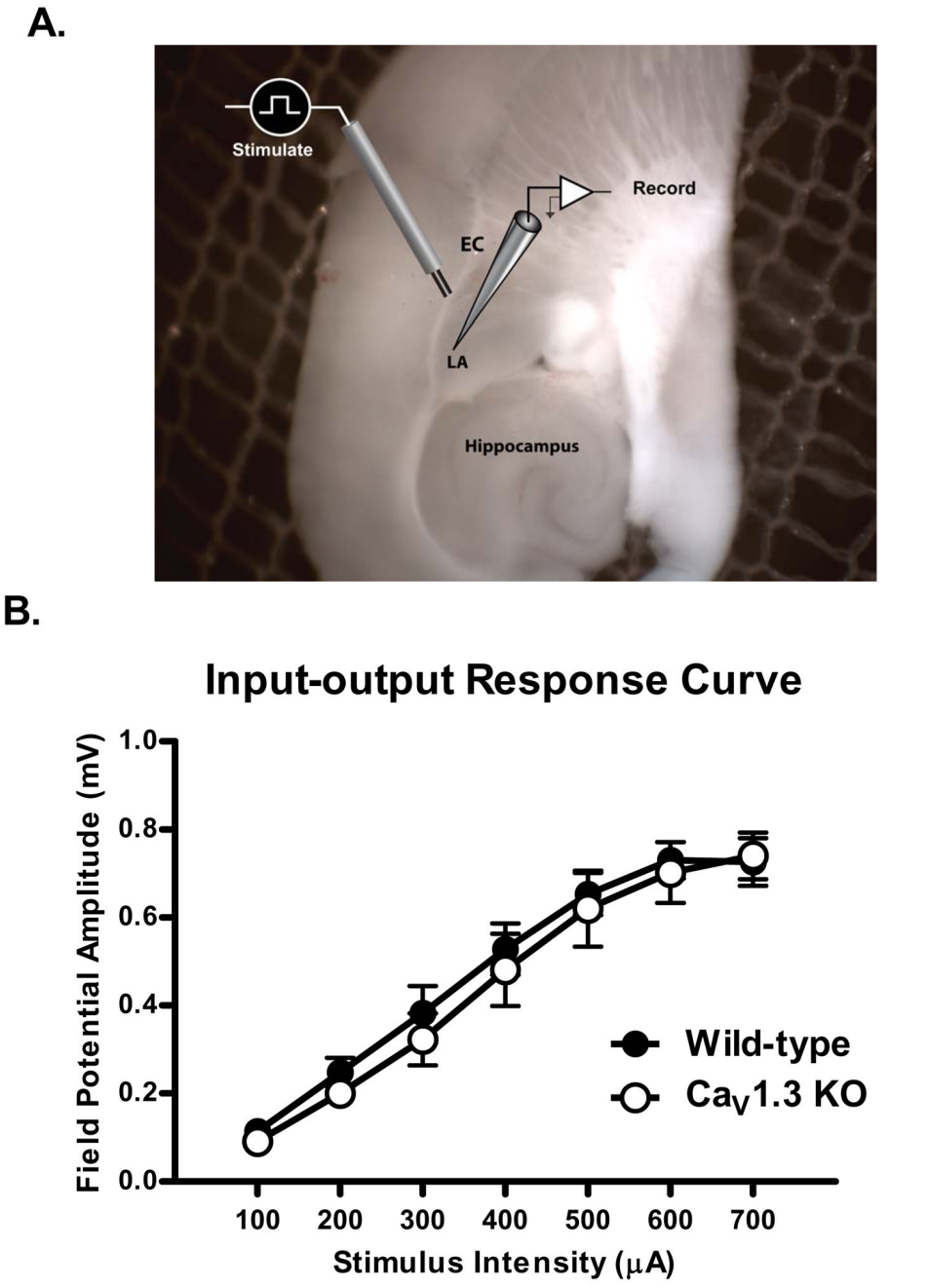

Figure 1.

Synaptic transmission in the EC-LA pathway does not differ between $Ca_V1.3$ knockout and wild-type mice. **(A)** A picture of a typical horizontal amygdala slice with stimulating and recording electrode placement depicted (EC; external capsule, LA; lateral amygdala). **(B)** Input/output curve plotted as a function of field potential amplitude (in mV) recorded in the LA verses amount of current delivered to the EC (in μA). Amplitude of field potentials recorded in the LA increases and plateaus with increasing stimulation strength in both $Ca_V1.3$ knockout and wild-type mice. All data are presented as mean ± S.E.M.

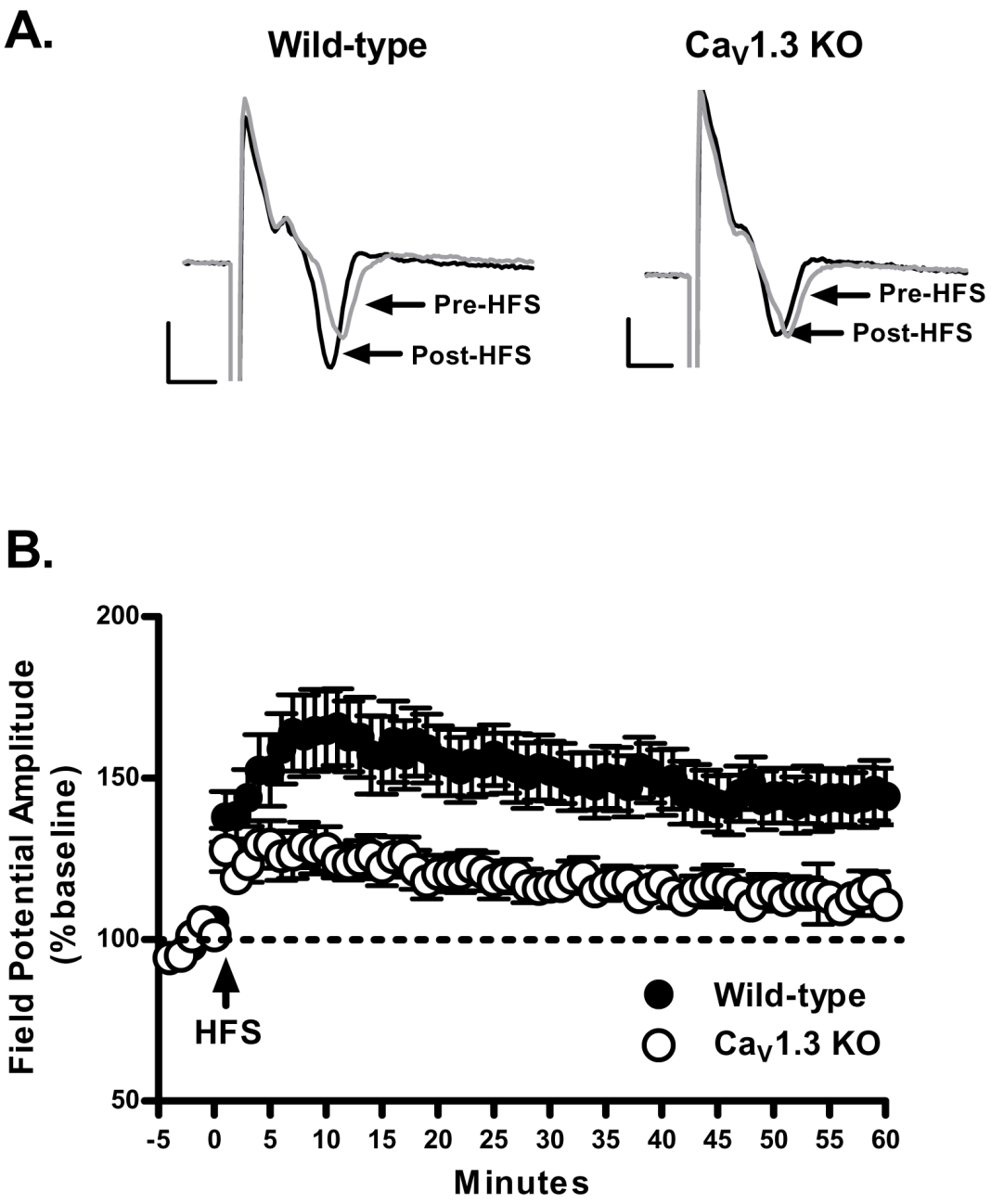

Figure 2.

Long-term potentiation of LA field potentials (LA-LTP) induced by high-frequency stimulation (HFS) of the EC is reduced in $Ca_V1.3$ knockout mice. **(A)** Representative traces of field potentials evoked by EC stimulation before and after HFS stimulation in wild-type mice (left traces) and $Ca_V1.3$ knockout mice (right traces). Scale bar 0.5 mV/5 msec. **(B)** Group data from all experiments. Significant LA-LTP is induced by HFS in slices prepared from both wild-type and $Ca_V1.3$ knockout mice, but LA-LTP was significantly reduced in $Ca_V1.3$ knockout mice. Data points represent averaged amplitudes normalized with respect to baseline. All data are presented as mean ± S.E.M.

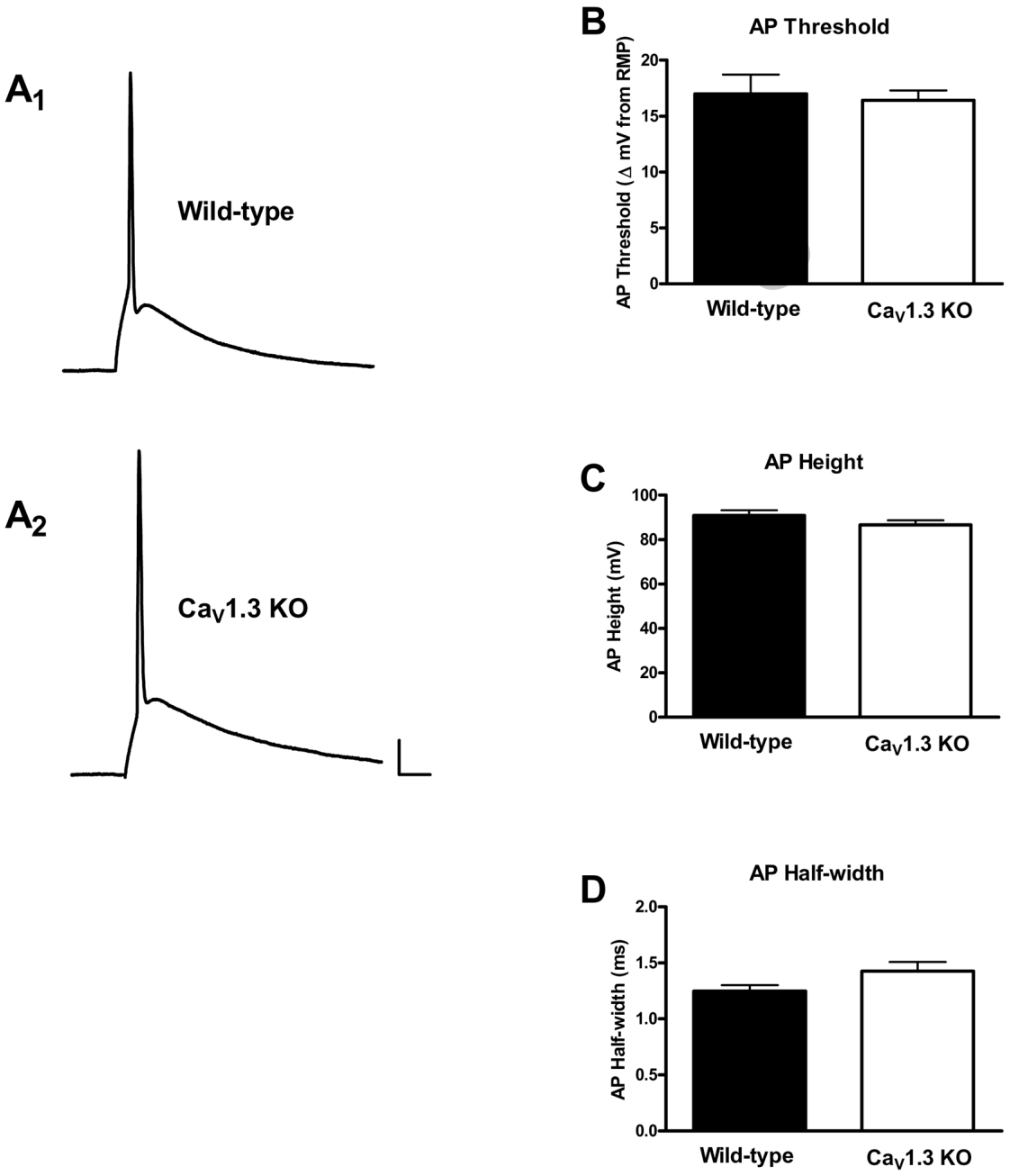

Figure 3.

Singe spike properties of BLA principal neurons recorded from $Ca_V1.3$ knockout mice were similar to those recorded in wild-type mice. (A) Representative single action potentials from a wild-type (A_1) and $Ca_V1.3$ knockout (A_2) BLA principle neuron. Scale bars: 10 mV/20 ms. (B) Action potential threshold, defined as the amount of membrane depolarization from resting membrane potential (RMP) required to elicit an action potential, was not significantly different in neurons from CaV1.3 mice when compared to wild-type neurons. Action potential height **(C)** and action half-width **(D)** was similar in neurons recorded from $Ca_V1.3$ knockout and wild-type mice. All data are presented as mean ± S.E.M.

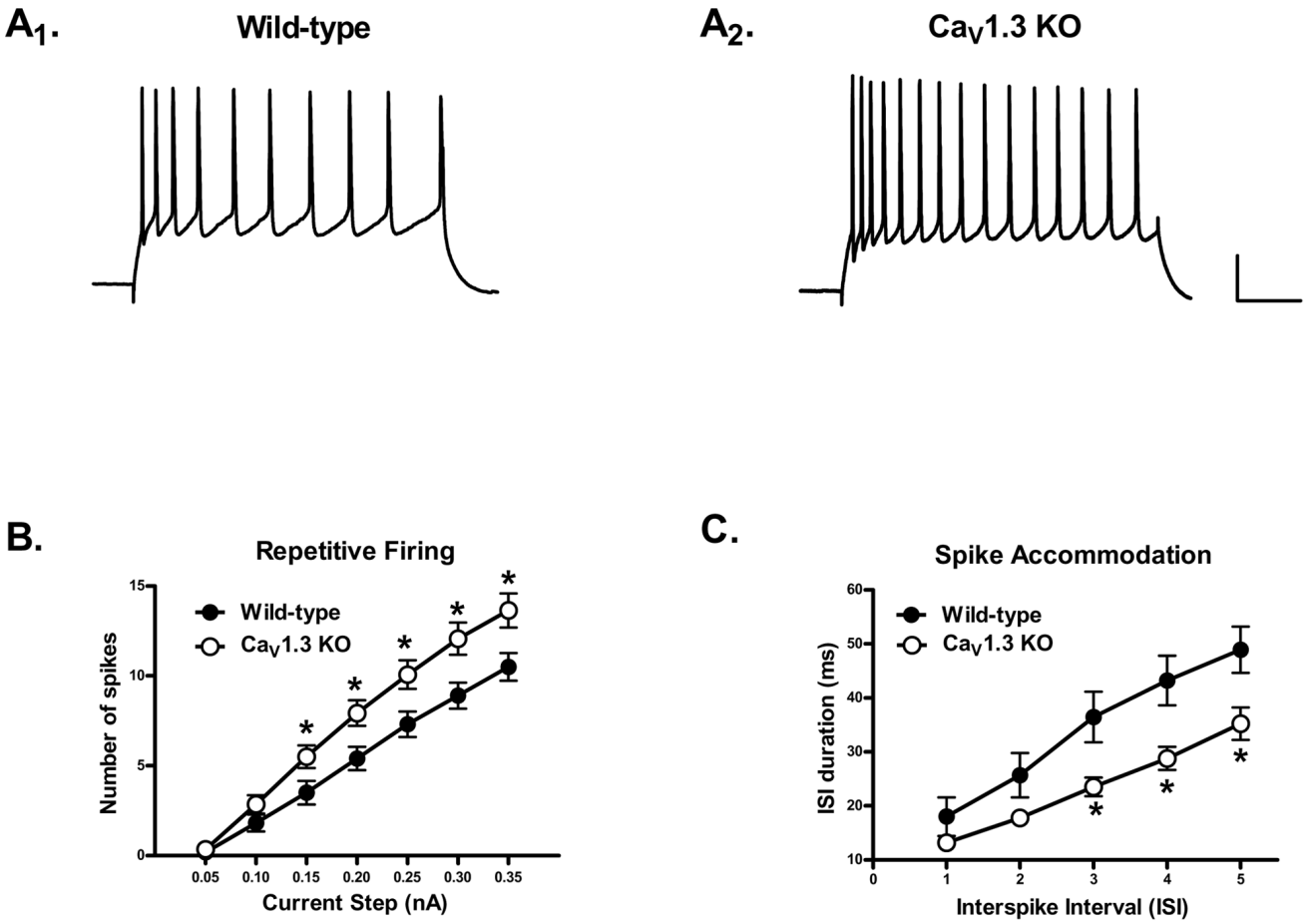

Figure 4.

BLA principal neurons from $Ca_V1.3$ knockout mice exhibit greater excitability than neurons from wild-type mice. **(A)** Representative traces from BLA principal neurons recorded in horizontal amygdala slices prepared from a wild-type **(A_1)** or $Ca_V1.3$ knockout **(A_2)** mouse during a 500 ms, 0.35 nA current injection. Scale bar: 20 mV/100 ms. **(B)** Current steps of increasing magnitude produced an increase in the average number of spikes in both groups; however BLA principal neurons recorded from $Ca_V1.3$ knockout fired significantly more action potentials as compared to wild-type BLA principle neurons for current steps larger than 0.10 nA. **(C)** Spike accommodation, as measured by the average duration of the interspike interval (ISI), was significantly reduced in BLA neurons from $Ca_V1.3$ knockout mice for ISI 3, 4 and 5. All data are presented as mean ± S.E.M. (*) $p < 0.05$ (Fisher's PLSD posthoc comparison).

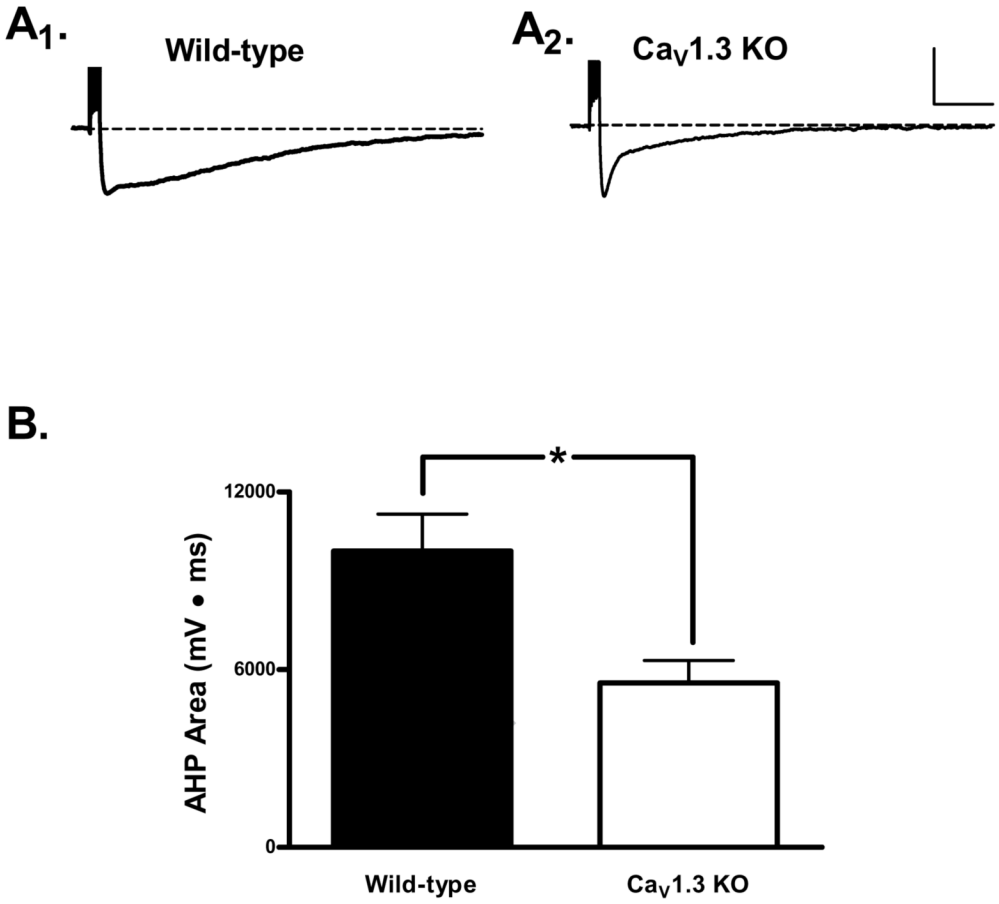

Figure 5.

Area of the AHP is significantly smaller in $Ca_V1.3$ knockout mice. **(A)** Representative traces of AHP recorded from a BLA neuron of a wild-type mouse **(A_1)** and $Ca_V1.3$ knockout mouse (A_2). In both traces the action potentials are cropped to increase the scale of the AHP. Scale bar: 1 mV/1000 ms. **(B)** Average AHP area area of the AHP in $Ca_V1.3$ knockout mice is ~50% of that in wild-type mice. Data are presented as mean ± S.E.M. (*) $p < 0.05$ (unpaired t-test).

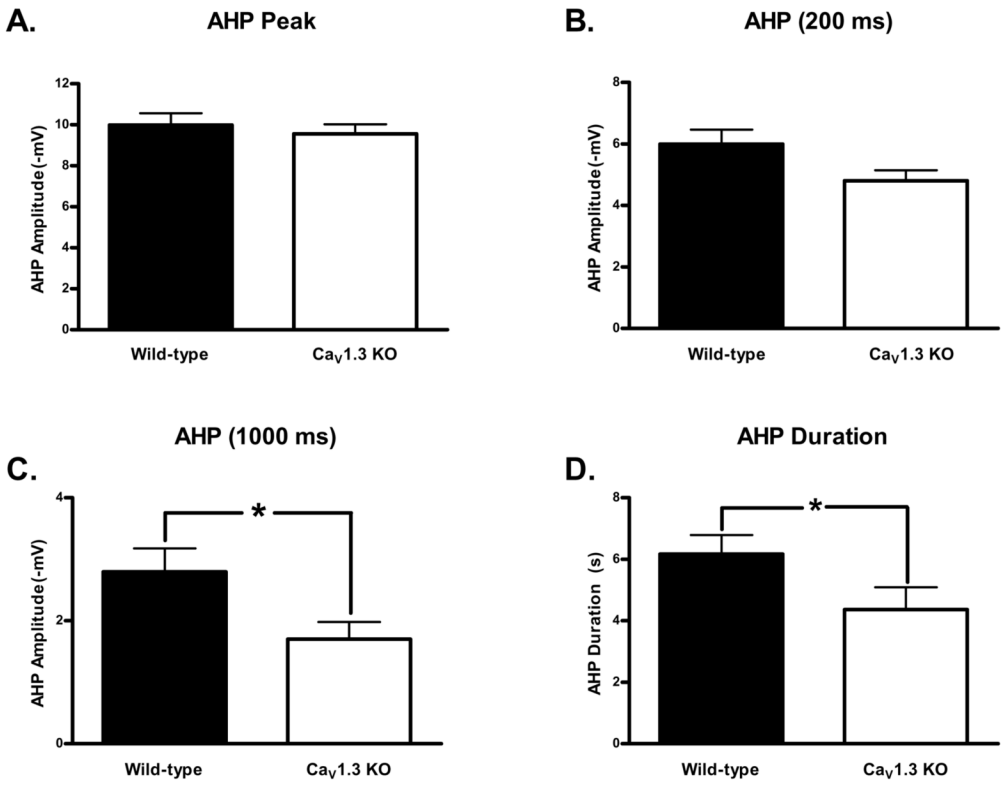

Figure 6.

Later components of the AHP are decreased in $Ca_V1.3$ knockout mice. **(A)** AHP peak amplitude does not differ between wild-type and $Ca_V1.3$ knockout mice. **(B)** While there a trend for a smaller AHP amplitude at 200 ms after stimulus offset in $Ca_V1.3$ knockout than wild-type mice this difference was not statistically significant ($p = 0.06$). **(C)** The AHP amplitude at 1000 ms after stimulus offset is significantly smaller in $Ca_V1.3$ knockout mice than wild-type mice. **(D)** The duration of the AHP is significantly shorter in $Ca_V1.3$ knockout mice than wild-type mice. All data are presented as mean ± S.E.M. (*) $p < 0.05$ (unpaired ttest).