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Sound Localization in Preweanling Mice Was More Severely Affected by Deleting the *Kcna1* Gene Compared to Deleting *Kcna2*, and a Curious Inverted-U Course of Development That Appeared to Exceed Adult Performance Was Observed in All Groups

JAMES R. ISON,[1,2] PAUL D. ALLEN,[3] BRUCE L. TEMPEL,[4] AND HELEN M. BREW[4]

[1]*Department of Brain and Cognitive Sciences, Meliora Hall, University of Rochester, Rochester, NY 14627, USA*

[2]*Department of Neuroscience and The Del Monte Neuromedicine Institute, University of Rochester Medical Center, Rochester, NY 14642, USA*

[3]*Department of Otolaryngology, University of Rochester Medical Center, Rochester, NY 14642, USA*

[4]*The Virginia Merrill Bloedel Hearing Research Center and the Departments of Otolaryngology-Head and Neck Surgery and Pharmacology, University of Washington School of Medicine, Seattle, WA 98195, USA*

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ABSTRACT

The submillisecond acuity for detecting rapid spatial and temporal fluctuations in acoustic stimuli observed in humans and laboratory animals depends in part on select groups of auditory neurons that preserve synchrony from the ears to the binaural nuclei in the brainstem. These fibers have specialized synapses and axons that use a low-threshold voltage-activated outward current, I_{KL}, conducted through Kv1 potassium ion channels. These are in turn coupled with HCN channels that express a mixed cation inward mixed current, I_H, to support precise synchronized firing. The behavioral evidence is that their respective *Kcna1* or *HCN1* genes are absent in adult mice; the results are weak startle reflexes, slow responding to noise offsets, and poor sound localization. The present behavioral experiments were motivated by an in vitro study reporting *increased* I_{KL} in an auditory nucleus in *Kcna2*$^{-/-}$ mice lacking the Kv1.2 subunit, suggesting that *Kcna2*$^{-/-}$ mice might perform better than *Kcna2*$^{+/+}$ mice. Because *Kcna2*$^{-/-}$ mice have only a 17–18-day lifespan, we compared both preweanling *Kcna2*$^{-/-}$ vs. *Kcna2*$^{+/+}$ mice and *Kcna1*$^{-/-}$ vs. *Kcna1*$^{+/+}$ mice at P12-P17/18; then, the remaining mice were tested at P23/P25. Both null mutant strains had a stunted physique, but the *Kcna1*$^{-/-}$ mice had severe behavioral deficits while those in *Kcna2*$^{-/-}$ mice were relatively few and minor. The in vitro increase of I_{KL} could have resulted from Kv1.1 subunits substituting for Kv1.2 units and the loss of the inhibitory "managerial" effect of Kv1.2 on Kv1.1. However, any increased neuronal synchronicity that accompanies increased I_{KL} may not have been enough to affect behavior. All mice performed unusually well on the early spatial tests, but then, they fell towards adult levels. This unexpected effect may reflect a shift from summated independent monaural pathways to integrated binaural processing, as has been suggested for similar observations for human infants.

Keywords: Deletion Kv1 subunits, acoustic startle reflex, noise offset, sound localization

INTRODUCTION

The ability to locate sounds and track their changing positions depends on rapid cochlear mechanics and acute sensory processing to follow the acoustic

Correspondence to: James R. Ison · Department of Brain and Cognitive Sciences, Meliora Hall · University of Rochester · Rochester, NY 14627, USA. email: JIson@ur.rochester.edu

fluctuations that vary with their different locations and then on maintaining the relative arrival times of neural information from both ears in the ascending monaural pathways at their binaural targets (Yin 2002). The mechanisms that maintain temporal coherence in these streams of sensory-neural activity include a low-voltage outward potassium current, I_{KL}, conducted through Kv1 channels to stabilize membrane potentials, limit neuronal excitability, and facilitate precise firing in synchrony with the acoustic stimuli to enhance auditory perception (Oertel et al. 2017). Deleting the *Kcna1* gene, which encodes the Kv1.1 subunit in the tetrameric Kv1 channels, reduces I_{KL} and increases scattered asynchronous neural reactivity in the auditory brainstem, shown by both in vitro and in vivo electrophysiology (Brew et al. 2003; Kopp-Scheinpflug et al. 2003; Gittelman and Tempel 2006; Karcz et al. 2012). Parallel behavioral experiments comparing *Kcna1*$^{-/-}$ mice to *Kcna1*$^{+/+}$ mice using reflex inhibition audiometry (Yerkes 1905; Marsh et al. 1978; Young and Fechter 1983; Lauer et al. 2017) found high levels of motor activity, weak startle reflexes, slow responsivity to noise offsets, and poor sound localization in *Kcna1*$^{-/-}$ mice (Allen and Ison 2012; Ison and Allen 2012; Karcz et al. 2015). These *Kcna1*$^{-/-}$ mice had normal auditory thresholds in evoked ABR potentials, despite non-synchronous firing (Allen and Ison 2012).

Here we study the behavioral effects of deleting *Kcna2*, the gene that encodes the Kv1.2 subunit that is another contributor to Kv1 channels and I_{KL}. When expressed in oocytes, Kv1.1 and Kv1.2 subunits conferred similar characteristics on the resulting channels except that Kv1.2 homomeric channels activated at less negative potentials with slower onsets and recovery from inactivation, and heteromers with higher Kv1.1/Kv1.2 ratios provided more negatively activating I_{KL}, (Akhtar et al. 2002; Hopkins et al. 1994). Both subunits are present in most of the monaural and binaural nuclei of the auditory brainstem (Grigg et al. 2000). The presence of I_{KL} together with other specialized neural features, such as large axons and synapses and rapidly marshaled neurotransmitters (Brew and Forsythe 1995; Trussell 2002; Golding 2012), supports the synchronous timing in parallel monaural pathways and is necessary for accurate neural computation in the target brainstem binaural nuclei; the LSO (the lateral superior olivary nucleus) and the MSO (the medial superior olivary nucleus) express I_{KL} (Grigg et al. 2000; Karcz et al. 2012). While these two subunits, and a third common Kv1 subunit, Kv1.6, are often combined in heteromeric channels, on balance, they are numerically dominated by Kv1.1 subunits (Brew et al. 2003, 2007): these several observations suggest that deleting *Kcna2* should not affect channel numbers as greatly as does deleting *Kcna1* and thus should not have the severe effects on behavior.

In the auditory brainstem neurons, the I_{KL} conductance may actually increase in the absence of Kv1.2 subunits, as is shown for the principal neurons of the medial nucleus of the trapezoid body (MNTB), which carries contralateral auditory signals to the LSO. There was increased I_{KL} in *Kcna2*$^{-/-}$ MNTB neurons due to a more negative voltage activation (Brew et al. 2007) that is consistent with the Kv1.1-dominated properties shown in the oocyte data. A similar negative shift in voltage activation for Kv1.1/Kv1.2 channels was shown in cultured murine spiral ganglion neurons using subunit-specific specific toxins (Smith et al. 2015). If I_{KL} were increased throughout *Kcna2*$^{-/-}$ auditory pathways, it could even reduce the physiological time constant and improve the temporal fidelity for those behaviors based on temporal cues.

Our experiments with *Kcna2*$^{-/-}$ mice were limited from the onset of hearing at about P12 to their premature death at P17–18 (Brew et al. 2007; Robbins and Tempel 2012), following seizures, possibly exacerbated by cardiac failure at the age when Kv1.2 is necessary to replace the declining levels of Kv1.4 in the development of cardiac myocytes (Xu et al. 1996): the same secondary consequence of cardiac failure is sometimes observed after an epileptic episode in human patients (van der Lende et al. 2016).

The two notable dimensions of acoustic stimuli affected in *Kcna1*$^{-/-}$ adult mice are sounds moving along the azimuth (Allen and Ison 2012; Karcz et al. 2015) and noise offsets with variable offset ramps (Ison and Allen 2012). The spatial protocol was used for both *Kcna1* and *Kcna2* mice, but only the *Kcna2* mice were tested on the offset protocol. Surviving mice were retested on the spatial experiment at P23-P25 to extend the data on the developmental course of sound localization.

METHODS AND PROCEDURES

Subjects

Sixteen mice, 8 *Kcna2*$^{+/+}$mice (3 male, 5 female) and 8 *Kcna2*$^{-/-}$ mice (4 male, 4 female), provided data from the two experiments for the *Kcna2* mice: one on spatial acuity following Allen and Ison (2012) and the second comparing the effects of abrupt offsets vs. ramped offsets following Ison and Allen (2012). The mice were obtained from 5 litters (mean = 3.2 mice/litter) generated on a C3HeB/FeJ background and derived from *Kcna2* breeding stock as described previously by Brew et al. (2007) and were bred in the AAALAC-approved Specific Pathogen-Free facilities of the University of Washington: Specific Pathogen-Free vivarium conditions are necessary to extend the

lifespan of the *Kcna2*$^{-/-}$ mice from ~ P13 to P17–18. Genotyping was performed at about P6, and mice were ear-punched for later identification following the protocol described by Brew et al. (2007). The *Kcna2*$^{+/-}$ mice were removed from the litters at approximately P9. Thirty-three mice, 18 *Kcna1*$^{+/+}$ mice (9 male, and 9 female) and 15 *Kcna1*$^{-/-}$ (6 males and 9 females), were tested for the experiment on spatial acuity. They were obtained from 15 litters ($n = 5.3$/ litter including +/− mice) generated on the same C3HeB/FeJ background, but the parent *Kcna1*$^{+/-}$ heterozygotic mice were initially obtained from The Jackson Laboratory (Bar Harbor, ME, USA). They were bred in the AAALAC-approved (but not SPF) vivarium of the University of Rochester. Genotyping and ear punching at Rochester followed the protocol of Brew et al. (2003) but was scheduled at weaning at about P25. Because none of the *Kcna1* mice at Rochester were identified until the experiment was finished, the typical litter size at Rochester was larger and the mice were smaller than the Washington mice. All of the litters were raised in a 12/12 light/dark cycle with food and water available ad libitum save during the approximately 15–30 min periods of the behavioral protocols described below. The pups were isolated only in these testing periods, and they were weighed generally every day to identify any of the mice that were obviously not thriving under these conditions. On preliminary testing, only 3 of 13 *Kcna2* mice responded to the startle-eliciting stimulus on P11 (details in following section) and 6 of the 8 *Kcna2*$^{-/-}$ mice died before the scheduled test on P18. The remaining two *Kcna2*$^{-/-}$ mice died before the test on P19.

Apparatus

The same testing apparatus was used at Washington and Rochester, but it was housed in the differently sized sound-attenuating chambers that were available at the two sites: 1 m on a side at Washington and about 2 m at Rochester. Both were lined with echo-attenuating acoustical foam (Sonex: Illbruck, Minneapolis, MN). One mouse was tested at a time while confined in an aluminum wire cage having free sound penetration. The eliciting stimulus (ES) for the acoustic startle reflex (ASR) was a 15-ms broad-band noise burst (rectangular-gated, 50 kHz bandwidth, 130 dB SPL) digitally generated using a Tucker-Davis Technology (TDT, Alachua, FL) RP2.1 Real-Time Processor. The ES was attenuated by a TDT PA5, then amplified with an Adcom GFA-535 II amplifier (East Brunswick, NJ), and broadcast from 15 cm above the test cage via a high frequency tweeter. These stimuli do not affect hearing thresholds in mice, as has been demonstrated in the normal neural thresholds in single cells of the inferior colliculus in mice after being tested in similar ASR experiments (personal communication from Professor J. Walton, University of South Florida). The prepulse stimuli for measuring sensitivity for spatial localization were 1–50 kHz bands of noise presented at 70 dB SPL, delivered by matched TDT-ES1 electrostatic speakers. The noise was digitally generated using a second TDT-RP2.1 processor with 100 kHz sample rate in real time. For the spatial experiment, two speakers were elevated to the height of the test cage and were variously placed around a semicircle with angular separations left to right of 180 °, 90 °, 45 °, 22.5 °, and 15 °. The semicircle had a 30-cm radius at Washington and a 50-cm radius at Rochester. The test stimulus for spatial acuity was the exchange of the continuous noise from one speaker to the second speaker across the midline. For the noise-offset experiment, a single test speaker was placed at the midpoint on the semicircle. The test cage was mounted at the center of the diameter of the semicircle on a 15-cm high pedestal that was bolted to a stiff suspended acrylic platform, and the cage was oriented so that the mouse faced the mid-line between the two speakers. The head of the mouse was not restrained in position because of the likelihood of causing stress, and at least for cats and primates, restraining the head reduces spatial accuracy (Tollin et al. 2005; Populin 2006). The startle speaker and its support, the pedestal and the acrylic shelf, and the table on which the apparatus was placed were all covered with foam or cotton batting to absorb echoes. An accelerometer was attached underneath the platform to capture the downward force of the ASR and spontaneous background motor activity (ACT), and its voltage output was sampled at 1 kHz by the first RP2.1 processor. The ASR amplitude was defined as the RMS (root mean square) of this output in the 100 ms period after the delivery of the ES, and the ACT amplitude was defined as the RMS of the output of the same RP2.1 processor in the 100 ms period immediately before the ES. The ASR measure described above was consistent with electromyogram data of the near-coincident forelimb flexor and extensor muscles of rats (Hammond et al. 1972). Strong startle responses in mice produce the same defensive posture that has been filmed in rats by Horlington (1968) and in humans by Landis and Hunt (1939), while small inhibited responses are better described as a momentary flinch of the neck and head. The ACT measure reflects the summation of muscle twitches, restlessness, or agitation in the test cage, and for *Kcna1*$^{-/-}$ mice, their exaggerated ACT levels (for example, in Allen and Ison 2012) may reflect the spontaneous

muscle twitching labeled as "myokymia" in episodic ataxia patients carrying a *KCNA1* mutation (Browne et al. 1994).

Procedure

The two behavioral tests were variants of "reflex inhibition audiometry" (Marsh et al. 1978; Young and Fechter 1983; Lauer et al. 2017). One procedure was analogous to the experiment used to determine the "minimal audible angle" (MAA) in human listeners (Mills 1958). This method does not provide absolute information about the perceived location of a sound, but instead, the MAA describes sensitivity to the relative spatial positions of the two nonsimultaneous sounds, or in the present context, if a change in the location from one speaker to the other had significantly inhibited the ASR below the control level, with $\alpha = 0.05$, for a 1-tail t-statistic. The second test was patterned on experiments by von Bekesy (1933/1960, p. 322–326) and Miller (1948) that were designed to quantify the "physiological" or "perceptual" decay in human listeners after the abrupt offset of a sound, by finding the match between the longest ramp in a set of ramps that could not be discriminated from the abrupt offset. Following this approach, a set of ramp offsets was compared with abrupt noise offsets as prepulses were presented before a probe startle stimuli, as in two recent studies which found differences between adult knockout mice and their control littermates ($Kcna1^{+/+}$ vs. $Kcna1^{-/-}$, Ison and Allen 2012; and $Hcn1^{+/+}$ vs. $Hcn1^{-/-}$ lacking the hyperpolarization activated channel subunit HCN1, Ison et al. 2017).

For the spatial localization experiment, each test trial began with the broad band continuous noise emitted from the speaker of the left of the mouse, then after a random time lasting between 15 and 25 s, the noise was turned off simultaneously with the onset of an identical noise in a second speaker on the right of the mouse. There were 11 blocks of 4 test trials: in random order, two of the ES alone, and one trial each with the ES following the change in the sound source by 10 ms or 60 ms. The noise offset experiment began with the same continuous noise, and after the same random time of 15 to 25 s, it was either abruptly turned off and the probe ES was presented in quiet after 1, 2, 4, or 10 ms after the offset or turned off gradually with linear ramps of 1, 2, 4, or 10 ms and the startle stimulus was presented in quiet at the end of the ramp. Both tests began with a 2-min "settling down" in the noise; then, 44 trials in the spatial experiment or 110 trials in the noise offset experiment were presented at an average intertrial interval of 20 s (15–25 s), the full testing period lasting for about 17 min for the spatial experiment or about 35 min for the noise offset experiment. Given the limitation on the number of tests possible in 1 day for overlapping litters of mice, a semiadaptive procedure was used for the spatial experiment. The first test began for each mouse with an angular separation estimated as being within its ability as given its earlier result, and a second or sometimes a third test could be given at either a smaller or a larger separation. As a result, the MAA could not be always determined by testing just two adjacent separations (e.g., with success at the larger separation and not for an adjacent smaller separation), but instead, it was a conservative estimate of the MAA as the smallest angular separation that had been detected, without having an observed failure with the next smaller adjacent angle.

Statistical Treatment of the Data

The means of the ASR and ACT amplitudes for each condition were calculated for each mouse on each test, after excluding the first block of trials to avoid possible effects of the initial novelty of the ES. In experiments with these juvenile mice, the ACT levels are used as a baseline for each mouse to verify that the control ASR values were significantly larger than its ACT values. Using $\alpha = 0.05$ for a two-sided t test: if the ASR values were not significantly different from the ACT values on the test run (as was common for the young $Kcna1^{-/-}$ mice), the data for that test run were included in the group results for the baseline ASR and ACT but not analyzed for prepulse inhibition (PPI) or the minimal audible angle (MAA). However, given a significant ASR, the level of PPI is stable across the different strength of ASR as demonstrated in experiments on circadian rhythms (Ison and Foss 1997), muscle weakness from chemical toxicants (Fechter and Young 1983), advanced age and individual differences (Ison et al. 1997), and the relative effects of single prepulses and combinations of two prepulses (Ison et al. 1975). Many other experiments have used PPI methods but focused on central attentional processes ("sensorimotor gating") following a finding of Braff et al. (1978) that PPI was reduced in schizophrenic patients, and later a report of Swerdlow et al. (1986), that the strength of PPI was reduced in rats following forebrain dopamine receptor stimulation. Subsequent experiments have tested the hypothesis that the differences in the strength of PPI may be a secondary effect of changes in the strength of ASR rather than "sensorimotor gating," a similar concern described for PPI here. For example, a very recent and substantial experiment (Shoji and Miyakawa 2018) directly tested this hypothesis using male C57BL/6J mice ($N = 1363$ mice) ranging from 2 to 12 months of age: indeed, there was an overall

significant correlation between ASR and PPI values ($p = 0.0016$) but the Spearman Rho was small (0.0852) and the effect size was negligible (0.0073): indeed, this small positive correlation may be the result of the age-related hearing loss in this strain reported by Zheng et al. (1999) and many others, as hearing loss could affect both PPI and ASR,

In the present experiment, the PPI values for each mouse on each accepted test run were based on the mean level of the ASR following the prepulse (ASRp), relative to the control level of the ASR with no preceding prepulse (ASRc), then calculated as PPI = 1 – (ASRp/ASRc). In adult mice, the PPI level for the 60 ms lead time is higher than that for a 10-ms lead time (Allen and Ison 2012; Ison et al. 2017), and that this relationship is different in early preweanling mice that suggests a difference in the neural base of PPI (Ison and Bowen 2000). The threshold for an angular separation (the MAA) was defined as the smallest tested angular separation that provided a significant difference between the responses on prepulse trials and control trials, as $\alpha < 0.05$, for a one-sided t test. The PPI data depicted in Fig. 4 include only the 45 ° condition because few *Kcna1*$^{-/-}$ mice were tested at angles less than 45 ° and few *Kcna2*$^{-/-}$ mice were tested at angles greater than 45 °, given the semiadaptive choice of the stimuli and the better overall performance of *Kcna2*$^{-/-}$ mice.

For the second experiment that measured the changing strength of the ASR for the variable noise offset ramps, our interpretation of the weakened ASR that immediately follows noise offset cannot possibly have the same neural basis as the similar outcome of "prepulse inhibition" as it is usually discussed in published literature, because of the rapidity of the effect. For example, for noise offset, the loss of the ASR is significant at a lead time of 1 ms and reaches its maximum in 4 ms: this is too fast to be processed in the midbrain neural structures for PPI as described, for example, by Koch (1999). Instead of PPI, we therefore describe this phenomenon as "ASR Depression" in Fig. 6, because it seems more likely to be a consequence of a more peripheral mechanism, such as the post-excitatory synaptic depression observed in the cochlear nucleus of the mouse by Wang and Manis (2008).

Descriptive statistics are reported as group means and the standard error of the mean (mean (SEM)), and the effect size for the t test and two-group differences is R^2, using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Appropriate parametric or non-parametric t tests on specific stimulus conditions within and between subjects were mostly performed separately within the *Kcna2* and *Kcna1* experiments, with other analyses between genes. When necessary, the ANOVA was calculated using SPSS v.12 software and the effect size was given as $_p\eta^2$ (partial-Eta-squared). The comparison of null-mutant vs. wild-type mice was always a between-S variable, but age, PPI, and MAA measures were necessarily also treated as between-*S* variables because these measures were not available across every day. This was because *Kcna2* mice could not be tested on all days because of time limitations, while 7 of 15 *Kcna1*$^{-/-}$ mice had weak startle reflexes on P12-P15 and did not provide PPI and MAA values.

RESULTS

Kcna1$^{-/-}$ and *Kcna2*$^{-/-}$ Mice Were Physically Stunted, but Both Gained Weight at a Normal Rate

Previous experiments have found that *Kcna1*$^{-/-}$ and *Kcna2*$^{-/-}$ mice were smaller than their control littermates (Smart et al. 1998; Brew et al. 2007), and this is affirmed in Fig. 1. The value of gathering the weights of the individual mice on each day was to identify mice that were systematically losing weight as this would suggest a physical illness: no mouse in this work failed the criterion. The differences between the null-mutant mice and their controls were significant, for the *Kcna1.1* mice, $F(1/31) = 8.97$, $p = 0.005$, $_p\eta^2 = 0.22$, and for the *Kcna1.2* mice, $F(1/14) = 15.462$, $p < 0.002$, $_p\eta^2 = 0.52$. Both *Kcna1.1* and *Kcna1.2* mice increased their weight over the 6 days: in order, $F(5/155) = 22.43$, $p < 0.001$, $_p\eta^2 = 0.42$, and $F(2/28) = 17.20$, $p < 0.001$, $_p\eta 2 = 0.55$. The percentage increase from P12 to P17 for each group was 11.2 % and 11.0 % for *Kcna1.1*$^{+/+}$ and *Kcna1.1*$^{-/-}$ mice and 10.8 % and 11.1 % for the *Kcna1.2*$^{+/+}$ and *Kcna1.2*$^{-/-}$ mice. An ANOVA directly comparing the *Kcna1* and *Kcna2* mice for their summed weights provided a significant mutation effect (+/+ vs. −/−), $F(1/45) = 18.82$, $p < .0.001$, $_p\eta^2 = 0.30$ and a significant gene effect (*Kcna1* vs *Kcna2*), $F(1/45) = 8.49$, $p = 0.006$, $_p\eta^2 = 0.16$, with no significant interaction, $F\ (1/45) < 1$, $_p\eta^2 = 0.0$. Overall, the effect of deleting either *Kcna1* or *Kcna2* on reducing body weight was near identical, both groups being 9 % lighter than their respective control groups.

The *Kcna2* mice were significantly heavier than the *Kcna1* mice, by about 8 %. This difference is reasonably attributed to the smaller litters at Washington, as the consequence of earlier genotyping protocols that made it possible to identify and remove the *Kcna2*$^{+/-}$ mice before the experiment began. Consistent with this, the *Kcna2* mice studied by Brew et al. (2007) included the three genotypes and when tested at P14 those mice approximated the weight of the groups of the present *Kcna1* mice at P14 and weighed less than the groups of the present *Kcna2*

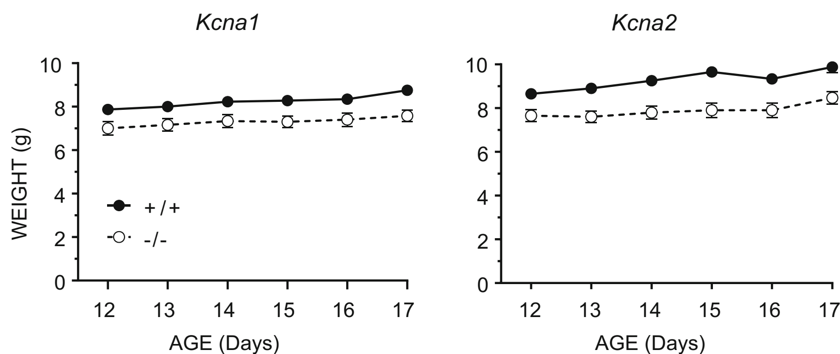

FIG. 1. The weight data are presented as mean and SEM, but many of SEM bars are hidden within the symbol for the mean. The *Kcna1* mice ($n = 18$+/+ and 15 −/−) mice and the *Kcna2* mice ($n = 8$+/+ and 8−/−) had the same deficit in body weight compared with their +/+ control littermates, but all groups had a similar increase in weight up to P17/P18

mice. A further salient effect of litter size on weight was observed on the comparison of the 60 % increase in weight from P17-P18 and P23-P25 for the *Kcna2*$^{+/+}$ mice in litters having then an average of 2.0 pups and the smaller 29 % increase in weight for the *Kcna1*$^{+/+}$ mice in litters having an average of 5.3 pups, but there were no overall differences in weight between male and female mice in either the Kv1.1 and the Kv1.2 groups, each having $t < 1$ and $R^2 = 0.00$ as the effect size.

The spontaneous activity measure, ACT, was increased in both groups of null-mutant mice, but much more for the *Kcna1*$^{-/-}$ mice.

As depicted in Fig. 2, the ACT data for both groups of null-mutant mice began at the same level with the control mice at P12 and P13; then, the *Kcna1*$^{-/-}$ mice sharply diverged from the *Kcna1*$^{+/+}$ mice at P14 to double its activity over its control group by P17. In contrast, the *Kcna2*$^{-/-}$ mice provided a small and transient increase over the *Kcna2*$^{+/+}$ mice at P15 and P16. An ANOVA of the summed data across all days provided a significant interaction of gene G: (*Kcna1* vs. *Kcna2*) and mutation (M: +/+ vs. −/−): for G × M, $F(1/45) = 17.78$, $p < 0.001$, ${}_p\eta^2 = 0.47$. The ACT score was 60 % higher for the *Kcna1*$^{-/-}$ mice over the *Kcna1*$^{+/+}$ mice and just 16 % for the *Kcna2*$^{-/-}$ mice over the *Kcna2*$^{+/+}$ mice. It is also interesting that there were no difference between the *Kcna1*$^{-/-}$ and *Kcna2*$^{-/-}$ groups and their respective *Kcna1*$^{+/+}$ and *Kcna2*$^{+/+}$ control groups for the ACT measure at P12 and P13, the ANOVA showing a near zero effect for the effects of M or the G × M interaction, $F(1/45) < 1$, ${}_p\eta^2 = 0.0$. This lag in the effect on motor activity of deleting either *Kcna2* or especially *Kcna1* suggests the presence of skeletal motor neurons that regulate motor activity but are not yet dependent on the presence of Kv1.

Deleting *Kcna1* weakened the ASR, but deleting *Kcna2* did not

The severe effect of deleting *Kcna1* on the amplitude on the ASR was most evident for 7 of 15 *Kcna1*$^{-/-}$ mice that failed the ASR criterion on P12 and P13, when their mean ASR was not significantly above the ACT measure. Only one mouse of the other groups missed the ASR criterion and only on 1 day. The ANOVA of

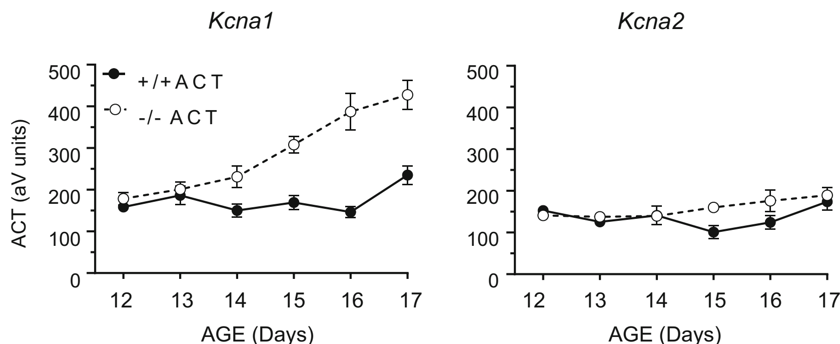

FIG. 2. The ACT measure is the spontaneous activity measured for 100 ms before the startle eliciting stimulus, presented as mean and SEM. A high level of motor activity is a common outcome in behavioral experiments with *Kcna1*$^{-/-}$ mice, and while *Kcna2*$^{-/-}$ mice had also a significantly increased ACT, this was relatively small and transitory. That these effects did not appear until P14 or P15 suggests a delayed developmental contribution of *Kcna1.1* and *Kcna1.2*

the *Kcna1* mice provided a significant main effect for mutation, $F(1/31) = 17.97$, $p < .001$, ${}_p\eta^2 = 0.367$, and age (A) $F(5/155) = 44.54$, $p < 0.001$, ${}_p\eta^2 = 0.59$, but the interaction of M × A was not significant, $F(5/155) < 1$, ${}_p\eta^2 = 0.02$.

The corresponding ANOVA for the *Kcna2* mice provided a significant effect of age, $F(5/65) = 3.008$, $p = 0.017$, ${}_p\eta^2 = 0.219$, but neither for mutation, $F(1/65) < 1$, ${}_p\eta^2 = 0$, nor for the M × A interaction, $F(1/65) < 1$, ${}_p\eta^2 = 0$. An ANOVA including the *Kcna2* mice and the *Kcna1* mice for the sum of the ASR across P12-P17 provided no significant effect for gene (G) $F(1/45) < 1$, ${}_p\eta^2 = 0$, a significant effect for M, $F(1/45) = 8.483$, $p = 0.006$, ${}_p\eta^2 = 0.15$, and a "near-significance" for the G × M interaction, F $(1/45) = 3.938$, $p = 0.053$, ${}_p\eta^2 = 0.08$. On P17, the ASR amplitudes of the $Kcna1^{+/+}$ and the $Kcna2^{+/+}$ mice were not different ($R^2 = 0.03$) but on P24, the ASR of the $Kcna2^{+/+}$ mice increased by 85 % while the ASR of the *Kcna1*+/+ mice increased by only 43 %. This may be the effect of the greater weight gain between P17-P18 and P23-P25 of 60 % for $Kcna2^{+/+}$ mice contrasted to the smaller 29 % increase for the $Kcna1^{+/+}$ mice.

The PPI Levels for Both the $Kcna1^{-/-}$ and the $Kcna2^{-/-}$ Mice Were Lower Than Their Respective Control Groups, but the Smaller Difference for the $Kcna2^{-/-}$ Mice Was Not Significant

Figure 4 describes the inhibitory effect of the 45 ° shift of the background noise from left to right across the midline, with the 10 ms and 60 ms lead times between the changing of the position of the noise before the ES. The change in the strength of PPI with age was expected to follow a monotonic growth function as had been previously found in mice for gap detection (Ison et al. 2017), but instead, all four groups provided roughly inverted U-shape PPI functions with increasing age, and, particularly unexpected, their performance peaks much exceeded the levels of PPI found in adult mice (Allen and Ison 2012). Except for P12 and P13, the PPI levels for the $Kcna1^{+/+}$ mice were significantly higher than those of the $Kcna1^{-/-}$ mice (all daily comparisons provided $p < 0.01$ and $R^2 > 0.20$), but the smaller difference between the PPI levels between the $Kcna2^{+/+}$ and $Kcna2-/-$ mice was never significant (all daily comparisons provided $p > 0.15$ and $R^2 < 0.02$). The PPI peaks at P13 to P15 for the 10 ms lead time for the $Kcna2^{+/+}$ group was higher than the peak at P13 for the $Kcna1^{+/+}$ group, $t(23) = 2.63$, $p < 0.05$, $R^2 = 0.23$, and the 60 ms peaks at P14-P15 for the $Kcna2^{+/+}$ mice compared to P13 and P14 peaks for the $Kcna1^{+/+}$ group had the same trend but were only "near significant", $t(20) = 1.81$, $p = 0.085$, $R^2 = 0.14$. Up to P17, the PPI differences at the 10 ms lead time were not notably different from the 60 ms lead time, but the difference was very apparent at P23–25. For the $Kcna1^{+/+}$ mice on the last test, there was a mean loss in PPI for the 60 ms lead time of 30 % and a 100 % loss at the 10 ms lead time (that is, PPI = 0), and for the $Kcna2^{+/+}$ mice, the PPI has a slight increase of 3 % for the 60 ms lead time but a loss of 45 % for the 10 ms lead time.

Deleting Either *Kcna1* or *Kcna2* Increased the MAA Threshold Relative to Their Control Groups, but for the *Kcna2* Groups the Difference Was Small and Was Not Significant

Figure 5 describes the non-monotonic maturation of the MAA that is consistent with the PPI in Fig. 4, the single expected outcome being the poor performance of the $Kcna1^{-/-}$ mice, as this effect replicated the deficit of the adult $Kcna1^{-/-}$ mice (Allen and Ison 2012). At P12, there were no significant differences in the MAA between any of the groups, but at P13, the poor MAA for the $Kcna1^{-/-}$ mice sharply departed from the other groups with its continuing loss of sensitivity with increasing age. The ANOVA for the $Kcna1^{+/+}$ and $Kcna1^{-/-}$ mice provided a main effect for mutation, $F(1/174) = 112.64$, $p < 0.001$, ${}_p\eta^2 = 0.39$, and for age, $F(5/174) = 7.88$, $p < 0.001$, ${}_p\eta^2 = 0.18$. In contrast, there was no significant difference for the MAA between the $Kcna2^{+/+}$ and $Kcna2^{-/-}$ mice, and the ANOVA for these data provided only a significant effect for age, $F(2/39) = 16.04$, $p < 0.001$, ${}_p\eta^2 = 0.45$. An ANOVA for the sum of each mouse across all days for each group provided a significant effect of gene (*Kcna1* vs. *Kcna2*), $F(1/45) = 92.75$, $p < 0.001$, ${}_p\eta^2 = 0.43$; for mutation, $F(1/45) = 54.5$, $p < 0.001$, ${}_p\eta^2 = 0.25$; and for the G × M interaction, $F(1/45) = 24.5$, $p < 0.001$, ${}_p\eta^2 = 0.11$. There is a suggestive difference between the $Kcna2^{+/+}$ and $Kcna2^{-/-}$ groups from P15 to P17, but it was not significant: $p = 0.23$, $R^2 = 0.10$.

One similar effect in the MAA data of the $Kcna1^{+/+}$ group and both groups of *Kcna2* mice is the shallow inverted U-shaped MAA function across days that suggest a very early but transient higher sensitivity to changes in the position of the noise. For the $Kcna1^{+/+}$ mice, the MAA improved from P12 to P13, $t(34) = 2.03$, $p = 0.05$, $R^2 = 0.10$, and then deteriorated from P13 to P17, $t(34) = 2.62$, $p = 0.02$, $R^2 = 0.24$. For the *Kcna2* mice (collapsed over the $Kcna2^{+/+}$ and $Kcna2^{-/-}$ mice), the MAA at P12/P13 improved to P14/P15, $t(29) = 2.523$, $p < 0.02$, $R^2 = 0.18$, then deteriorated from P14/P15 to P16/P17, $t(29) = 3.97$, $p < 0.001$, $R^2 = 0.35$. The paradox in the inverted U-shaped maturation of the PPI and MAA is that this apparent loss of sensitivity should not be thought as a behavioral deficit because their performance was moving towards the normal values for adult mice found by Allen and Ison (2010, 2012) and is based on

temporary monaural neural activity prior to binaural hearing.

The Strength of the ASR Depression by Abrupt Noise Offsets vs. Ramped Noise Offsets and Longer Lead Times Was Equal in *Kcna2*$^{+/+}$ and *Kcna2*$^{-/-}$ Mice

The depression of the ASR by abrupt vs. ramped offsets at lead times from 1 to 10 ms for both the *Kcna2*$^{+/+}$ and *Kcna2*$^{-/-}$ mice is shown in Fig. 6 for P12 + P13, P14 + P15, and P16 + P17.

The effect of the abrupt offset on the ASR was very rapid, providing significant depression within a lead time of just 1 ms, and it closely approached its asymptotic level after 4 ms. The ANOVA of these data found that both of the two stimulus factors were each significant with large effect sizes: lead time (LT), $F(3/99) = 116.01$, $p < 0.001$, ${}_p\eta^2 = 0.78$; and the abrupt and the ramped offset type (OT), $F(1/33) = 75.06$, $p < 0.001$, ${}_p\eta^2 = 0.70$; and their interactions with age were significant: LT × age, $F(6/99) = 2.83$, $p = 0.02$, ${}_p\eta^2 = 0.15$ and OT × age, $F(2/33) = 6.55$, $p = 0.004$, ${}_p\eta^2 = 0.28$: but overall, there were neither main effects for age, $F(2/33) = 1.49$, $p = 0.24$, ${}_p\eta^{22} = 0.08$, nor the main effect for mutation, (M), $F(1/33) < 1$, and there were no significant interactions with M: LT × M, $F < 1$; OT × M, $F < 1$; or M × GD, $F < 1$. The effect size between these *Kcna2*$^{+/+}$ and *Kcna2*$^{-/-}$ mice was just ${}_p\eta^2 = 0.002$, compared to an earlier effect size of ${}_p\eta^2 = 0.27$ between adult *Kcna1*$^{+/+}$ and *Kcna1*$^{-/-}$ mice reported by Ison and Allen (2012).

DISCUSSION

The present investigation compared the behavioral consequences for temporal and spatial auditory processing after deleting *Kcna2* or *Kcna1* in preweanling mice to test the implications of the contrasting results of in vitro experiments that had deleted or blocked one or the other of these two genes in mice (Brew et al. 2003, 2007; Smith et al. 2015).

The first comparison is the effect of physical development, an outcome perhaps not consequential for electrophysiological experiments but potentially important for behavior if, for example, it were a source of illness, stress, or physical weakness. The stunted physique and low weight is a common deficit for both *Kcna1*$^{-/-}$and *Kcna2*$^{-/-}$ mice as shown in Fig. 1 and reported earlier in mice lacking Kv1.1 (Smart et al. 1998) and Kv1.2 (Brew et al. 2007). McDaniel et al. (2001) described an "anorexic effect" of deleting *Kcna* genes, being a loss of transport of nutrients because of intestinal and mesenteric vasoconstriction. However, although both strains of the null mutant mice studied here had always had lower weights than the control mice, both had increased their weight at the same rate as their controls. This suggests that they were healthy and reasonably able to compete for nutrition with their littermates. Only two *Kcna1* mice, one control and one mutant mouse, registered a small variation of 1 to 2 g up or down that could be attributed to irregular times of measuring their weight or possible measurement error.

The large increase in the ACT score for the *Kcna1*$^{-/-}$ mice in Fig. 2 as observed previously in adult *Kcna1*$^{-/-}$ mice (Allen and Ison 2012) may have a parallel in the intrinsic fine-muscle twitching observed in episodic ataxia type 1 patients carrying a *KCNA1* mutation (Browne et al. 1994). At the extreme, *Kcna1*$^{-/-}$ mice exhibit "neuromyotonia" after swimming in cold water, attributed to the temperature-sensitive nerve backfiring in vitro in the absence of Kv1.1 (Zhou et al. 1998). This protocol had not induce neuromyotonia in *Kcna2*$^{-/-}$ mice (Brew et al. 2007), suggesting generally a smaller anomaly than seen in *Kcna1*$^{-/-}$ mice, and was consistent with our observation of a relatively small 16 % increase of ACT in *Kcna2*$^{-/-}$ mice contrasting with the 60 % increase in *Kcna1*$^{-/-}$ mice.

If a lesser ASR was a consequence of low weight and muscular weakness then both of the null mutant groups might have had low ASR levels, but only the *Kcna1*$^{-/-}$ mice had weak startle reflexes. In addition, Karcz et al. (2015) had found that the stronger ASR amplitudes for *Kcna1*$^{+/+}$ mice were observed only with binaural acoustic stimuli, with no difference between the *Kcna1*$^{+/+}$ and *Kcna1*$^{-/-}$ mice with monaural acoustic presentation. The ASR data shown in Fig. 3 support the conclusion that the weak ASR in the *Kcna1*$^{-/-}$ mice is not a result of their small stature but primarily the loss of binaural synchrony in the reflex pathways of the ASR: and from these data, we have presumed that the ASR of the *Kcna2*$^{-/-}$ mice must benefit from a higher level of synchrony in the bilateral reflex pathways than the *Kcna1*$^{-/-}$ mice. But the large increment in weight coupled with the higher ASR between P17/18 and P23/25 in the *Kcna2*$^{+/+}$ compared to the *Kcna1*$^{+/+}$ mice suggests that during development, a large difference in body mass does add to the synchrony in the bilateral reflex pathways leads to a stronger ASR. Up to P17, the normal development of the ASR for both *Kcna2*$^{-/-}$ and *Kcna2*$^{+/+}$ mice in Fig. 3, and their sensitivity to changes in the location of the background shown in PPI in Fig. 4 and the MAA in Fig. 5, all contrast with the findings for the *Kcna1*$^{-/-}$ mice beyond P13. Further, as is shown in Fig. 6, the rapid effect on the ASR by the abrupt and ramped offsets was observed in both preweanling *Kcna2*$^{-/-}$ and *Kcna2*$^{+/+}$ groups, as

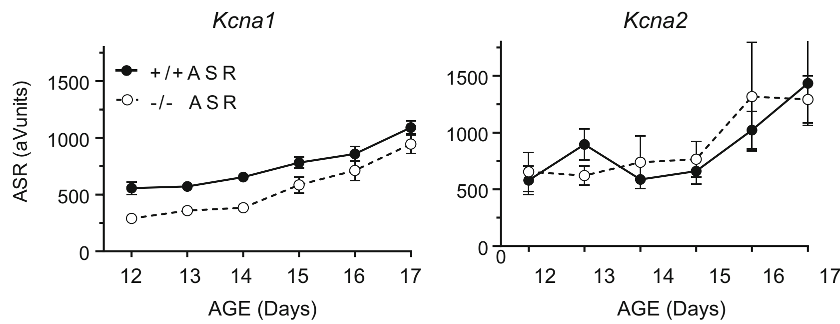

FIG. 3. The ASR measure captures the downward force of the startle reflex for 100 ms beginning with the onset of the startle stimulus. The SEM bars of the *Kcna1* mice are relatively small because these groups are larger ($n = 15, 18$) than the *Kcna2* mice (nominally 8 and 8, but 3–7 on some days). On P12-P15, only 8/15 of the *Kcna1*$^{-/-}$ mice had a criterion ASR that was significantly different from the ACT score

had been seen in adult *Kcna1*$^{+/+}$ mice but not *Kcna1*$^{-/-}$ mice (Ison and Allen 2012).

The PPI and MAA measures were higher for both *Kcna2* groups than the *Kcna1*$^{+/+}$ mice. This may have been an effect of their smaller litter size and better nutrition, but possibly, the acoustic characteristics of the smaller testing chamber may have increased the interaural intensity differences. However, one surprising observation in this experiment was the strong PPI for a change of the 45° position of the noise in all of the groups on P12 and P13 in Fig. 4 even including the *Kcna1*$^{-/-}$ mice: this latter effect suggests that the P12 and P13 performance did not depend on I_{KL}. Then, PPI increased to a high level of about 60 % inhibition for all of the preweanling mice at P13-P14 (save for the *Kcna1*$^{-/-}$ group); this followed by declines in PPI in a few days later: this was a second surprising effect. This relatively lower performance of about 25 % or 35 % at ~ P17 and ~ P24 at the 60 ms lead time, and near zero at the 10 ms lead time must be understood in the light of spatial performance in the behavior of adult mice, that is closer to the lower PPI in P17 and P 24 to the older preweanling mice and not to the higher PPI of younger preweanling mice (Allen and Ison 2010, 2012). Their higher PPI at about P14–16 appear to represent not “spatial performance” as seen in adult mice, but instead, it is an effect of independent onset and offset of the noise,

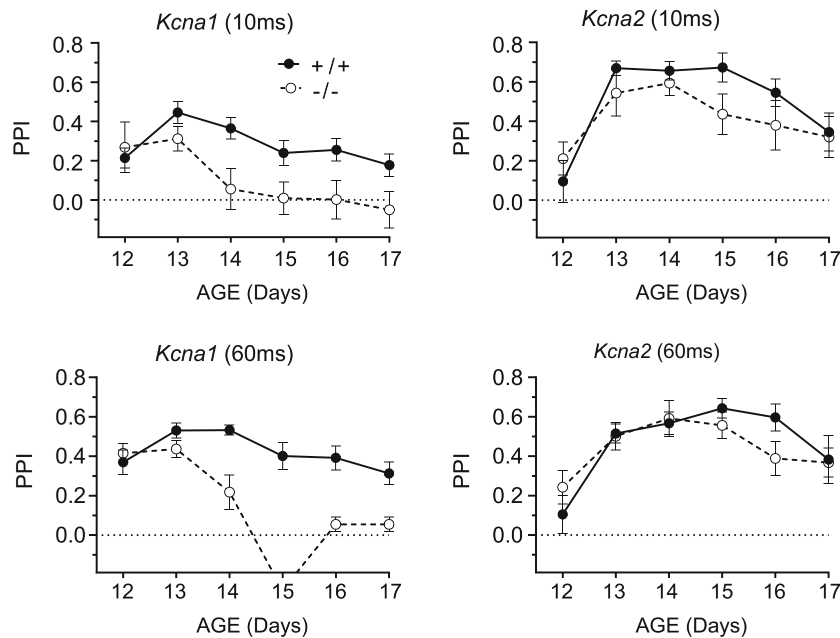

FIG. 4. The PPI measure is the relative degree to which the ASR is inhibited by the prepulse; in these data, the background noise is broadcast from a speaker that is 22.5° to the left of the mouse for 15–30s and then the sound switches over to the second speaker at 22.5° right to the mouse: the 45° shift in the noise is the prepulse. On the upper graphs, the change was presented 10 ms before the startle stimulus; on the lower graphs, the prepulse was presented 60 ms before the startle stimulus. On the left side of the figure, the PPI of the *Kcna1*$^{-/-}$ mice falls significantly away from the PPI of the *Kcna1*$^{+/+}$ mice beginning at P14. On the right, the PPI of *Kcna2*$^{-/-}$ mice is never significantly different from the PPI of the *Kcna2*$^{+/+}$ mice

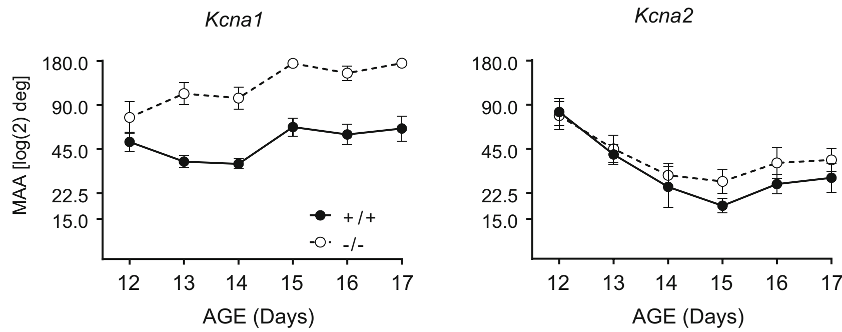

FIG. 5. The MAA, the "minimum audible angle," is the smallest angle through which the prepulse noise moves from left to right of the mouse with the 60 ms lead time that provides a significant PPI, constrained by the set of alternative angles from 180°, 90°, 45°, 22.5°, and 15°. On P12, there is a small non-significant deficit in *Kcna1*$^{-/-}$ mice compared to the *Kcna1*$^{+/+}$ mice, but by P15, most of these mice were detecting only180° if at all. The behavior of the *Kcna2*$^{-/-}$ mice was similar to that of the *Kcna2*$^{+/+}$ mice

because these higher PPI levels at around P14–16 represent the results of experiments of noise onsets and offset (Allen and Ison 2010): and also, the data shown here in Fig. 6 provided high levels of "PPI", or, rather, response depression. The early data are better understood as near-coincident responses to independent noise onsets on one ear and noise offsets on the other, prior to the maturation of the integrated

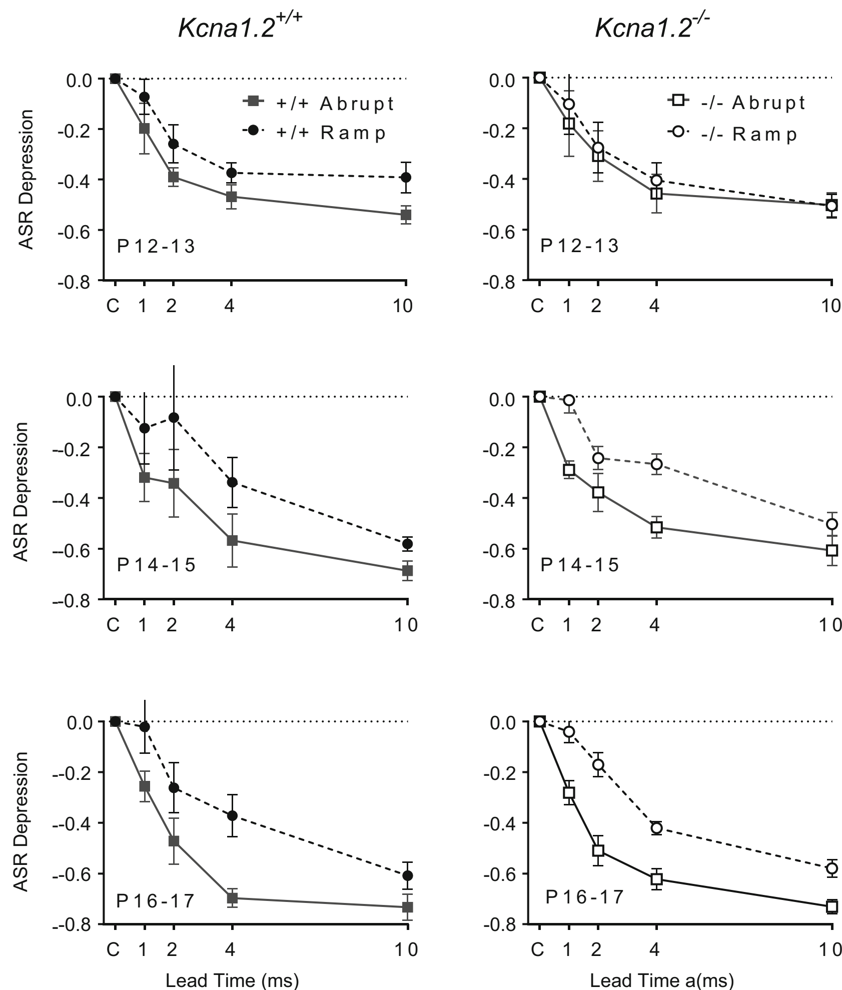

FIG. 6. An abrupt noise offset presented before the ASR provides a strong and rapid depressive effect on the ASR. The effect increases with the lead time with the abrupt offset (1, 2, 4, and 10 ms) before the startle stimulus, and the ramped offset of 1, 2, 4, and 10 ms delayed the strength of the depression. Overall, the growth of the depression in both offset conditions was the same for both *Kcna2*$^{+/+}$ and *Kcna2*$^{-/-}$ mice

binaural pathways. This apparently incongruous comparison of the ASR (that is certainly binaural) and the "spatial" performance (that is possibly monaural at this age) may be compatible with the temporary contralateral ascending inhibitory input to the inferior colliculus in preweanling rats just after the initial onset of hearing (Clopton and Silverman 1977).

There are other examples of a non-monotonic maturation path for sound localization in human infants reviewed by Muir et al. (1989), reporting relatively high levels of orientation to sounds in neonates followed by a decrement in performance with a recovery at 4–5 months of age with continuing further improvement. These authors suggested that the early performance in human neonates was based on caudal monaural mechanisms then followed by a loss of acuity, and after a lag, an increasing performance being a reflection of stronger rostral binaural mechanisms. While there is a sharp distinction in methodology between the observation of a newborn's orientation towards a novel sound and our observation of the change in the strength of the startle reflex when a sound is shifted from one location to a second, there may be some comparative development of sound localization across mammalian species from monaural to binaural processing.

In conclusion, our study did not find any substantial behavioral abnormality in either spatial localization or the response to noise offset data in the *Kcna2*$^{-/-}$ mice, especially contrasted with the severe deficits of the *Kcna1*$^{-/-}$ mice. These data, combined with the observation that there are more Kv1.1 subunits than Kv1.2 subunits in the auditory brainstem in the mice that may substitute for the missing Kv1.2 subunits, could suggest that the Kv1.2 subunits do not have any special quantitative contribution to I_{KL} that is different from the Kv1.1 subunits. However, one unique benefit of Kv1.2 units may be their "managerial" role over Kv1.1 subunits, as demonstrated by Smith et al. (2015), that Kv1.2 subunits modulate the activity of Kv1.1 subunits. This important function for *Kcna2* was observed in the monaural spiral ganglion neuron, though as yet, there is no similar evidence in the monaural and binaural nuclei of the auditory brainstem. These two effects that increase the effectiveness of Kv1.1 subunits after deleting *Kcna2* may be responsible for the in vivo increase in I_{KL} observed by in Brew et al. (2007). But relevant to the present behavioral experiments, there are possibly two other conflicting outcomes of deleting Kv1.2 subunits in the MNTB. As shown in Fig. 4A in Brew et al. (2007), the hypo-excitability after deleting *Kcna2* has two contrary effects: one is the better synchronous stimulus/responding in the *Kcna2*$^{-/-}$ cells compared to the *Kcna2*$^{+/+}$ cells, but this advantage is at the cost of the reduction of multiple spiking and thus, better synchrony but less excitation may be transmitted to the upstream binaural nuclei. This hypothesis may yield to an in vivo electrophysiology study akin to the *Kcna1* experiment of Kopp-Scheinpflug et al. (2003), but a behavioral experiment would be too blunt a technique to settle the matter.

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COMPLIANCE WITH ETHICAL STANDARDS

All protocols were approved by the IACUCs of the Universities of Washington and Rochester and were in accord with USPHS regulations and the Federal Animal Welfare Act.

Conflict of Interest The authors declare that they have no conflict of interest.

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