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KCNQ4 potassium channel subunit deletion leads to exaggerated acoustic startle reflex in mice

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The potassium voltage-gated channel subfamily Q member 4 (KCNQ4) subunit forms channels responsible for M-current, a muscarine-sensitive potassium current regulating neuronal excitability. In contrast to other KCNQ subunits, its expression is restricted to the cochlear outer hair cells, the auditory brainstem and other brainstem nuclei in a great overlap with structures involved in startle reflex. We aimed to show whether startle reflexis affected by the loss of KCNQ4 subunit and whether these alterations are similar to the ones caused by brainstem hyperexcitability. Young adult KCNQ4 knockout mice and wild-type littermates, as well as mice expressing hM3D chemogenetic actuator in the pontine caudal nucleus and neurons innervating it were used for testing acoustic startle. The acoustic startle reflex was significantly increased in knockout mice compared with wild-type littermates. When mice expressing human M3 muscarinic (hM3D) in nuclei related to startle reflex were tested, a similar increase of the first acoustic startle amplitude and a strong habituation of the further responses was demonstrated. We found that the acoustic startle reflex is exaggerated and minimal habituation occurs in KCNQ4 knockout animals. These changes are distinct from the effects of the hyperexcitability of nuclei involved in startle. One can conclude that the exaggerated startle reflex found with the KCNQ4 subunit deletion is the consequence of both the cochlear damage and the changes in neuronal excitability of startle networks.

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Introduction

The potassium voltage-gated channel subfamily Q member 4 (KCNQ4) is one of the subunits forming ion channels for M-current, a voltage-gated potassium current that opens at subthreshold potentials and determines neuronal excitability [1,2]. KCNQ4 is found in certain peripheral locations; most importantly in the outer hair cells of the cochlea [3–5]. Dominant negative mutation of the subunit leads to hereditary nonsyndromic hearing loss (DFNA2) [3,4]. Its background is a progressive outer (and consequential inner) hair cell degeneration with age [5].

In contrast to other KCNQ subunits, KCNQ4 expression is restricted to certain brainstem nuclei, including the auditory system, the vestibular nuclei, some components of the reticular activating system and trigeminal nuclei [3].

The KCNQ4 subunit expression pattern is in a great overlap with the network responsible for startle reflex, 'a sudden motor response to potentially threatening intense stimuli' [6]. Acoustic, vestibular or tactile startle can be distinguished in mice [6]. Enhancement or inhibition of the acoustic startle reflex and its modulation was described in various diseases or conditions affecting the cochlea or the central nervous system [7–12].

The acoustic startle reflex of 2-month-old KCNQ4 knockout mice and wild-type littermates, as well as mice expressing hM3D in the pontine caudal nucleus and neurons projecting to the area, was investigated. We found that the acoustic startle reflex is increased in both cases but is differentially affected by habituation.

Materials and methods

Animal experiments were conducted in accordance with the appropriate institutional guidelines, and national and international laws (EU Directive 2010/63/EU for animal experiments) on the care of research animals. The experiments below were approved by the Committee of Animal Research of the University of Debrecen (19/2019/DEMÁB). The KCNQ4 KO strain (*Kcnq4*$^{-/-}$) was a kind gift from Prof. Thomas Jentsch (MDC/FMP, Berlin, Germany). Heterozygous animals were bred in our animal facility and 2-month-old knockout animals and wild-type littermates were employed (n = 8 wild

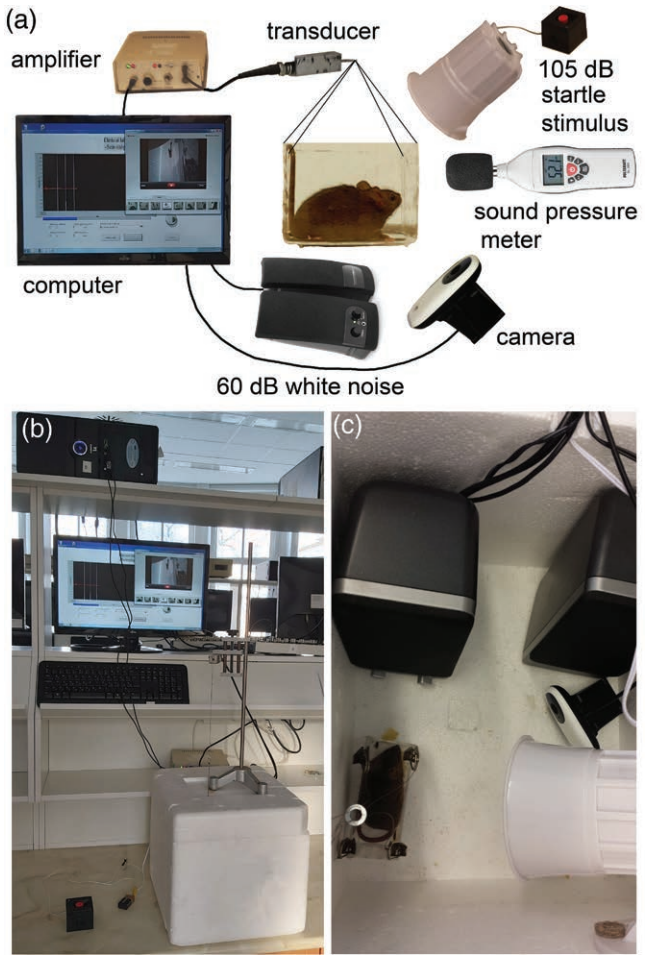
type and 14 knockouts). Further wild-type animals from the same breeding were used for virus injections ($n = 12$).

Young adult (7–8 weeks old) mice from both sexes were subjected to stereotaxic injection of ready-to-use retrogradely spreading adeno associated virus (AAV)

virus carrying plasmids encoding human M3 muscarinic (hM3D)(Gq) chemogenetic actuator and mCherry tag (pAAV-hSyn-hM3D(Gq)-mCherry; a gift from Bryan Roth; Addgene viral prep #50474-AAVrg; http://n2t.net/addgene:50474; RRID: Addgene_50474; $n = 6$) or solely mCherry tag as control expressed in neurons (pAAV-hSyn-mCherry, a gift from Karl Deisseroth; Addgene viral prep #114472-AAVrg; http://n2t.net/addgene:114472; RRID: Addgene_114472; $n = 6$).

Mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) injections and warmed to 37 °C. Stereotaxic injection of viruses (200 nl) was performed bilaterally 6 mm from bregma and 0.6 mm from the midline at 5 mm depth. After 2 weeks of virus expression, mice were involved in startle test.

Fig. 1



The arrangement of the startle setup. (a) Components of the startle setup. (b) The force measuring setup and the polystyrene box containing the experimental animal, (c) the loudspeakers, the siren and the camera.

Acoustic startle recordings were performed in a setup built in our laboratory (Fig. 1). The animal was placed in a perforated and transparent plastic chamber (3.5 × 8 × 5.5 cm) in which its horizontal movements were restricted. One week before the experiment, the chamber was placed in the mouse cage to make it familiar to the experimental animals. During recording, it was hanged on a force measurement setup including a mechanoelectric transducer, an amplifier and a computer with a force recording program (National Instruments, Austin, Texas, USA). The animal box was hanged 3–5 mm from the ground to provide its free movements. A continuous 60 dB white noise was employed, whereas a 105 dB sound stimulus with a frequency alternating between 500 and 1500 Hz was used by a siren to elicit startle response. The movement of the mouse was continuously monitored with a web camera and the intensity of sound stimuli was checked with a sound pressure gauge (Conrad Electronics, Budapest, Hungary). Five consecutive stimuli were employed with 60-s intervals. The first responses, the average responses and the course of the consecutive responses were analyzed. The whole experimental setup was placed in a polystyrene box (with pores for ventilation) to insulate the behavioral test from the environment.

For virus-injected mice, the injection site and the areas of mCherry expression were evaluated post hoc. For this, mice were transcardially perfused with 4% paraformaldehyde and 80-µm slices were cut with vibratome (Campden, Loughborough, UK) and postfixed for 24 h. Slices were mounted on a coverslip by using 4',6 diamidino-phenylindol-containing mounting medium and tile-scanned with confocal microscope (Zeiss LSM 700 Live; Carl Zeiss AG, Oberkochen, Germany).

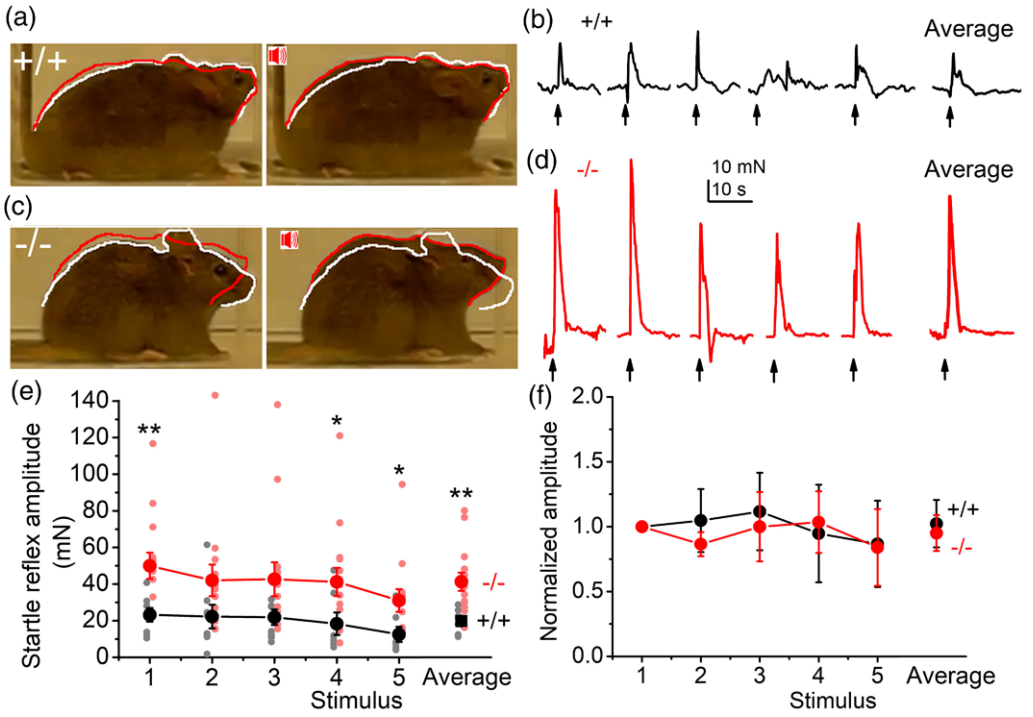
All data represent mean ± SEM. Student's *t*-test was applied for assessing statistical significance (level of significance = $P < 0.05$).

Results

In the first set of the experiments, five stimuli eliciting acoustic startle were employed concomitantly with 1-min gaps on wild-type and KCNQ4 knockout animals (Fig. 2a–d). In knockout mice, the startle amplitudes were greater during all five stimuli, with a significant difference at the first, fourth and fifth stimuli (wild type: 1st: 23.22 ± 3.74, 2nd: 22.22 ± 6.47, 3rd: 21.86 ± 4.23, 4th: 18.3 ± 6.13, 5th: 12.52 ± 4.14 mN; knockout: 1st: 49.96 ± 7.15, 2nd: 42.02 ± 8.65, 3rd: 42.62 ± 9.26, 4th: 41.15 ± 7.75, 5th: 30.99 ± 6.19 mN; 1st: $P = 0.007$; 4th: $P = 0.035$; 5th: $P = 0.028$). The average of the five startle responses was also significantly different (wild type: 19. 76 ± 2.14 mN; knockout: 41.27 ± 4.97 mN; $P = 0.0025$; Fig. 2e).

The startle reflex during the trains of stimuli did not show habituation in any genotype (startle amplitudes

Fig. 2



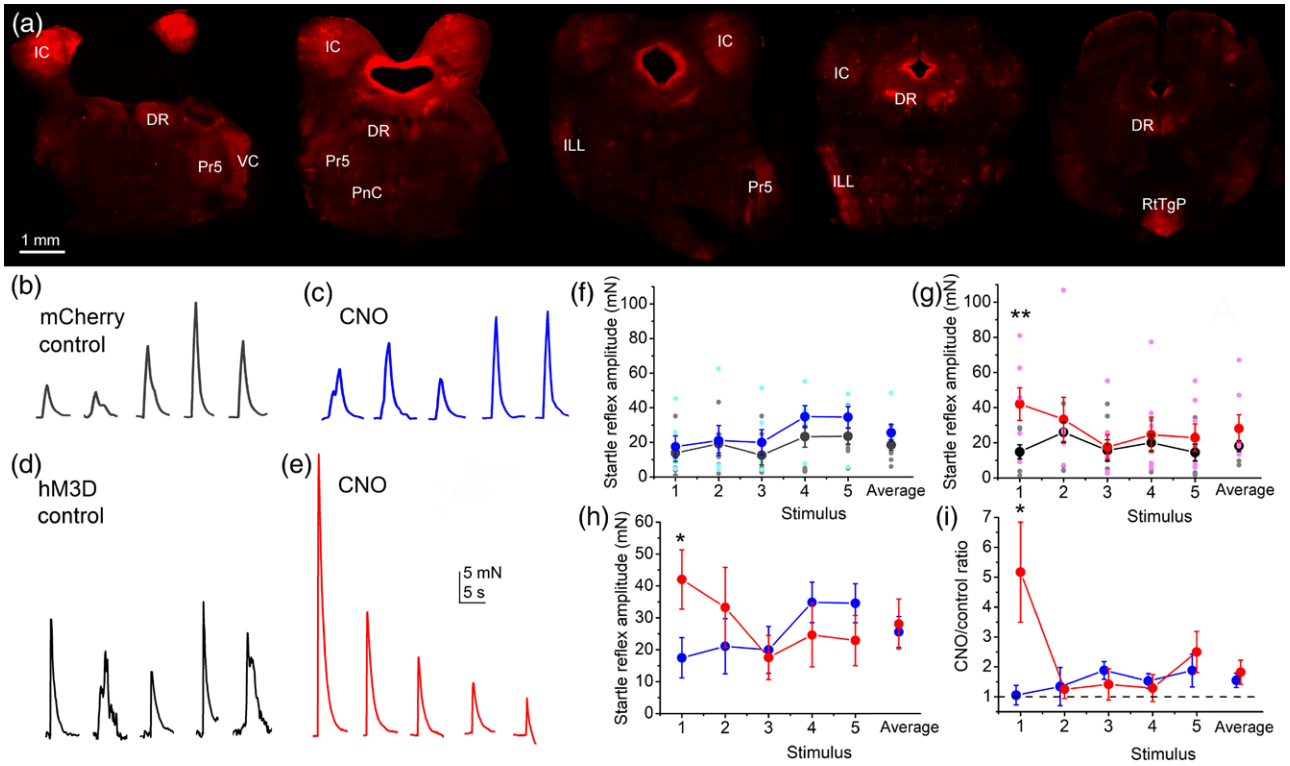
Acoustic startle reflex is facilitated by the deletion of KCNQ4 subunit. (a) Acoustic startle response of a wild-type (+/+) mouse before (left) and during the startle response (right) White contour: before startle, red contour: during startle. (b) Five consecutive startle responses and the average of them recorded on a wild-type animal. (c) Acoustic startle response of a KCNQ4 knockout (–/–) animal with the same arrangement as on panel (a). (d) Consecutive startle responses of a KCNQ4 knockout animal and the average of them. (e) Comparison of startle reflex amplitudes measured from the wild type (+/+, black) and knockout (–/–, red) animals (gray dots: individual data from wild type; pink dots: individual data from knockout animals). (f) Startle reflex amplitudes normalized on the first amplitude (black: wild type, red: knockout) *$P < 0.05$; **$P < 0.01$. KCNQ4, potassium voltage-gated channel subfamily Q member 4.

normalized to the first peaks were as follows: wild type: 2nd: 1.04 ± 0.24, 3rd: 1.12 ± 0.29, 4th: 0.954 ± 0.37, 5th: 0.84 ± 0.29; knockout: 2nd: 0.86 ± 0.09, 3rd: 1 ± 0.27, 4th: 1.03 ± 0.27, 5th: 0.84 ± 0.29; averages: wild type: 1.02 ± 0.18, knockout: 0.95 ± 0.14; Fig. 2f).

In the next set of experiments, a model of startle network hyperexcitability was tested. Five mice expressing hM3D with mCherry tag in neurons of the caudal pontine reticular nucleus (PnC) and areas projecting to it (ventral cochlear nucleus, principal sensory trigeminal nucleus, superior paraolivary nucleus, lateral and medial superior olive, laterodorsal tegmental nucleus, dorsal raphe nucleus, central nucleus of the inferior colliculus, reticulotegmental nucleus, latero- and medioventral nucleus of the pons, dorsal, intermediate and ventral nucleus of the lateral lemniscus, and pedunculopontine nucleus) were involved in the experiment (Fig. 3a and b). As a control, another five mice expressing solely mCherry tag in the same areas were employed (Fig. 3c–f). Startle reflex amplitudes were compared after intraperitoneal physiological saline or clozapine-N-oxyde (CNO) injections. In mCherry-expressing mice, CNO did not elicit significant change in startle reflex amplitudes (physiological saline: 1st: 13.78 ± 4.93, 2nd: 19.19 ± 5.39, 3rd: 12.55 ± 5.68, 4th: 23.31 ± 6.14, 5th: 23.52 ± 4.62 mN; CNO: 1st: 17.47 ± 6.29, 2nd: 21.07 ± 8.66, 3rd: 19.96 ± 7.38, 4th: 34.85 ± 6.35, 5th: 34.58 ± 6.11 mN; averages: 18.47 ± 3.09 with saline and 25.59 ± 4.85 mN with CNO; Fig. 3g). In contrast, in hM3D-expressing mice, a significant increase of the first amplitude was seen (physiological saline: 1st: 14.85 ± 3.98, 2nd: 25.97 ± 5.14, 3rd: 15.63 ± 6.08, 4th: 20.01 ± 4.53, 5th: 14.48 ± 4.8 mN; CNO: 1st: 42.05 ± 9.26, 2nd: 33.3 ± 12.5, 3rd: 17.55 ± 6.9, 4th: 24.61 ± 9.97, 5th: 22.89 ± 7.87 mN; $P = 0.009$ for the 1st amplitude; averages: 18.19 ± 3.13 with saline and 28.08 ± 7.83 mN with CNO; Fig. 3h). When the first startle amplitudes from mCherry- and hM3D-expressing mice with CNO treatment were compared, the hM3D-expressing mice had significantly increased startle amplitude ($P = 0.028$; Fig. 3i).

A strong habituation of startle amplitudes was seen in hM3D-expressing mice with CNO treatment. When responses during CNO treatment were normalized to the ones with physiological saline injection, a significant increase in the first amplitude was detected in hM3D-expressing mice ($P = 0.04$) followed by a rapid decline of this proportion (1st: 5.17 ± 1.67, 2nd: 1.25 ± 0.31, 3rd: 1.41 ± 0.52, 4th: 1.29 ± 0.45, 5th: 2.5 ± 0.68; average: 1.82 ± 0.4). This decline was not seen in control mice expressing only mCherry tag (1st: 1.05 ± 0.33, 2nd: 1.34 ± 0.64, 3rd: 1.88 ± 0.29, 4th: 1.52 ± 0.25, 5th: 1.87 ± 0.55; average: 1.55 ± 0.24). Datasets belonging to

Fig. 3



Chemogenetic stimulation on the PnC and nuclei innervating it causes a similar increase in acoustic startle amplitude. (a) Injection sites and mCherry tag expression. (b and c) Five consecutive startle responses of an mCherry-expressing animal under control conditions (black) and after CNO injection (blue). (d and e) Startle responses of an hM3D-expressing animal under control conditions (black) and during CNO injection (red). (f) Comparison of startle response amplitudes in mCherry-expressing animals (black: before CNO application, blue: during CNO application, gray dots: individual control data, light blue dots: individual data with CNO). (g) Comparison of startle response amplitudes in hM3D-expressing animals (black: before CNO application, red: during CNO application, gray dots: individual control data, pink dots: individual data with CNO). (h) Comparison of average startle responses with CNO treatment of mCherry- (blue) and hM3D-expressing mice (red). (i) Averages of the ratios of CNO-treated and control cases of mCherry- (blue) and hM3D-expressing mice (red). CNO, clozapine-N-oxyde; DLL, dorsal nucleus of the lateral lemniscus; DR, dorsal raphe nucleus; hM3D, human M3 muscarinic; IC, inferior colliculus; ILL, intermediate nucleus of the lateral lemniscus; LDT, laterodorsal tegmental nucleus; PnC, pontine caudal nucleus; PnR, pontine raphe nucleus; Pr5, principal trigeminal nucleus; SOC, superior olivary complex; SPTG, subpeduncular tegmental nucleus; VC, ventral cochlear nucleus.

the first peaks of mCherry- and hM3D-expressing mice were significantly different ($P = 0.04$; Fig. 3j).

Discussion

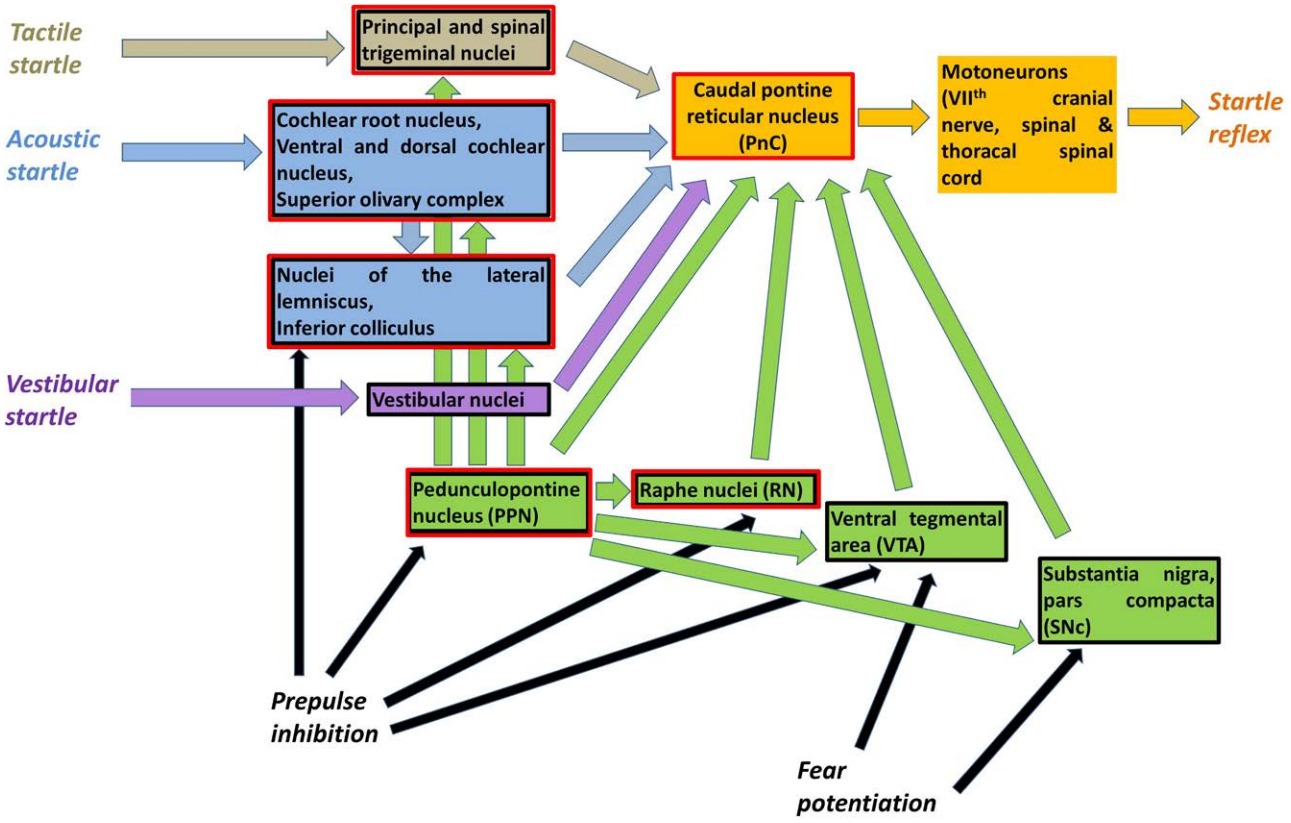
In this project, a setup measuring startle reflex was built to record acoustic startle response and its habituation. We found that deletion of KCNQ4 subunit leads to exaggerated startle reflex, but this increase can not be fully modeled by hyperexcitability of brainstem structures for startle.

The startle response is a sudden motor response for protecting the individual from potentially harmful stimuli [6,9]. Changes of the startle reflex can be related to cochlear damage by aging, noise exposure or ototoxic drugs [10–13]. Minimal cochlear damage caused increase in startle reflex, whereas severe damage is associated with the declination of it [10]. The main symptom of KCNQ4 knockout mice, as well as patients suffering from DFNA2 (caused by dominant negative mutation of KCNQ4), is 60 dB hearing loss progressing with age. Its background is the degeneration of the cochlear outer (and, in a lesser extent, the inner) hair cells [5]. Neuronal hyperexcitability affecting the auditory system or caused by impaired glycinergic inhibition also leads to increase in the amplitude of the startle response [7,8,11].

The possibility that the KCNQ4 ion channel subunit is involved in the startle reflex was raised by previous studies and is not excluded by the findings of other laboratories, including our own. The expression pattern of the subunit has a great overlap with brainstem structures for startle [4], and the presence and functional significance of the subunit was additionally shown in the pedunculopontine nucleus, which is involved in startle [14,15]. Besides the well-characterized cochlear damage, the lack of the subunit causes the hyperexcitability of brainstem nuclei involved in startle reflex (Fig. 4).

To provide a model of brainstem hyperexcitability without cochlear damage, we employed a chemogenetic model using retrogradely spreading AAV as a vector to deliver the plasmid encoding hM3D chemogenetic actuator. With this model, we were able to elicit a similar increase in the initial startle response but with a stronger short-term habituation

Fig. 4



Overlaps of KCNQ4 expression, startle pathways and nuclei involved in chemogenetic stimulation. Black squares: KCNQ4-positive areas, red squares: mCherry-expressing areas in this study. Yellow symbols: main startle reflex pathway, brown symbols: tactile startle pathways, blue symbols: acoustic startle pathways, purple symbols: vestibular startle pathways, green symbols: pathways modulating startle reflex [3,6]. KCNQ4, potassium voltage-gated channel subfamily Q member 4.

as seen in KCNQ4 knockout animals. The reason that this method led to differences in startle response might be that the cochlear damage is also present in KCNQ4 deleted animals and more nuclei are affected by the mutation than the injection (Fig. 4). In addition, CNO itself also exerts mild inhibition on acoustic startle response, which possibly leads to the decrease of the amplitudes [16].

The consequence of KCNQ4 subunit deletion was considered as 'nonsyndromic' hearing loss for a long time. However, this view is challenged as elevated mechanosensitivity in Meissner corpuscles and, in a subset of hair follicles, was demonstrated in KCNQ4 knockout animals [17]. Similarly, adaptation of the circadian rhythm to alterations of outer light-darkness conditions is also affected by the loss of the subunit in the reticular activating system [15]. Although the hearing loss is still the dominating symptom of this disease, with this work we demonstrated that an exaggerated acoustic startle is also present. This increase in the startle amplitude is a consequence of the cochlear damage and brainstem hyperexcitability occurring in KCNQ4-deleted animals.

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Conflicts of interest

There are no conflicts of interest.

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