Respiratory dysfunction progresses with age in *Kcna1*-null mice, a model of sudden death in epilepsy

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Abstract

Objective—Increased breathing rate, apnea and respiratory failure is associated with sudden unexpected death in epilepsy (SUDEP). We recently demonstrated the progressive nature of epilepsy and mortality in $Kcna1^{-/-}$ mice a model of temporal lobe epilepsy and SUDEP. Here, we tested the hypothesis that respiratory dysfunction progresses with age in $Kcna1^{-/-}$ mice, thus increasing risk of respiratory failure and sudden death (SD).

Methods—Respiratory parameters were determined in conscious mice at baseline and following increasing doses of methacholine (MCh) using non-invasive airway mechanics (NAM) systems. $Kcna1^{+/+}$, $Kcna1^{+/-}$ and $Kcna1^{-/-}$ littermates were assessed during three age ranges when up to ~30%, ~55% and ~90% of $Kcna1^{-/-}$ mice have succumbed to SUDEP: postnatal day (P) 32-36, P40-46 and P48-56, respectively. Saturated arterial O_2 (SaO_2) was determined with pulse oximetry. Lung and brain tissues were isolated and $Kcna1$ gene and protein expression were evaluated by RT-qPCR and Western blot techniques. Airway smooth muscle responsiveness was assessed in isolated trachea exposed to MCh.

Results—$Kcna1^{-/-}$ mice experienced an increase in basal respiratory drive, chronic oxygen desaturation, frequent apnea-hypopnea (A-H), an atypical breathing sequence of A-H-tachypnea-A-H, increased tidal volume and hyperventilation induced by MCh. The MCh-provoked hyperventilation was dramatically attenuated with age. Interestingly, only $Kcna1^{-/-}$ mice developed seizures following exposure to MCh. Seizures were provoked by lower concentrations of MCh as $Kcna1^{-/-}$ mice approached sudden death (SD). MCh-induced seizures experienced by a subset of younger $Kcna1^{-/-}$ mice triggered death. Respiratory parameters of these younger $Kcna1^{-/-}$ mice resembled older near-SD $Kcna1^{-/-}$ mice. $Kcna1$ gene and protein were not expressed in $Kcna1^{+/+}$ and $Kcna1^{+/-}$ lungs and MCh-mediated airway smooth muscle contractions exhibited similar EC_{50}'s in isolated $Kcna1^{+/+}$ and $Kcna1^{-/-}$ trachea.

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Significance—The *Kcna1*$^{-/-}$ model of SUDEP exhibits progressive respiratory dysfunction which suggests a potential increased susceptibility for respiratory failure during severe seizures that may result in sudden death.

Keywords

Kv1.1 knockout; mortality; survival; hypopnea; seizures; apnea

Introduction

The lifetime incidence of epilepsy is 1:26 people.[1] Approximately one third of people with epilepsy have seizures that are refractory to current anti-seizure drugs and the estimated risk for Sudden Unexpected Death in Epilepsy (SUDEP) in the refractory population is dramatically increased from 1:1000 to ~1:150 people each year.[2,3] The pathological mechanisms mediating SUDEP events are not understood, however, risk factors include uncontrolled seizures, generalized tonic-clonic (GTC) seizures, age and cardiac arrhythmias. [2,4–7]

The mechanisms underlying SUDEP are elusive primarily because it is rarely clinically witnessed. Of these witnessed cases, the majority report a partial or GTC seizure,[4–7] which is associated with dysfunctional respiratory response reflexes[8], lowered blood oxygen saturation followed by a period of increased breathing rate, cardiac arrhythmia/bradycardia, apnea, respiratory arrest and asystole.[4–7] Although it is clear that respiratory arrest contributes to the terminal SUDEP event, it is unknown whether a progressive respiratory dysfunction develops in susceptible individuals that may contribute to the pathology that culminates in death.

The *Kcna1* gene encodes for the Kv1.1 alpha subunit of the delayed rectifier potassium channel, which participates in the repolarization of the neuronal membrane potential, action potential properties, neurotransmitter release, synaptic integration and network oscillatory behavior.[9,10] *Kcna1*-null (*Kcna1*$^{-/-}$) mice develop spontaneous recurrent seizures including myoclonic seizures, clonus and generalized tonic-clonic seizures around the third week of their life.[9,11,12] We have recently demonstrated that seizures progressively become more frequent and severe until the mice die at the early age of postnatal day (P) 42 ± 1.3[12], although the background strain of the mouse influences the severity of seizures and mortality.[11] A genetic variant in *Kcna1* has been identified in a clinical SUDEP case.[13] *Kcna1*$^{-/-}$ mice phenocopy human SUDEP and experience a GTC seizure, cardiac arrhythmias and asystole prior to death.[14] While chemically-provoked seizures elicited minimal cardiorespiratory effects in wild type mice, similarly provoked seizures in *Kcna1*$^{-/-}$ mice often result in peri-ictal apneas, decreased blood oxygen saturation and sudden spreading depolarization in the dorsal medulla followed by death.[15] These studies indicate that the respiratory responses of *Kcna1*$^{-/-}$ mice ultimately fail following the final GTC. Here, we tested the hypothesis that *Kcna1*$^{-/-}$ mice exhibit progressive respiratory dysfunction which is associated with age-dependent susceptibility to sudden death (SD).

Material and Methods

Animals

Kcna1-null mice on a C3HeB/FeJ congenic background were bred and housed in the Animal Resource Facilities at Creighton University School of Medicine in a temperature (25°C) and humidity (50-60%) controlled, pathogen-free environment on a 12-hr light/dark cycle. Food and water were provided ad libitum. Male and female mice were used. No gender differences were detected in this study. Genotype was determined (Transnetyx Inc., Cordova, TN, U.S.A.). *Kcna1*$^{-/-}$ mice seize and thus experiments are not blinded to the experimenter. Procedures were in accordance with National Institutes of Health guidelines, the EU Directive 2010/63/EU and were approved by the Institutional Animal Care and Use Committee at Creighton University School of Medicine.

Respiratory Measurements

Non-invasive airway mechanics techniques were used to measure respiratory parameters in non-anesthetized mice (NAM; Buxco Finepointe/DSI Inc, Minneapolis, MN, USA). *Kcna1*$^{+/+}$, *Kcna1*$^{+/-}$ and *Kcna1*$^{-/-}$ littermates were assessed at three age bins: P32-36, P40-46 and P48-56 when up to ~30%, ~55% and ~90% of *Kcna1*$^{-/-}$ have succumbed to SUDEP.[12] Mice were allowed to acclimate in the NAM chamber for 10-15 min, exposed to nebulized phosphate buffered saline (PBS), then challenged with increasing doses of MCh (6, 12, 24, 48, 100 and 200 mg ml^{-1}, 45μl for 30 sec). Each challenge was followed immediately by a 3-min recording period (Buxco FinePointe acquisition software V2.3.1.9). The independent measurement of both nasal and thoracic air flow allows the NAM to measure several more respiratory parameters than whole body plethysmography. Accordingly, peak expiratory flow (PEF), peak inspiratory flow (PIF), time of inspiration (T_i), time of expiration (T_e), frequency of breathing (f), minute ventilation (V_E), tidal volume (V_T), respiratory drive (V_T/T_i) and specific airway resistance (sRaw) were determined. To account for varying body weights, f, PIF, PEF, V_E and V_T were normalized to individual animals' weights.

Seizure Monitoring

The NAM chamber physically restricted the ability to conduct electroencephalography (EEG) recordings, therefore only convulsive seizures were quantified. Seizures were video-monitored during recording sessions and continued for 20-30 min after removal from the NAM. Seizures were scored using a modified Racine scale (0-normal; 1-immobility; 2-head bobbing; 3-forelimb/hindlimb clonus and tail extension; 4- generalized clonus with forelimb tonic extension; 5-severe tonic-clonic seizures with hindlimb tonic extension) as we have previously described.[11,12,16,17]

Apnea/Hypopnea Analysis

Respiratory cycles were determined for each animal. The duration of two respiratory cycles was calculated for each individual animal at baseline using the equation: (frequency of breaths per min/60 sec) * 2. An apnea event was defined as the decrease of airflow   90%

for a period   two complete respiratory cycles. Hypopnea was defined as the reduction of airflow   30% for a period   two complete respiratory cycles (i.e. shallow breathing).

Oximetry

Blood oxygen saturation was measured noninvasively using MouseOx Plus infrared pulse oximetry (Starr Life Sciences Corp, Oakmont, PA, USA). The hair around the neck was removed and a CollarClip Sensor (Starr Life Sciences Corp) was fitted in $Kcna1^{+/+}$ and $Kcna1^{-/-}$ mice (P40-42). Eight parameters including oxygen saturation (SaO_2), pulse rate and respiratory rate were acquired at 5 Hz and mice were recorded for 1 hr. Only measurements that were error-free for all parameters were included in the analyses. Error-free measurements proved more difficult to obtain for $Kcna1^{-/-}$ mice resulting in a lower, but reasonable, number of usable data points compared to $Kcna1^{+/+}$ mice (1411 ± 600 vs. 2939 ± 214, n = 4, $p = 0.079$, unpaired t-test). The total number of measurements for individual $Kcna1^{-/-}$ mice did not correlate with the SaO_2 ($R^2 = 4 \times 10^{-6}$, p = 0.998, Pearson correlation coefficient) or the percent of measurements considered hypoxic ($< 90\%$ SaO_2) ($R^2 = 0.037$, p = 0.81) indicating that the difference in error-free measurements between $Kcna1^{+/+}$ and $Kcna1^{-/-}$ mice did not skew the results. State of vigilance was video recorded and scored as active or rest (immobile and eyes closed) by a trained technician.

Assessment of airway smooth muscle reactivity

$Kcna1^{+/+}$ and $Kcna1^{-/-}$ mice (P39-41) were sacrificed, tracheas were removed and placed in Krebs' solution (composition in mM: NaCl-120; KCl-5.5; $CaCl_2$-2.5; NaH_2PO_4-1.4; $MgCl_2$-1.2; $NaHCO_3$-25; dextrose-11.1; $CaNa_2$-EDTA-0.027) equilibrated with 95% O_2-5% CO_2 at pH 7.4. Tracheas were cut into 2-mm ring segments, mounted via 2 stainless steel pins through the lumen, and placed in water-jacketed glass muscle chambers containing Krebs' solution maintained at 37°C and gassed with 95% O_2-5% CO_2, pH 7.4. One pin was attached to a Grass FT.03 force transducer (Quincy, MA) for measurement of isometric contraction. After 1-hr equilibration at 300 mg resting tension, ring segments were challenged with a maximal contractile concentration (60 mM) of KCl, washed for 15 min. This was repeated until the response was within 10% of the previous contraction. After 30 min of washing, cumulative concentration-response curves for MCh-induced contraction were obtained. Airway reactivity was determined as the contractile sensitivity to MCh, which was quantified as the half-maximal effective concentration (EC_{50}) for MCh-induced contraction as previously described.

Reverse transcriptase qPCR

Tissue was homogenized in Trizol, RNA was extracted via phenol chloroform, treated with DNase, suspended in 30μL of nuclease free H_2O and quantified (NanoDrop 2000 spectrophotometer, Thermo Scientific, USA). cDNA synthesis (4μg) was completed using Improm II reverse transcriptase kit (Promega, Madison, WI) and a C1000™ Thermo Cycler (25°C-5 min, 42°C-60 min and 72°C-15 min, Bio-Rad Labs, Hercules, CA). Quantitative PCR was performed for $Kcna1$ and housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (100 ng), 10μl SYBR green PCR master mix (Agilent Technologies, Santa Clara, CA) and 1μl of forward and 1μl of reverse primers (10 pmol/μl) (Integrated DNA Technologies, Coralville, IA), 7.9μl nuclease free water, mixed together

and using a AriaMx Real Time PCR instrument (Agilent Technologies, Santa Clara, CA). Primers[14] were *Kcna1* forward, 5'-GCATCGACAACACCACAGTC-3'; *Kcna1* reverse, 5'-CGGCGGCTGAGGTCACTGTCAGAGGCTAAGT-3'; *Gapdh* forward, 5'-TGAAGGTCGGTGTGAACGGATTT GGC-3'; *Gapdh* reverse, 5'-ATGTAGGCCATGAGGTCCACCAC-3' (IDT's Primer Quest software2). Cycling conditions: 95°C-10min, 40cycles of 95°C-30sec, 72°C-30sec. Each real-time PCR was performed using three biological samples in technical triplicates and melt curve analyses were completed to ensure the specificity of amplification. Calculations of the *Kcna1* gene expression relative to *Gapdh* were based on the differences in threshold cycles using the $2^{-\Delta\Delta Ct}$ method.[18]

Immunofluorescent western blot

Mouse brain and lungs were isolated and flash frozen in liquid nitrogen. Tissue was homogenized in buffer (in mM: 4-HEPES pH-7.4, 320-sucrose, 2-EGTA, 10-Na pyrophosphate, 1-Na orthovanadate, 10-NaF, 0.1-PMSF, phosphatase inhibitor cocktail). Following centrifugation, protein (15μg) (in Laemmli loading buffer and β-mercaptoethanol) was separated by electrophoresis (12% Mini-PROTEAN TGX gels; Biorad, Hercules, CA), transferred to Immobilon-FL PVDF membranes (EMD Millipore, Billerica, MA, U.S.A.) and blocked with Odyssey blocking buffer (OBB; Li-Cor Biosciences, Lincoln, NE, U.S.A.)/PBS at a 1:1 ratio. Membranes were incubated overnight with primary antibodies rabbit anti-Kv1.1 (1:1000; ab32433, Abcam) or mouse anti-beta actin (1:8000; 926-42212 LiCor Biosciences) at 4°C. Following PBS-T washes, membranes were incubated in secondary antibodies for one hour: goat anti-rabbit (1:5,000; 926-32221, Li-Cor Biosciences) or goat anti-mouse (1:20,000-1:40,000; 926-32210, Li-Cor Biosciences). Samples were run in duplicates, images were captured on an Odyssey FC (Licor, Lincoln, NE). Kv1.1 protein signal was normalized to β-actin values.

Reagents and Statistical Analysis

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich. Data are presented as the mean ± S.E. Statistical significance was determined with Prism6 software (Graphpad Software Inc., La Jolla, CA, U.S.A.) using either an unpaired t-test, one-way ANOVA or two-way ANOVA for genotype and age with an appropriate *post hoc* test. EC_{50} values for MCh were calculated from concentration-response curves by nonlinear regression analysis.

Results

Kcna1^{−/−} mice have increased respiratory drive and blood oxygen desaturation with increased probability of SD

We determined respiratory endpoints during three age ranges: P32-36, P40-46 and P48-56 when up to ~30%, ~55% and ~90%, respectively, of *Kcna1*^{−/−} mice have succumbed to SD[12]. For simplicity, we refer to these groups as SD30, SD55 and SD90. Most baseline breathing parameters were similar. The most notable change occurred in the SD90 *Kcna1*^{−/−} mice cohort which had the highest probability of imminent SD at the time of the respiratory experiments[12]. V_T was significantly increased in SD90*Kcna1*^{−/−} mice when compared to

age-matched $Kcna1^{+/+}$ controls ($p < 0.01$; $Kcna1^{+/-}$ resembled $Kcna1^{+/+}$; Figure 1A). Also as the probability of SD rose, $Kcna1^{-/-}$ respiratory drive (V_T/T_i) and peak inspiratory flow was significantly increased ($p < 0.01$ and $p < 0.05$, respectively; Figure 1B and C).

An increase in respiratory drive can be triggered by blood gas instability. To assess stability we measured SaO_2 by oximetry over one hour in freely moving mice. SaO_2 was reduced by ~4% in SD55 $Kcna1^{-/-}$ mice when compared to age-matched $Kcna1^{+/+}$ mice ($92.8 \pm 0.7\%$ vs. $97 \pm 0.5\%$, n = 4, $p < 0.001$) (Figure 1D). SaO_2 was reduced by a similar degree during both rest and activity. Plotting all of the raw data revealed that a large number of $Kcna1^{-/-}$ measurements were hypoxic (i.e. SaO_2 drops below 90%) (Figure 1E). The percent of hypoxic measurements was determined for each subject. $Kcna1^{+/+}$ mice rarely experienced hypoxia (with only $0.6 \pm 0.3\%$ of measurements having $SaO_2 < 90\%$), whereas $26 \pm 0.7\%$ of the measurements from $Kcna1^{-/-}$ mice were considered hypoxic (n = 4, Figure 1F). These data suggest that $Kcna1^{-/-}$ mice experience frequent hypoxia.

Apnea and hypopnea is severe in $Kcna1^{-/-}$ mice

Hypoxia can be caused by apnea or hypopnea (referred to as A-H). A-H frequently occur during/after generalized tonic-clonic seizures and during the final events leading up to SUDEP in humans and in the $Kcna1^{-/-}$ model.[6,15] We hypothesized that $Kcna1^{-/-}$ mice are more prone to A-H events at ages with increased probability SD. Raw traces of eupnea, apnea and hypopnea are depicted in Figure 2A-C, respectively. In the SD30 group, there were no differences in the number of apnea events among genotypes. A trend emerged in the SD55 group (p = 0.09; $Kcna1^{+/+}$ vs. $Kcna1^{-/-}$). SD90 $Kcna1^{-/-}$ mice experienced significantly more apnea events when compared to age-matched $Kcna1^{+/+}$ and $Kcna1^{+/-}$ littermates (Figure 2D). Interestingly, hypopnea was highest in the SD30 $Kcna1^{-/-}$ cohort and significantly diminished with age (Figure 2E). A-H rarely occurred in $Kcna1^{+/+}$ and $Kcna1^{+/-}$ mice. $Kcna1^{-/-}$ mice spent 8-18% of time monitored in A-H, compared with only 1-4% for $Kcna1^{+/+}$ and $Kcna1^{+/-}$ mice (Figure 2F). It is important to note that these measurements occurred in the absence of seizures. These data suggest a critical period exists and the potential conversion of hypopnea events to apnea may indicate a dysfunction in circuitry and systems responsible for preventing apnea onset.

Respiratory responses surrounding A-H events are dysregulated in $Kcna1^{-/-}$ mice

We examined the respiration parameters prior to and following each A-H event. We were able to detect bronchoconstriction prior to approximately half of all A-H events, as indicated by an increase in sRaw values ($Kcna1^{+/+}$ $21 \pm 4\%$; $Kcna1^{+/-}$ $42 \pm 10\%$; $Kcna1^{-/-}$ $33 \pm 3\%$ higher than baseline sRaw). Irrespective of whether bronchoconstriction occurred, we classified three distinct respiratory patterns surrounding all A-H events (Types 1-3). The incidence of each Type was similar across ages (data not shown) and differed among genotypes. As depicted in Figure 3A, Type 1 A-H events were preceded by tachypnea (rapid breathing and increased V_T), and were then followed by eupnea (normal breathing). Type 2 A-H events were preceded and followed by eupnea (Figure 3B) and Type 3 events were preceded by eupnea then followed by tachypnea (Figure 3C). A majority of $Kcna1^{+/+}$ and $Kcna1^{+/-}$ A-H events were Type 1 (29/33 $Kcna1^{+/+}$ and 32/35 $Kcna1^{+/-}$ events; Figure 3D).

In contrast, $Kncal^{-/-}$ mice had two differences in their A-H phenotypes. $Kncal^{-/-}$ mice had significantly fewer Type 1 A-H events (42%) and a greater number of Types 2 and 3 A-H events (35% and 23% of events, respectively; Figure 3D). Interestingly, in $Kcnal^{-/-}$ mice it was not uncommon to observe an A-H event that was either preceded or followed by tachypnea, to then be followed by another A-H event, in a repeating pattern (Figure 3E). Collectively, these data suggest that $Kcnal^{-/-}$ mice are more prone to A-H events and the typical respiratory responses surrounding apnea events may be dysregulated or these responses are compensatory in $Kcnal^{-/-}$ mice.

MCh paradoxically provokes hyperventilation and seizures in $Kcna1^{-/-}$ mice

Respiratory endpoints were assessed following exposure to increasing doses of nebulized MCh, a muscarinic cholinergic receptor agonist that induces bronchoconstriction. To compare responses, we normalized each response to its corresponding PBS baseline. $Kcna1^{+/+}$ and $Kcna1^{+/-}$ mice responded similarly with hypoventilation across all ages: indicated by reduced breathing rates, tidal volume, minute ventilation, peak inspiratory and expiratory flow and respiratory drive; while inspiration time, expiration time and specific airway resistance were increased. Because there were no differences across ages, data were combined across age for both $Kcna1^{+/+}$ and $Kcna1^{+/-}$ mice for each endpoint (depicted in grey in Figure 4).

In contrast, MCh provoked hyperventilation in $Kcna1^{-/-}$ mice: indicated by increased breathing rate, minute ventilation, peak inspiratory and expiratory flow and respiratory drive, along with a decrease in inspiration time and expiration time (Figure 4A-I). Interestingly, the overall response was attenuated as $Kcna1^{-/-}$ mice aged. MCh provoked seizures in only $Kcna1^{-/-}$ mice in a concentration-dependent manner. All $Kcna1^{-/-}$ mice experienced seizures by 200 mg ml^{-1} MCh (Figure 5A).

$Kcna1^{-/-}$ mice become more sensitive to MCh-induced tachypnea and seizures with age

$Kcna1^{-/-}$ mice developed greater sensitivity to MCh as they aged. The EC_{50} for seizure induction in the SD30 group was 40.8 mg ml^{-1}. This significantly decreased to 11.9 mg ml^{-1}, representing a 71% overall reduction with age ($p < 0.0001$; Figure 5A). In the SD30 cohort, the EC_{50}s for inspiration time, expiration time and breathing rate were ~40-45 mg ml^{-1} and decreased 35-50% as mice approached SD ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$; Figure 5B-D).

MCh-induced seizures resulted in the death of one SD30 and two SD55 $Kcna1^{-/-}$ mice. These three mice died at P36, P45 and P46 after experiencing severe seizures. This presented the opportunity to determine whether these susceptible mice had distinguishing respiratory characteristics, the hypothesis being that they would resemble the SD90 cohort. One baseline parameter that distinguished SD90 $Kcna1^{-/-}$ mice from age-matched $Kcna1^{+/+}$ and $Kcna1^{+/-}$ mice was tidal volume. The tidal volume of the $Kcna1^{-/-}$ mice that died young was 15.0 ± 1.3 μl g^{-1}, resembling the SD90 cohort (16.3 ± 1.4 μl g^{-1}). In addition, the sensitivity to MCh between the two cohorts were similar. The EC_{50}s for expiration time was 24.9 ± 1.3 and for breathing rate was 34.7 ± 1.3 mg ml^{-1}, similar to the SD90 group ($26.4 \pm$

1.3 and for f was 30.6 ± 1.3 mg ml^{-1}). Collectively, these data indicate that $Kcna1^{-/-}$ mice at higher risk of SUDEP have greater respiratory sensitivity to MCh.

K$_v$1.1 is not expressed in lung tissue and does not participate in airway smooth muscle reactivity

$Kcna1$ expression in lung tissue was investigated to determine the potential contribution of K$_v$1.1 to the intrinsic function of this organ. We used brain tissue as a positive control. RT-qPCR and western blots revealed that $Kcna1$ mRNA and protein were highly expressed in both $Kcna1^{+/+}$ and $Kcna1^{+/-}$ brain (Figure 6A, B). The loss of one allele resulted in a ~70% reduction of mRNA and ~60% reduction of protein in $Kcna1^{+/-}$ brain. $Kcna1$ mRNA or protein were not detectable in $Kcna1^{+/+}$ or $Kcna1^{+/-}$ lung tissue. There was no $Kcna1$ mRNA or protein in $Kcna1^{-/-}$ brain or lung.

In vitro experiments with isolated trachea from $Kcna1^{+/+}$ and $Kcna1^{-/-}$ mice were conducted to determine whether airway smooth muscle responsiveness to increasing concentrations of MCh differed between genotypes. Mean concentration-response curves for MCh-stimulated contraction of isolated tracheas were constructed and EC$_{50}$ values calculated (Figure 6C). $Kcna1^{+/+}$ and $Kcna1^{-/-}$ trachea had similar EC$_{50}$s (0.85 ± 0.4 μM v.0.65 ± 0.2 μM, respectively; n = 4 each genotype), consistent with muscarinic-cholinergic receptor activation. These data indicate that the absence of K$_v$1.1 does not affect airway smooth muscle reactivity to MCh.

Discussion

This is the first study demonstrating that changes in respiratory function are progressive with age in $Kcna1^{-/-}$ mice which may promote respiratory failure prior to SD. (1) $Kcna1^{-/-}$ mice experience chronic blood gas instability as evidenced by frequent seizures, chronic oxygen desaturation, frequent A-H, the unusual breathing sequence of repeating patterns of tachypnea-A-H, increased tidal volume and the paradoxical MCh-induced hyperventilation. Blood gas instability triggers a compensatory increase in respiratory drive. Thus, the increase in basal respiratory drive as $Kcna1^{-/-}$ mice aged may indeed be compensatory. (2) The MCh challenge unveiled further respiratory abnormalities in $Kcna1^{-/-}$ mice. Following MCh, the hyperventilation response of $Kcna1^{-/-}$ mice was dramatically attenuated with age. Interestingly, only $Kcna1^{-/-}$ mice developed seizures following exposure to MCh and only $Kcna1^{-/-}$ mice became more sensitive to MCh as they approached sudden death. Indeed, MCh induced sudden death in a subset of SD30 and SD55 $Kcna1^{-/-}$ mice that exhibited respiratory parameters resembling SD90 $Kcna1^{-/-}$ mice.

$Kcna1^{-/-}$ mice exhibit chemoresponsive instability

Limited data are available concerning the role of Kv1.1 in respiratory peripheral organs including the trachea and lungs. We did not detect $Kcna1$ mRNA or Kv1.1 protein in lung tissue and there were no differences in contractility of $Kcna1^{+/+}$ $Kcna1^{-/-}$ trachea. Our data further support previous findings. Despite $Kcna1$ mRNA expression in trachea-bronchial airway smooth muscle cells, it does not contribute to contractile function.[19,20] K$_v$1.1 alpha subunit protein is located in neural tissue and its presence and function is established in the

vagal and phrenic nerves.[14,21] Hyperexcitable phrenic nerves of $Kcna1^{-/-}$ mice may contribute to increased respiration rate, an endpoint that was similar across age. We focused on endpoints that changed with age as these may implicate additional factors and have relevance to SUDEP susceptibility.

Our data is consistent with the notion that $Kcna1^{-/-}$ mice exhibit chemoresponsive instability. $Kcna1^{-/-}$ mice experience chronic oxygen desaturation, frequent seizures and A-H, both of which have been shown to be associated with elevated CO_2 (hypercapnia) and hypoxia.[4,8,22–24] In the seminal report by Ryvlin et al. the terminal GTC seizure was followed by a brief period of tachypnea, then bouts of apnea and finally complete respiratory arrest[9]. A similar breathing sequence of increased breathing rate then apnea was detected in $Kcna1^{-/-}$ mice. Tachypnea is increased respiration rate (reflected by elevated breathing rate, and shortened inspiration and expiration time). Hyperventilation is an increase in respiration rate and depth (reflected by increased tidal volume and peak inspiratory and expiratory flow). Tachypnea or hyperventilation is typically followed by arousal, not apnea. Hyperventilation-induced apnea is rare and is the result of chemoresponsive instability. Hyperventilation-induced apnea has been reported to occur in people with central apnea or respiratory failure.[25–28] This suggests that chemoresponsive instability may occur in patients prior to SUDEP and if it occurs early, hyperventilation-induced apnea may provide a novel marker of SUDEP risk.

A previous study has reported that $Kcna1^{-/-}$ mice have normal (and slightly elevated) response to hypoxia which is thought to be due to the absence of $K_v1.1$ in the peripheral carotid bodies.[29] Loss of Kv1.1 did not change the hypercapnia ventilator response, [29] which is thought to be centrally mediated. These data suggest a central mechanism may be responsible for chemoresponsive stability in SD90 $Kcna1^{-/-}$ mice.

Respiratory drive is an endogenous tool that is increased to promote blood gas stability. For example, a buildup of CO_2 in the lungs will elevate blood CO_2 levels to signal peripheral and central chemoreceptors to increase respiration rate which in turn stabilizes blood gasses. Indeed, as $Kcna1^{-/-}$ mice approach SD, basal respiratory drive and peak inspiratory flow are uniquely increased. Respiratory drive reflects the volume of air which enters and exits the lungs in one cycle normalized to inspiration time. Increased peak inspiratory flow indicates deeper inspiration. Respiratory drive and inspiration can be influenced by central input to the medulla where pre-inspiratory, inspiratory and expiratory neurons control the timing and air flow of inspiration and expiration. Orexin neurons in the lateral hypothalamus project to medullary pre-inspiratory and inspiratory neurons and are upstream of intercostal muscle-, phrenic nerve- and diaphragm-ventilator responses, influencing volume, inspiration and overall respiratory drive.[30–33]

Chemoresponsive orexin neurons increase firing in response to hypercapnia and hypoxia to increase breathing rate.[34–37] We have previously found that orexin protein levels are elevated in the lateral hypothalamus of $Kcna1^{-/-}$ mice.[40] Therefore, increased orexin neuronal activation may be a central driver of increased respiratory responses of $Kcna1^{-/-}$ mice. Indeed, orexin crosses the blood brain barrier and is elevated in the plasma of patients with epilepsy and in patients with hypercapnic respiratory failure.[38,39] Elevation of orexin may be

a compensatory mechanism to increase respiratory drive in people with respiratory failure. Elevated orexin levels in people with epilepsy and in *Kcna1*−/− mice may also be reflective of an endogenous compensatory mechanism to increase respiratory drive.[40] Ongoing studies are exploring the potential role of orexin in the respiratory dysfunction of *Kcna1*−/− mice.

MCh challenge unveils abnormal responses in *Kcna1*−/− mice

MCh is routinely used to diagnose asthma in humans and animals and does not cause seizures in normal humans or animals. However, the MCh-challenge is contraindicated for people with epilepsy suggesting an abnormal, potentially dangerous response. MCh is a charged quarternary amine which prevents it from crossing the blood brain barrier (BBB). Seizures can make the BBB permeable and we have found *Kcna1*−/− BBB to be leaky[40]; therefore, it is possible for MCh to enter the *Kcna1*−/− brain. Administration of a mechanistically similar drug, pilocarpine is used to induce seizures in animal models. Though, it is unlikely that the relatively small amount of MCh inhaled reaches concentrations in the CNS necessary to elicit a seizure. A more probable cause for MCh-induced seizures is the effect of hyperventilation on blood gases. Hyperventilation can lower seizure threshold and cause seizures in people with epilepsy. In fact, it is commonly used as a diagnostic tool.

MCh increases bronchoconstriction and airway resistance.[18] *Kcna1*+/+, *Kcna1*+/− and *Kcna1*−/− mice exhibited similar, non-asthmatic airway resistance responses to MCh consistent with our *in vitro* trachea data and previous reports indicating that the absence of Kv1.1 does not affect airway smooth muscle responses to MCh.[19,20] *Kcna1*+/+ and *Kcna1*+/− mice responded to increasing MCh with expected respiratory depression. In stark contrast, MCh triggered hyperventilation and increased respiratory drive in *Kcna1*−/− mice. Furthermore, the MCh EC_{50} decreased with age, indicating increased sensitivity to MCh as *Kcna1*−/− mice approached SD. Hyperventilation is triggered by blood gas instability. Thus, the hyperventilation further supports the notion of a compensatory response to endogenous blood gas instability. Interestingly, this potentially compensatory response to MCh is significantly attenuated in SD90 *Kcna1*−/− mice. This loss of or reduced ability to trigger deep breathing (hyperventilation), may indicate the compensatory mechanism becomes less reliable as *Kcna1*−/− mice approach sudden death.

Conclusion

One SUDEP case study with long-term monitoring reported a change in heart rate variability preceding the final seizure and sudden death when compared to within subject data seven months prior.[7] Although it was not clear how early the change occurred, it suggests a temporal window to detect within subject changes may occur in people at high risk for SUDEP. Data reported herein indicate a temporal window of opportunity may exist to identify chemoresponsive instabilities to possibly identify/develop/implement treatments to postpone or prevent SUDEP, however, additional pre-clinical and clinical studies are required.

Acknowledgments

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Key Bullet Points

- *Kcna1*$^{-/-}$ mice experience chronic blood gas instability, which triggered a compensatory increase in respiratory drive as mice aged.

- The MCh challenge unveiled respiratory abnormalities in *Kcna1*$^{-/-}$ mice.

- Only *Kcna1*$^{-/-}$ mice developed seizures following exposure to MCh and only *Kcna1*$^{-/-}$ mice became more sensitive to MCh as they approached sudden death.

- The progression of respiratory dysfunction with age may promote respiratory failure when challenged by a severe seizure, thereby increasing susceptibility of *Kcna1*$^{-/-}$ mice to sudden death.

- Data reported herein indicate a temporal window of opportunity may exist to identify chemoresponsive instabilities to possibly identify/develop/implement preventative treatments to postpone or prevent SUDEP
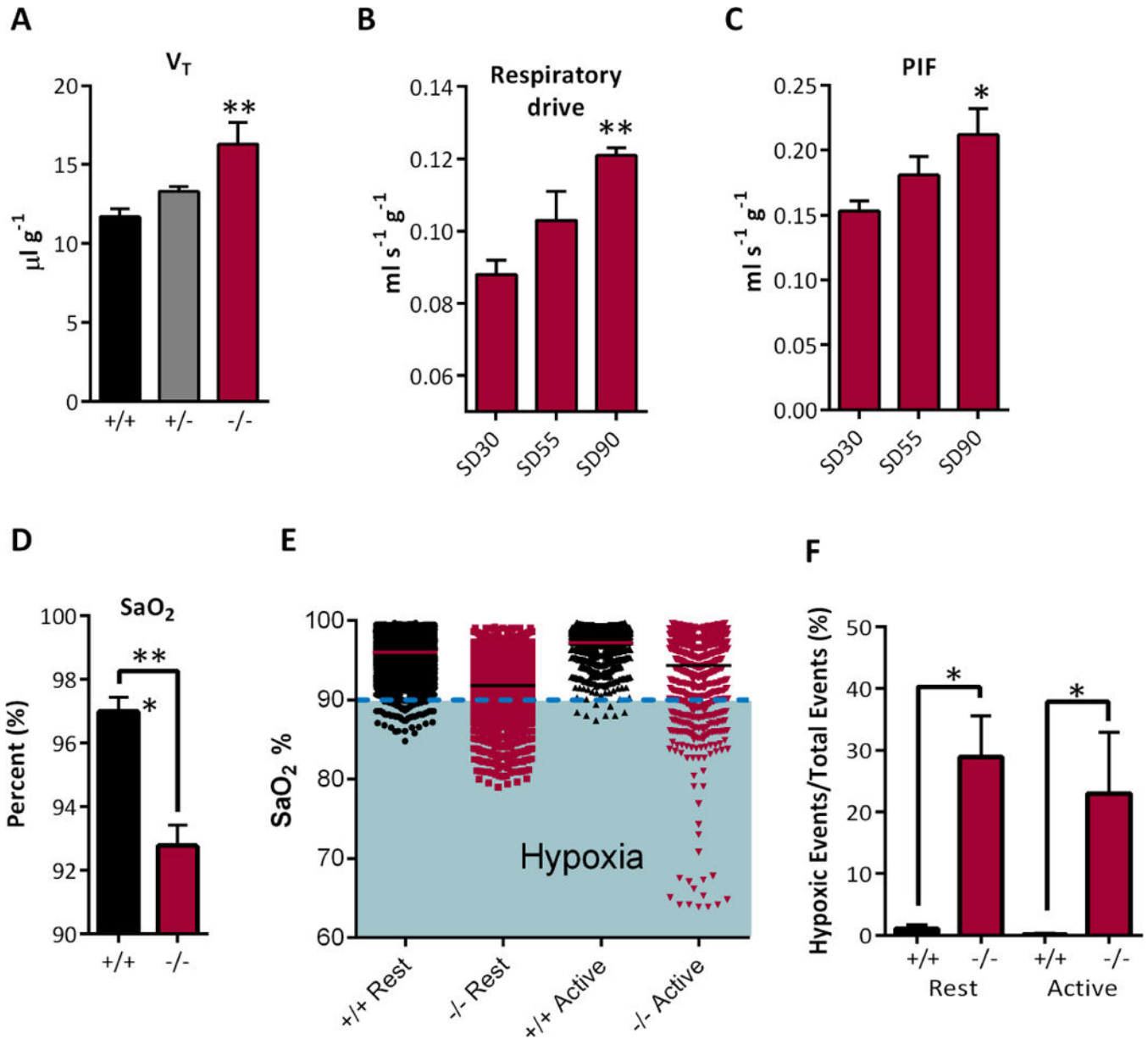
Figure 1. Basal respiration and chemoresponsive instability in _Kcna1_$^{-/-}$ mice

A) Tidal volume (V_T) is increased in SD90 _Kcna1_$^{-/-}$ mice (F $(2,14)$ = 6.9). (**B**) As _Kcna1_$^{-/-}$ mice age respiratory drive (V_T/T_i: F $(2,14)$ = 8.9) and (**C**) PIF (F $(2,14)$ = 3.5) are elevated. Significance was determined by two way ANOVA with Dunnett's multiple comparisons test, n = 5-7. (**D**) Pulse oximetry indicates that arterial oxygen saturation (SaO_2) is lower in SD55 _Kcna1_$^{-/-}$ mice compared to age matched _Kcna1_$^{+/+}$ mice (data expressed as mean per animal ± SEM, n = 4 mice). (**E**) Plotting all SaO_2 measurements indicates oxygen desaturation during different behavioral states by ~4% and illustrates frequent excursions into hypoxia when SaO_2 < 90%. (**F**) Quantification of the percentage of measurements with SaO_2 < 90%, unpaired Mann-Whitney test; n = 8, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
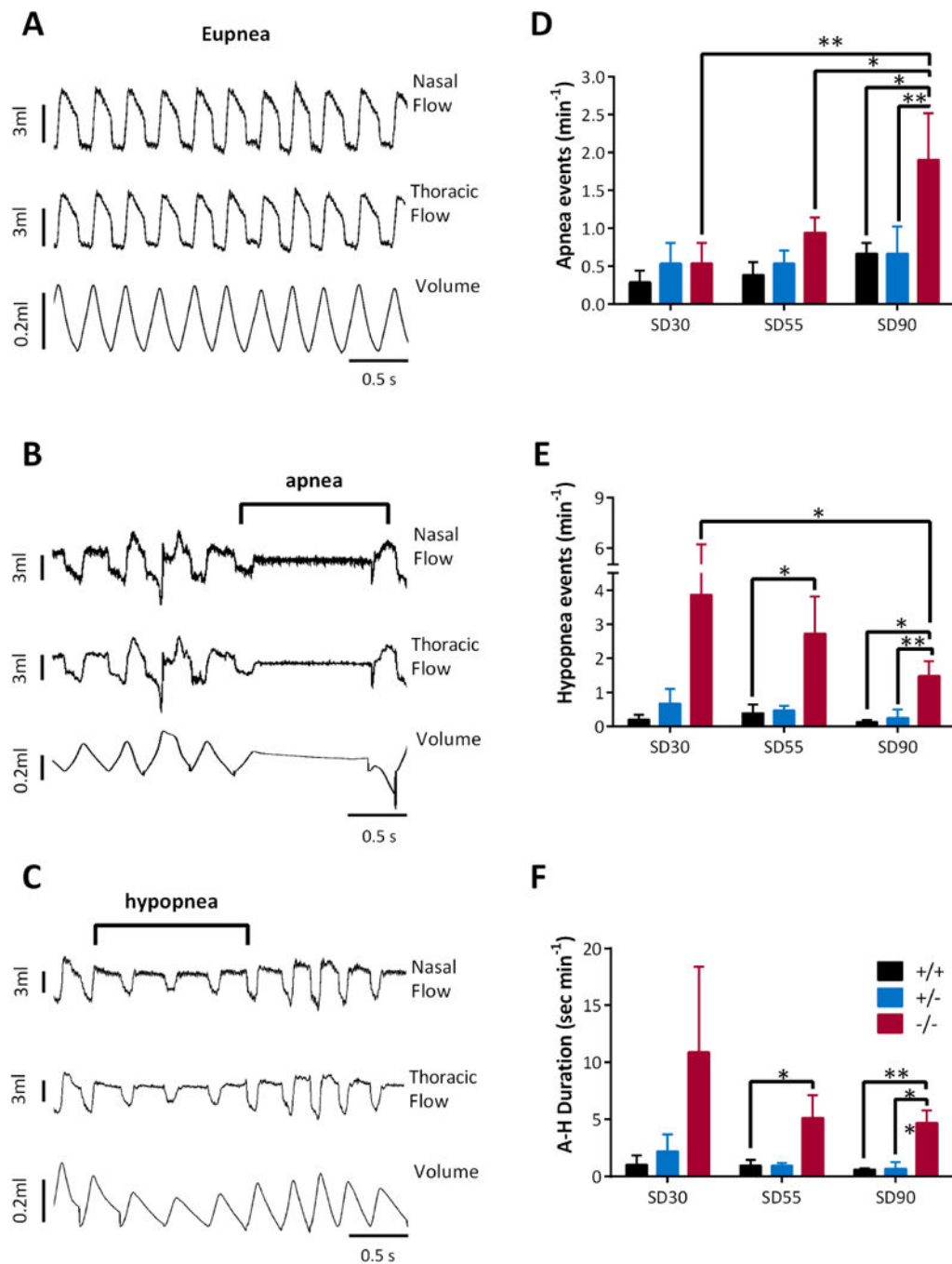
Figure 2. *Kcna1*^{−/−} mice experience more apnea and hypopnea events

Example traces of (**A**) normal breathing (eupnea), (**B**), apnea and (**C**) hypopnea. *Kcna1*^{−/−} mice display (**D**) more apnea events and (**E**) fewer hypopnea events as they age. (**F**) *Kcna1*^{−/−} mice spend significantly more time in apnea-hypopnea (A-H). Significance was determined by a two-way ANOVA for age and genotype followed by Holm-Sidak's multiple comparisons test, n = 4-7, * $p < 0.05$, ** $p < 0.01$.
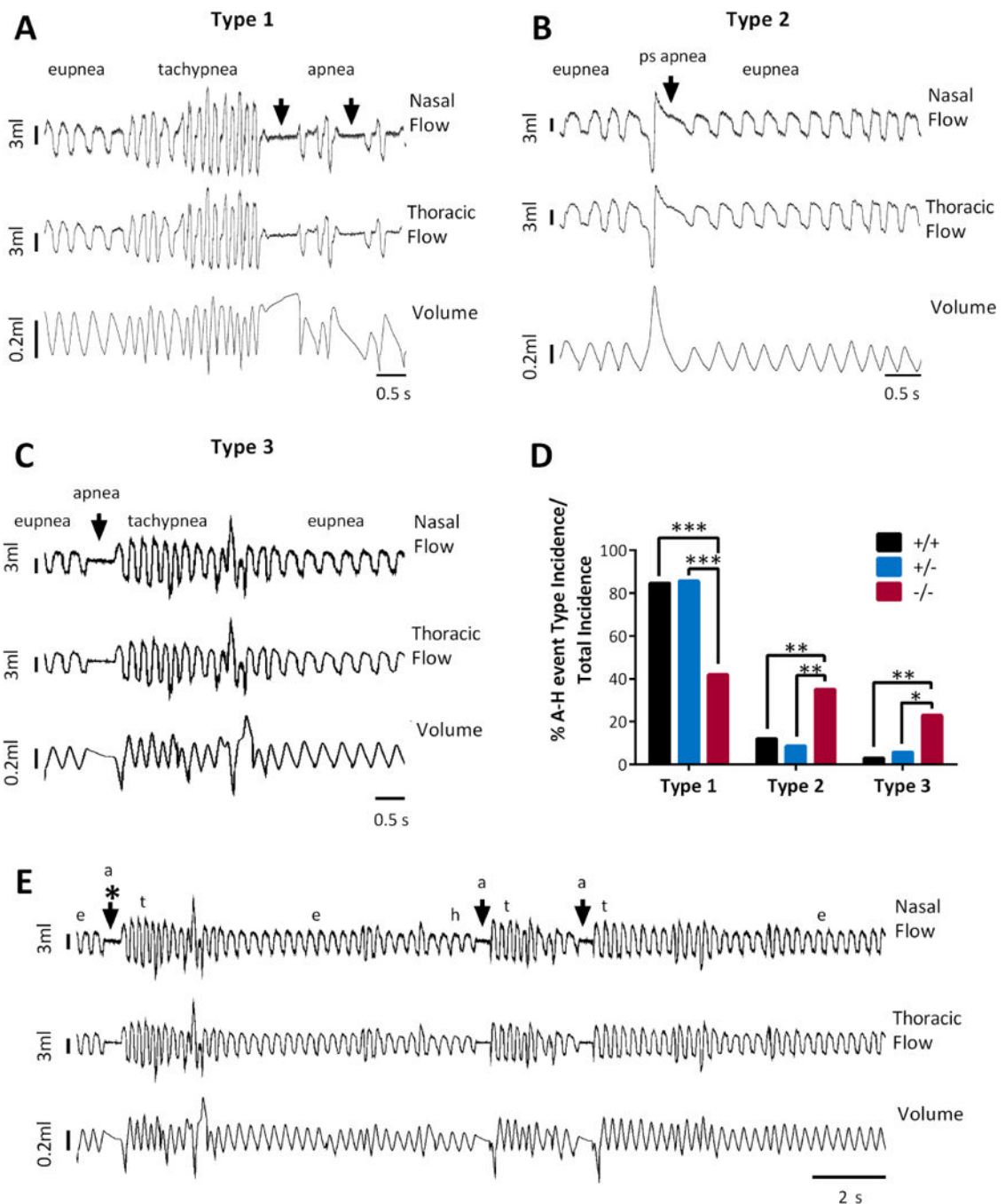
Figure 3. All genotypes display three types of Apnea-Hypopnea events
(**A**) An example of a Type 1 event which begins with eupnea, then tachypnea, then apnea or hypopnea. (**B**) Type 2 events are preceded and followed by eupnea. Shown is an example of a post sigh (*ps*) apnea. (**C**) Type 3 events are preceded by eupnea and followed by tachypnea. (**D**) In comparison to *Kcna1*^{+/+} and *Kcna1*^{+/−} mice, *Kcna1*^{−/−} mice had decreased Type 1 events and increased Type 2 and 3 events. Significance determined by Fisher's exact test, [*] $p < 0.05$, [**] $p < 0.01$, [***] $p < 0.001$. (**E**) Example trace of sequential A-H events.

Eupnea (*e*), apnea (*a*), tachypnea (*t*). Arrows indicate apnea events. Asterisk indicates the Type 3 event shown in C on a shorter time scale.
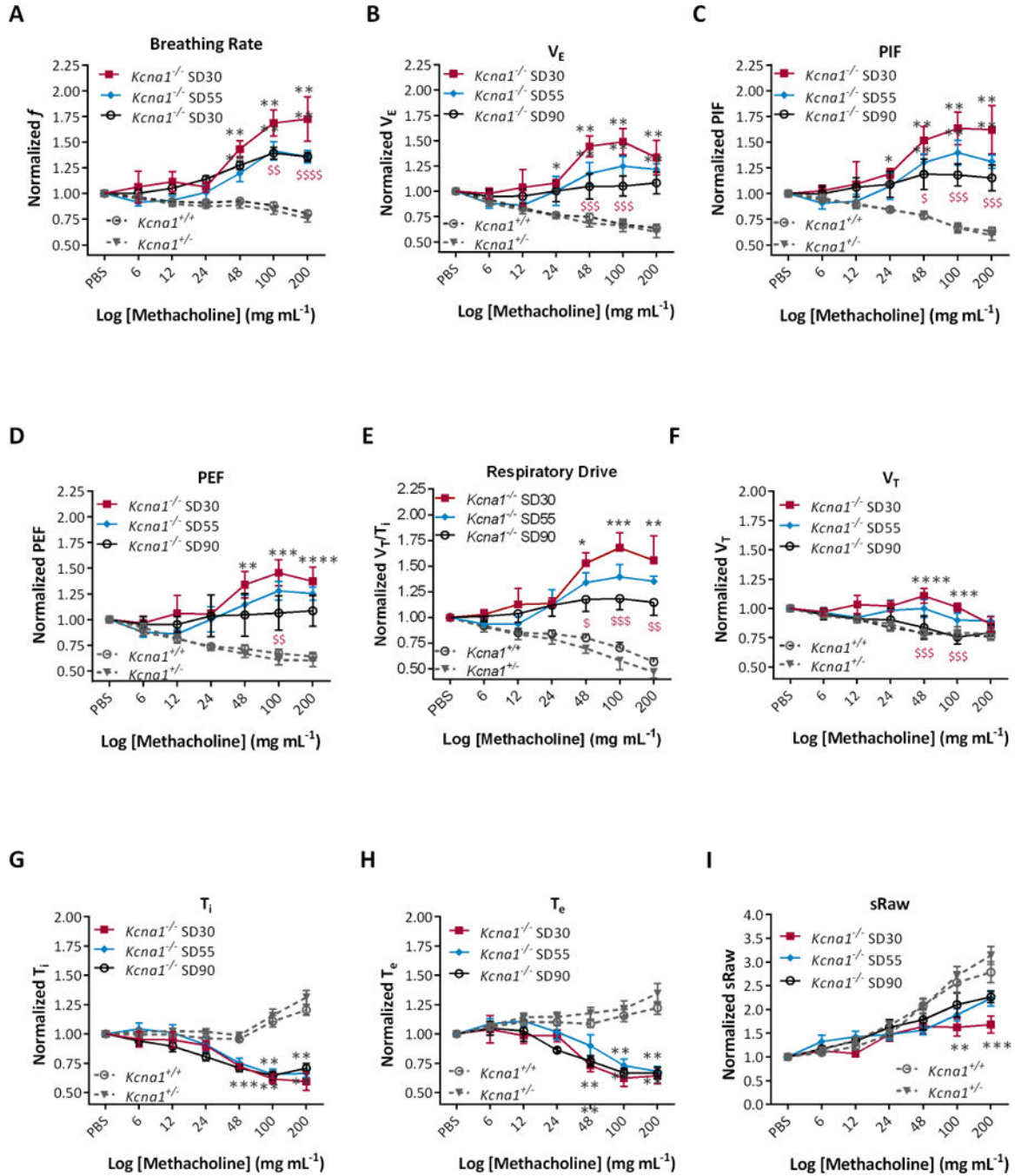
Figure 4. Paradoxical hyperventilation provoked by MCh attenuates with age in *Kcna1*−/− mice

In *Kcna1*−/− mice, exposure to increasing concentrations of nebulized increased (**A**) respiration rate (*f*: F (2,203) = 105.5, *p* < 0.0001), (**B**) minute ventilation (V_E: F (2,203) = 64.3, *p* < 0.0001), (**C**) peak inspiratory flow (PIF: F (2,203) = 73.9, *p* < 0.0001), (**D**) peak expiratory flow (PEF F (2,203) = 41.2, *p* < 0.0001) and (**E**) respiratory drive (V_T/T_i: F (2,203) = 31.9, *p* < 0.0001); and decreased (**F**) tidal volume (V_T: F (2,203) = 22.5, *p* < 0.0001), (**G**) inspiratory time (T_i: F (2,203) = 54.8, *p* < 0.0001) and (**H**) expiratory time (T_e: F (2,203) = 41.3, *p* < 0.0001), with (**I**) little effect on specific airway resistance (sRaw: F

(2,203) = 5.7, $p < 0.005$). Significance was determined by two-way ANOVA with repeated measures followed by Tukey's multiple comparisons test; n = 5-7, grey * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates P32-36 and P48-56 *Kcna1*$^{-/-}$ cohorts differ from *Kcna1*$^{+/+}$. Red $^\$$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$, $^{\$\$\$\$}$ $p < 0.0001$ indicates P48-56 differs from P32-36 *Kcan1*$^{-/-}$ mice.
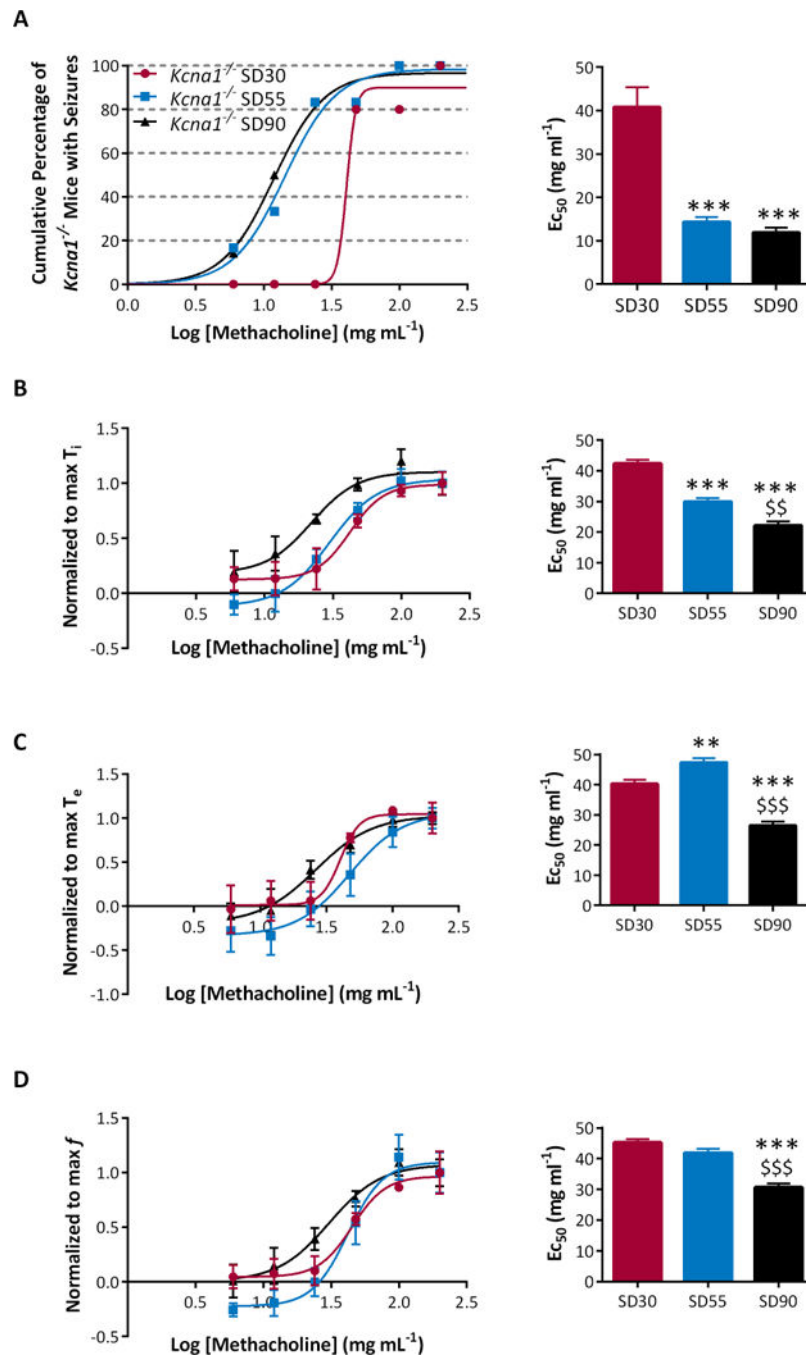
Figure 5. Seizures are triggered by lower MCh concentrations in SD90 *Kcna1*−/− mice

(**A**) MCh induced seizures at lower concentrations in older *Kcna1*−/− mice. (**B**) MCh concentration response curves exhibited a leftward shift in SD90 *Kcna1*−/− mice for inspiratory time (T_i), (**C**) expiratory time (T_e), and (**D**) and respiration rate (*f*). This resulted in significantly reduced MCh EC_{50}'s (*right*). Significance was determined by a one-way ANOVA followed by Tukey's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$ v. P32-36 and $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ v. P42-46, n = 5-6.
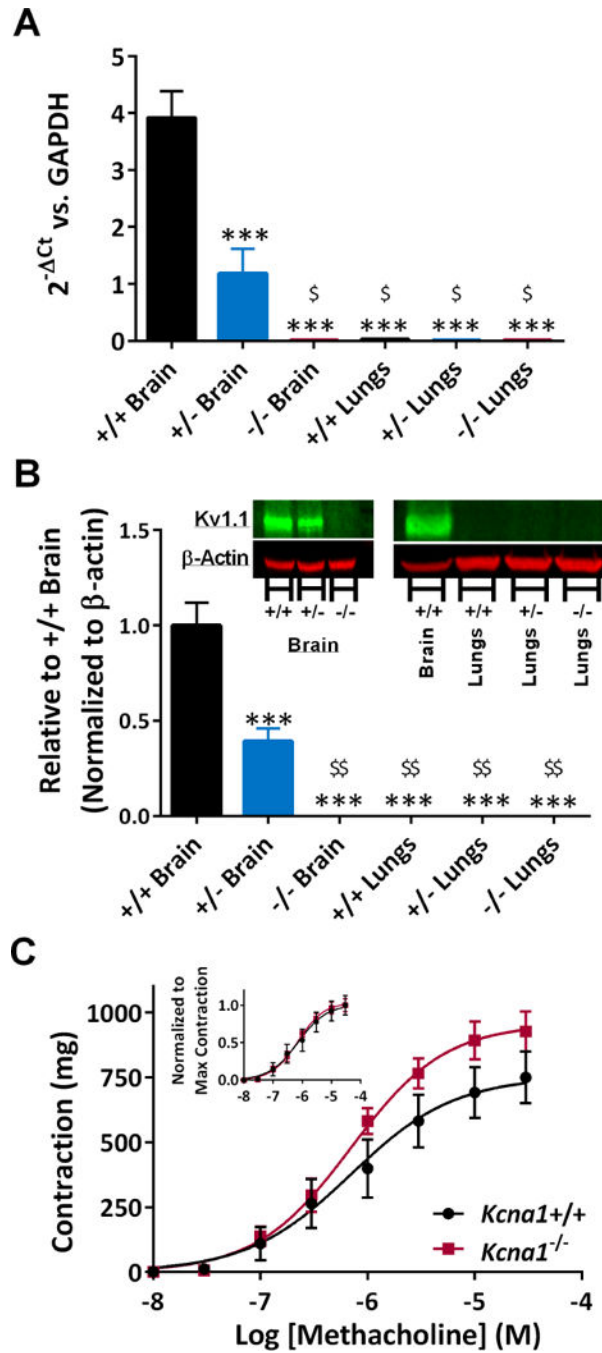
Figure 6. *Kcna1* mRNA and protein is not present in lung, and does not participate in airway smooth muscle reactivity to MCh

Kcna1 (**A**) mRNA and (**B**) protein were expressed to ~30-40% of wild-type levels in *Kcna1*^{+/−} brain tissue and absent in *Kcna1*^{−/−} mice brains. In contrast, *Kcna1* mRNA and protein were not detected in lung tissue from all three genotypes. Significance was determined by a two-way ANOVA for tissue and genotype followed by Tukey's multiple comparisons test, [***] $p < 0.001$ v. *Kcna1*^{+/+} and [$] $p < 0.05$, [$$] $p < 0.01$ v. *Kcna1*^{+/−}, n = 3 per genotype. (**C**) Concentration-response curves demonstrating the maximal contractile response and the sensitivity to MCh of isolated trachea did not differ between *Kcna1*^{+/+} and

Kcna1$^{-/-}$ mice (n = 4). *Inset*, concentration response curves normalized to the maximum contraction for each genotype. Significance was determined by a two-way ANOVA repeated measures for MCh concentration and genotype followed by Tukey's multiple comparisons test.