

The Absence of Brain-Specific Link Protein Bral2 in Perineuronal Nets Hampers Auditory Temporal Resolution and Neural Adaptation in Mice

J. POPELÁŘ¹, M. DÍAZ GÓMEZ², J. LINDOVSKÝ¹, N. RYBALKO¹, J. BURIANOVÁ¹, T. OOHASHI³, J. SYKA¹

¹Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech Republic, ²Medical Electronics (MED-EL), Madrid, Spain, ³Department of Molecular Biology and Biochemistry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

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Summary

Brain-specific link protein Bral2 represents a substantial component of perineuronal nets (PNNs) enwrapping neurons in the central nervous system. To elucidate the role of Bral2 in auditory signal processing, the hearing function in knockout Bral2^{-/-} (KO) mice was investigated using behavioral and electrophysiological methods and compared with wild type Bral2^{+/+} (WT) mice. The amplitudes of the acoustic startle reflex (ASR) and the efficiency of the prepulse inhibition of ASR (PPI of ASR), produced by prepulse noise stimulus or gap in continuous noise, was similar in 2-week-old WT and KO mice. Over the 2-month postnatal period the increase of ASR amplitudes was significantly more evident in WT mice than in KO mice. The efficiency of the PPI of ASR significantly increased in the 2-month postnatal period in WT mice, whereas in KO mice the PPI efficiency did not change. Hearing thresholds in 2-month-old WT mice, based on the auditory brainstem response (ABR) recordings, were significantly lower at high frequencies than in KO mice. However, amplitudes and peak latencies of individual waves of click-evoked ABR did not differ significantly between WT and KO mice. Temporal resolution and neural adaptation were significantly better in 2-month-old WT mice than in age-matched KO mice. These results support a hypothesis that the absence of perineuronal net formation at the end of the developmental period in the KO mice results in higher hearing threshold at high frequencies and weaker temporal resolution ability in adult KO animals compared to WT mice.

Key words

Critical period • Acoustic startle reflex • Prepulse inhibition • Auditory brainstem response • Perineuronal nets

Corresponding author

J. Popelář, Institute of Experimental Medicine of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Fax: +420 241 062 787. E-mail: jpopelar@biomed.cas.cz

Introduction

Perineuronal nets (PNNs) are aggregates of extracellular matrix molecules, lecticans and link proteins, that surround a subset of neurons in the central nervous system. PNNs were found in more than 100 brain regions and are often associated with specific cell types and nuclei (Seeger *et al.* 1994, Costa *et al.* 2007). PNNs have been shown in many brain areas to surround GABAergic interneurons preferentially (Härtig *et al.* 1992). These GABAergic interneurons are further distinguished by immunoreactivity to parvalbumin (Celio and Chiquet-Ehrismann 1993) and by presence of Kv3.1b subunits in voltage-gated potassium channels implicated in high frequency neural firing (Sekirnjak *et al.* 1997, Rudy *et al.* 1999).

The molecular organization of PNNs consists of hyaluronan complexed with chondroitin sulfated proteoglycans, tenascin-R, and link proteins (Bral1,

Bral2, Crtl1 and Lp3). This quaternary complex is organized in region- and cell type-dependent patterns (for review see Sonntag *et al.* 2015). The proteoglycan brevican is expressed mainly in the perisynaptic space tightly associated with both the pre- and postsynaptic membranes (Frischknecht and Seidenbecher 2012, Blosa *et al.* 2013, Blosa *et al.* 2015). The expression and function of link proteins addressed in this article mainly concern a new family of link proteins termed Hapln1/Crtl1, Hapln2/Bral1 and Hapln4/Bral2 (Bekku *et al.* 2003, Hirakawa *et al.* 2000, Ogawa *et al.* 2004, Spicer *et al.* 2003, Oohashi *et al.* 2015). The interaction between hyaluronan and lecticans is stabilized by Haplns. The link protein Bral1 is associated with extracellular matrix at the nodes of Ranvier, Crtl1 and Bral2 are expressed exclusively in neurons bearing a PNN (Bekku *et al.* 2003, Carulli *et al.* 2006, Galtrey *et al.* 2008, Rauch *et al.* 2004) and are essential for the proper assembly of the PNN structure. The Bral2 protein was found in PNNs mainly in the brainstem and cerebellum (Bekku *et al.* 2003, Bekku *et al.* 2012), where it binds extracellular matrix in PNNs (Oohashi *et al.* 2002, Bekku *et al.* 2003, Bekku *et al.* 2010).

It is hypothesized that PNNs play an important role during critical developmental period. The critical period is a temporal window of 4-6 postnatal weeks, in which experience has profound effects on synapse refinement and the wiring of neuronal circuits. The function of PNNs in the termination of the critical period is to stabilize the nervous structure following development by formatting synaptic contacts, by controlling synaptic activity and central nervous system plasticity, by protecting neurons from oxidative damage, and by binding molecules for transport into cells (Celio *et al.* 1998, Morawski *et al.* 2004, Bekku *et al.* 2012, Sonntag *et al.* 2015, Kantor *et al.* 2004, McRae *et al.* 2007, Vigetti *et al.* 2008, Hrabetová *et al.* 2009, Oohashi *et al.* 2015).

The potential role of brain-specific link protein Bral2 in the development of hearing function and in auditory stimulus processing was investigated in our study by comparing the behavioral reflexive responses in Bral2-deficient (KO) mice and wild type Bral2^{+/+} (WT) mice. To assess possible permanent changes of the auditory system of KO mice, electrophysiological evoked responses to auditory stimuli were further recorded in adult animals.

Behavioral reactivity to loud auditory stimulations was examined in KO and WT mice by

recording the acoustic startle response (ASR) during the early postnatal development (between 2 – 8 weeks of life). Along with the ASR, its modulation induced by a preceding brief stimulus (prepulse), known as prepulse inhibition (PPI), was also monitored in this period. Unlike conditioning behavioral paradigms, recording of ASR and PPI of ASR is not difficult and time consuming, it produces reliable results, and can be used in young mice from onset of hearing because it does not require prior training. Moreover, it is important that structural basis of these auditory reflexive responses is well-known.

We examined the PPI of ASR in KO and WT mice using two different types of prepulse stimuli: a brief noise in quiet and a gap in noise. The PPI induced by a brief noise allows to characterize the sensitivity to near-threshold and moderate-level sounds (Hoffman and Ison 1980, Willot 2001). Thus, in combination with the measurement of ASR alone, behavioral responses to a wide range of stimulus levels can be determined. Measurement of the PPI induced by a gap in noise provides information about gap detection ability and state of temporal processing (e.g. Bowen *et al.* 2003).

To evaluate the possible differences in hearing thresholds, neural adaptability and neural temporal resolution ability between adult (two-month-old) KO and WT mice, recordings of the auditory brainstem responses (ABRs) reflecting the evoked potentials in the auditory nerve and brainstem auditory nuclei were used.

Methods

Animals

Individual groups of 6-10 WT and KO mice were behaviorally tested at the age of 2 weeks, 1 month and 2 months. ABRs were recorded in a group of 10 KO mice and 10 WT mice at the age of 2 months. All the animals were housed under standard laboratory conditions in a constant environment and a 12/12 h normal light/dark cycle; food and water were available ad libitum.

The care and use of animals were approved by the Ethics Committee of the Institute of Experimental Medicine, and followed the European Community Directive 2010/63EU.

Generation of Bral2-deficient mice

A 20-kb genomic fragment harboring the entire mouse Bral2/Hapln4 gene was isolated from a 129/Sv genomic library as described previously (Hirakawa *et al.*

2000). The Bral2/Hapln4 gene was disrupted by inserting a promoterless IRES-tauLacZ gene and a floxed neomycin cassette into the NotI site of exon3, leading to disruption of the immunoglobulin-like module (Rodriguez *et al.* 1999). The targeting vector was linearized by XhoI digestion, electroporated into R1 embryonic stem cells, and subjected to G418 selection. The correctly targeted embryonic stem cells were injected into C57BL/6 blastocysts, and the injected blastocysts were transferred into pseudopregnant foster mothers. The resulting male chimeras were tested for germline transmission and used to establish 129Sv inbred lines and 129Sv/C57BL/6 outbred lines. After six intercrosses of male homozygous offspring with wild-type C57BL/6 females, the heterozygous offspring were crossed to obtain homozygous animals. F6 generations of 129Sv/C57BL/6 backgrounds were used for the present study. A more detailed description of Bral2-deficient mice generation can be found in Hirakawa *et al.* 2000, Bekku *et al.* 2003, Bekku *et al.* 2012.

Behavioral testing

Apparatus and behavioral procedure

ASRs were measured in a sound attenuated chamber (Coulbourn Habitest, model E10-21) localized in a soundproof room. During the testing procedure, the mouse was confined to a small wire mesh cage on a motion-sensitive load-cell platform. An amplified voltage signal was acquired and processed using a TDT system III with Real-Time Processor RP 2 (Tucker Davis Technologies, Alachua, FL) and custom-made software in a Matlab environment. Startle responses were evaluated in a 100 ms window beginning from the onset of the startling stimulus. The magnitude of the ASR was measured as the maximal peak-to-peak amplitude of transient voltage occurring in the response window. Acoustical stimuli were generated by the TDT system (Real-Time Processor RP 2) and presented *via* a loudspeaker (SEAS, 29AF/W) placed inside the chamber above the animal. Stimulus presentation and data acquisition were controlled by a custom-made application in a Matlab environment. Calibration of the apparatus was performed for frequencies between 4 kHz and 32 kHz by a ¼ inch Brüel & Kjaer 4939 microphone connected to a Brüel & Kjaer ZC 0020 preamplifier and a B&K 2231 sound level meter. The calibrating microphone was positioned at the location of the animal's head in the test cage.

Acoustic startle responses

Startle reactivity was measured using startling stimuli which were broadband noise (1.5 – 45 kHz) bursts (50 ms duration, 1 ms rise/fall times) of varying levels. A test session consisted of 5 sets and each set was composed of 9 trials (startle stimuli of different intensity: 60, 70, 75, 80, 90, 100, 110, 120 dB SPL and a trial without the startle stimulus). To minimize the possibility of habituation or predictability, we used randomized presentation of startling stimuli and a variable inter trial interval (15-30 s).

Prepulse inhibition of ASR

The PPI of the ASR was induced either by noise bursts of variable intensity or by gap-in-noise of variable duration. For both PPI measurements the ASR was elicited by a 110-dB SPL, broadband noise burst (50 ms duration, 1 ms rise/fall times); the inter-trial interval in each test session varied randomly between 15 – 30 s. The PPI efficiency was expressed as a percent reduction of the startle amplitude: (ASR amplitude with prepulse/ASR amplitude without prepulse) x 100 %; smaller values of relative ASR amplitude reflect a stronger PPI.

The session for measuring the startle PPI induced by a broadband noise burst (1.5 – 45 kHz, 50 ms duration, 1 ms rise/fall times) consisted of 6 sets of 9 trials including a startle stimulus without noise-prepulse, 7 combination of the startle stimulus and a noise prepulse at different intensities (10, 20, 30, 40, 50, 60 or 70 dB SPL) and a trial without the startle stimulus and the prepulse. The interval between startle and prepulse was 50 ms.

The PPI of ASR induced by gap was measured using a gap of different durations embedded in a continuous broadband noise (1.5 – 45 kHz) of 65 dB SPL. A test session consisted 6 sets of 7 trials including a startle stimulus without gap-prepulse, 5 combinations of the startle and a gap-prepulse of different duration (5, 10, 15, 30 or 50 ms) and a trial without startle and prepulse stimuli. The interval between startle stimulus and gap-prepulse (on-on) was 70 ms.

ABR recording

ABRs were recorded in animals placed in a sound proof and anechoic room. The walls and ceiling inside the room were covered by cones from phono-absorbent material. Acoustic measurements demonstrated that the attenuation inside the room against noise level outside the room was 55 dB at 250 Hz and

60 – 70 dB for frequencies above 500 Hz.

ABRs were recorded in 2-month-old mice anesthetized with an intramuscular injection of 50 mg/kg body weight of ketamine (Narkamon 5 %, Spofa) and 8 mg/kg body weight of xylazine (Sedazine, Fort Dodge, IA). The ABRs were recorded by three stainless-steel needle electrodes, placed subdermally over the vertex (active) and right and left mastoids (reference and ground electrodes) of the animal. The signal from the electrodes was amplified by a TDT RA16PA RA4LI preamplifier and processed with a TDT data acquisition system RX5-2 Pentusa Base Station (Tucker-Davis Technologies, Gainesville, FL, 16-bit A/D converter, sampling rate 50 kHz) using BioSig software. Acoustic stimuli for the ABR recordings were generated by a PC-based TDT system and presented in free-field conditions *via* ribbon tweeter RAAL 70-20XR and mid-bass woofer Selenium 6W4P, placed 70 cm in front of the animal's head.

Four different types of ABR stimuli were used to evaluate the mouse's hearing function:

- Tone pips, duration 5 ms, 2-ms rise/fall times, frequency range 2 – 40 kHz, presented in one octave steps with decreasing intensity by 5 dB steps to assess the hearing threshold. The threshold at each frequency was determined as the minimal tone intensity that still evoked a visually noticeable potential peak in the expected time window of the recorded signal. Usually ABR wave III was taken as a dominant wave for determining the hearing threshold.
- Acoustic monopolar clicks, duration 0.1 ms, presented with decreasing intensity by 5 dB steps to assess the hearing threshold to clicks and input-output functions for individual ABR waves.
- A series of four clicks with variable inter-click interval (ICI) ranging from 1 ms to 50 ms to test neural adaptability. The peak-to-peak amplitude of ABR wave III was measured to assess the neural adaptability.
- Two broadband noise stimuli, duration 25 ms, separated by a variable gap of 1 – 50 ms duration to test temporal resolution ability. The peak-to-peak amplitude of ABR wave III was measured to assess the temporal resolution ability.

During electrophysiological recordings, the mice were placed on a heating pad that automatically maintained body temperature at 38 °C.

Statistical analyses

Statistical analyses were performed using Prism (GraphPad Software, Inc., CA). To compare results of behavioral tests and ABR data in WT and KO mice, two-way ANOVA with Bonferroni *post hoc* test was used. Amplitudes and latencies of individual ABR waves in WT and KO mice were tested using two-tailed t-test.

Results

Acoustic startle reflex

Acoustic startle reflex (ASR) was measured in 2-week-, 1-month- and 2-month-old KO mice and compared with age-matched WT controls.

An example of the ASR evoked by a broad-band noise burst, 110 dB SPL, is demonstrated in Figure 1A, ASR amplitude-intensity functions at various animal age are demonstrated in Figure 1B-D. In 2-week-old KO mice, the ASR amplitude is similar to that measured in age-matched WT mice ($p=0.2495$, $F_{(1,12)}=1.464$, two-way ANOVA, Fig. 1B), but in 1-month-old ($p<0.0001$, $F_{(1,18)}=28.16$, two-way ANOVA, Fig. 1C) and in 2-month-old ($p=0.0003$, $F_{(1,14)}=22.83$, two-way ANOVA, Fig. 1D) WT mice the ASR amplitudes significantly exceed the ASR amplitudes measured in KO mice at all intensities higher than 80 dB SPL ($p<0.05-0.001$, two-way ANOVA, Bonferroni *post test*).

Prepulse inhibition (PPI) of ASR induced by noise burst

In the PPI procedure used in our study, the startle reaction is inhibited by a low-level broad band noise burst that shortly precedes the intense startle stimulus (schematically shown in Fig. 2A). The results demonstrated in Figure 2B, C, D indicate that the dependence of the PPI of the ASR on prepulse intensity is almost identical in 2-week-old WT and KO mice ($p=0.9645$, $F_{(1,13)}=0.002$, two-way ANOVA) (Fig. 2B). In 1-month-old mice (Fig. 2C) the dependence of the PPI of the ASR on prepulse intensity decreased more evidently in WT mice in comparison with KO mice and the difference between PPI efficiency in 1-month-old WT and KO mice is significant ($p=0.0436$, $F_{(1,11)}=5.197$, two-way ANOVA). In 2-month-old mice (Fig. 2D) the PPI efficiency tended to be better in WT mice than in KO mice, but the difference between both groups of mice is not statistically significant ($p=0.2350$, $F_{(1,14)}=1.540$, two-way ANOVA), probably due to a larger interindividual variability in 2-month-old mice.

Acoustic startle reflex (ASR)

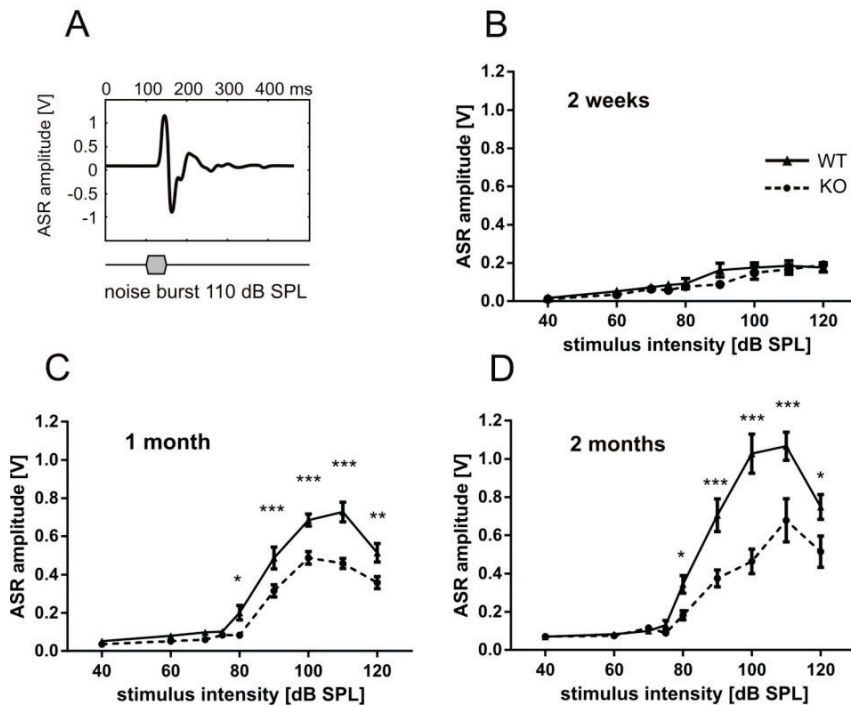


Fig. 1. (A) an example of the ASR evoked by a broad-band noise burst, 110 dB SPL. (B, C, D) amplitude-intensity functions of ASR measured in 2-week-old (B), 1-month-old (C) and 2-month-old (D) WT and KO mice. Bars represent \pm SEM. Significant differences marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Prepulse inhibition of ASR by noise

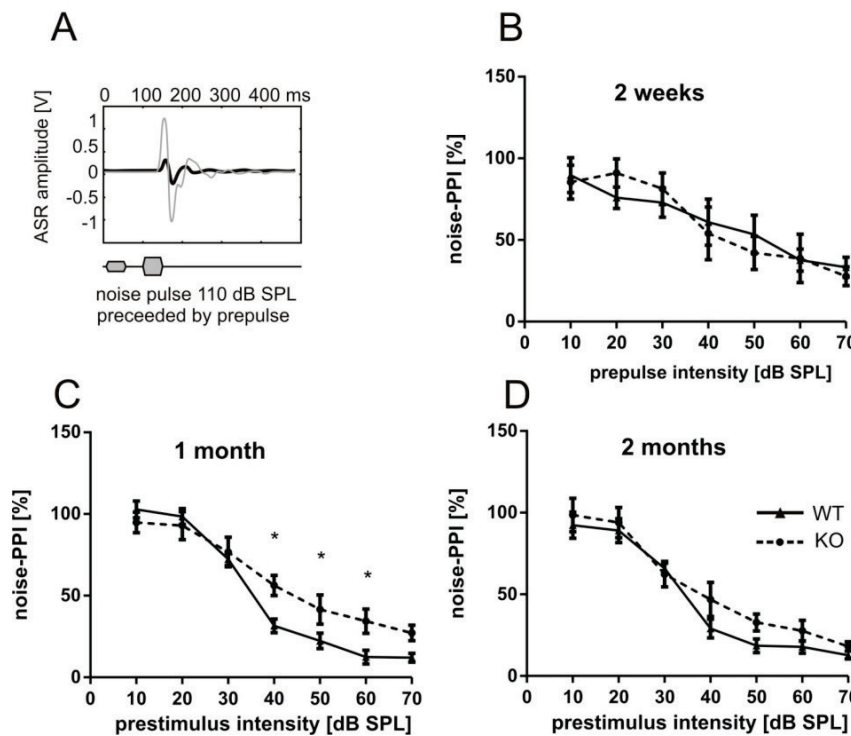


Fig. 2. (A) an example of the PPI of ASR produced by noise burst as a prepulse stimulus (thin grey curve – the response to startle stimulus only, thick black curve – the response suppressed by PPI). (B, C, D) dependence of the PPI of ASR on prepulse intensity measured in 2-week-old (B), 1-month-old (C) and 2-month-old (D) WT and KO mice. Bars represent \pm SEM. Significant differences marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The effect of changing gap duration on the degree of PPI of ASR

The temporal resolution of the hearing system was tested with the PPI of the ASR produced by a gap-in-noise of variable duration shortly preceding the

startle stimulus (schematically shown in Fig. 3A). The results presented in Figure 3B demonstrate that the dependences of the PPI of the ASR on gap duration are almost identical in 2-week-old WT and KO mice. In 1-month-old (Fig. 3C) and 2-month-old animals (Fig. 3D)

the gap-induced PPI of ASR is significantly less efficient in KO mice in comparison with WT mice, as documented by significantly different PPI-functions measured in 1-month-old and 2-month-old WT and KO mice ($p=0.0018$, $F_{(1,13)}=15.21$ for 1-month-old mice, and $p=0.0376$, $F_{(1,11)}=5.582$ for 2-month-old mice, two-way ANOVA).

The effect of changing prepulse intensity during development of PPI of ASR

The development of the PPI of ASR, produced by stimulation with broad-band noise (noise-PPI) or by a gap-in-noise (gap-PPI), measured in 2-week-old, 1-month-old and 2-month-old WT and KO mice, is demonstrated in Figure 4. In WT mice, the efficiency of noise-PPI (Fig. 4A) and gap-PPI (Fig. 4C) is significantly higher at the end of 4th postnatal week in comparison with 2nd postnatal week ($p=0.0078$, $F_{(1,12)}=10.19$ for noise-PPI, and $p<0.0001$, $F_{(1,12)}=22.88$ for gap-PPI, two way ANOVA). There is still a tendency for further improvement of the PPI efficacy of gap-PPI during the second postnatal month, but the difference between the efficacy of gap-PPI in 1-month-old and 2-month-old

WT mice is not statistically significant. The PPI efficacy of noise-PPI measured in 1-month-old and 2-month-old WT mice was not different.

In contrast to WT mice, in KO mice the efficacy of noise-PPI and gap-PPI did not change during their 2-month postnatal period and the noise-PPI functions and gap-PPI functions are almost identical in all three-age categories (Fig. 4B, D). These results demonstrate the different development of the PPI of ASR over the 2-month postnatal period in WT and KO mice.

Hearing thresholds

Hearing thresholds were assessed on the basis of ABR recording. Average hearing thresholds measured in 2-month-old KO and WT mice to clicks and pure tones are demonstrated in Table I. Average hearing thresholds to clicks and low-frequency tones up to 16 kHz are similar in WT and KO mice, but at higher frequencies (26, 32 and 40 kHz) hearing thresholds in KO mice are significantly higher in comparison with WT mice ($p<0.01$ for 25 kHz, $p<0.001$ for 32 and 40 kHz, two-way ANOVA with Bonferroni *post hoc* tests).

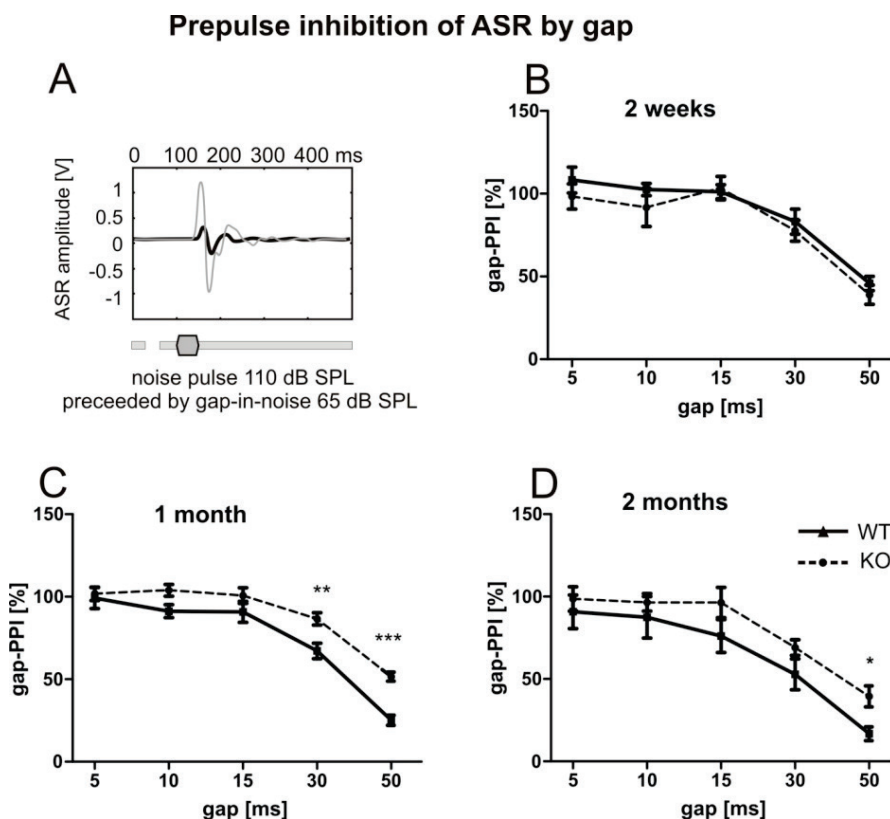


Fig. 3. (A) an example of the PPI of ASR produced by gap-in-noise as a prepulse (thin grey curve – the response to startle stimulus only, thick black curve – the response suppressed by PPI). (B, C, D) the dependence of the PPI of ASR on gap duration measured in 2-week-old (B), 1-month-old (C) and 2-month-old (D) WT and KO mice. Bars represent \pm SEM. Significant differences marked with asterisks (* $p<0.05$, ** $p<0.01$; *** $p<0.001$).

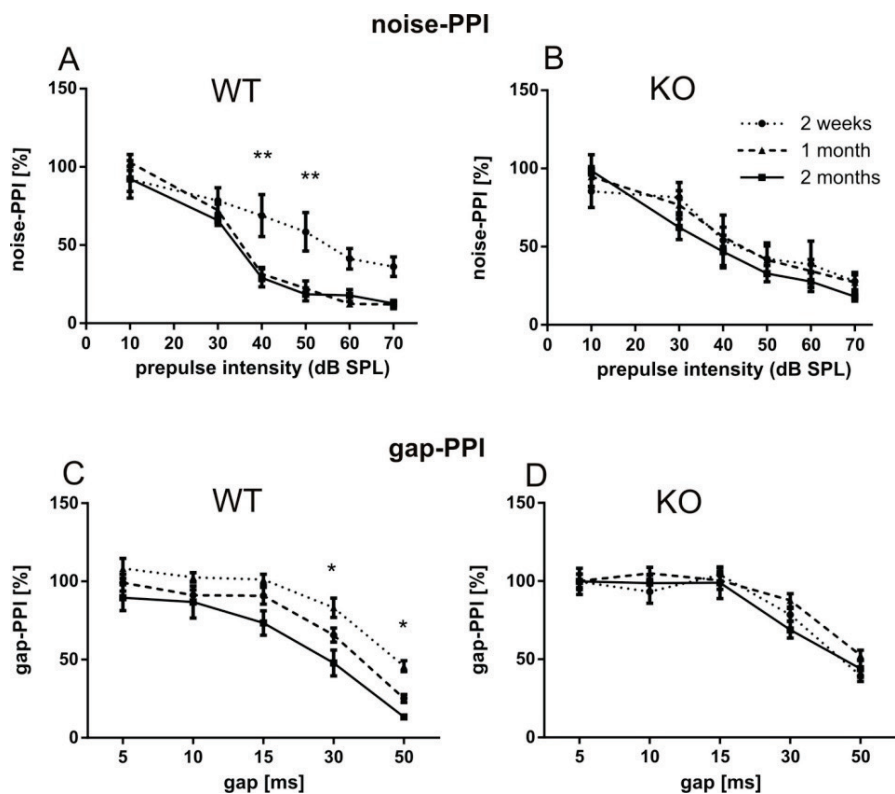


Fig. 4. The development of noise-PPI (A, B) and gap-PPI (C, D) functions measured in 2-week-old, 1-month-old and 2-month-old WT (A, C) and KO (B, D) mice. Bars represent \pm SEM. Significant differences marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 1. Hearing thresholds in WT and KO mice (in dB SPL).

<i>F</i> (kHz)	Click	4	8	16	26	32	40
				WT			
Mean (dB SPL)	25.0	42.9	24.3	20	31	43.6	73.6
SD	3.2	7.9	6.2	4.6	3.7	5.8	6.9
				KO			
Mean (dB SPL)	24.4	42.5	26.9	17.5	43.8	70	95.6
SD	3.0	7.9	4.3	6.6	6	11.5	5.8
<i>p</i>	n.s.	n.s.	n.s.	n.s.	**	***	***

Average hearing thresholds in dB SPL measured in 2-month-old KO and WT mice to clicks and pure tones. (** $p < 0.01$, *** $p < 0.001$).

Click-evoked ABR

Click-evoked ABR consisted of 5 waves reflexing generators of neuronal activity from the auditory nerve (wave I), cochlear nuclei (wave II), superior olivary complex (wave III), lemniscus lateralis (wave IV) up to the inferior colliculus (wave V). Examples of ABR, evoked by clicks of 70 dB SPL in 2-month-old WT and KO mice, are shown in Figure 5A. The ABR analysis demonstrated that peak-to-peak amplitudes of individual ABR waves (Fig. 5B) and peak latencies of individual ABR waves (Fig. 5C) are similar in both WT and KO mice.

Neural adaptation

Recording the ABR evoked by a series of 4 clicks with variable inter-click interval (ICI) can be used to test neural adaptation. An example of ABR recordings to stimulation with the series of 4 clicks with inter-click intervals (ICI) of 5 ms, 10 ms and 30 ms is shown in Figure 6A. When the ICI was short, the amplitudes of ABR to the 2nd click, 3rd click and 4th click were much smaller in comparison with ABR to the 1st click. The longer the ICI, the larger ABR amplitudes to the 2nd, 3rd and 4th click were observed. The dependence of the relative peak-to-peak wave III ABR amplitudes to the 2nd click with respect to the

relative wave III ABR amplitudes to the 1st click (taken as 100 %) on ICI, evaluated in WT and KO mice, are demonstrated in Fig. 6B. The wave III ABR amplitudes to the 2nd click in WT mice reached the 100 % level of 1st click amplitude at ICI 15 ms, whereas in KO mice the 2nd click function cross the 1st click 100 % level at ICI between 20-25 ms. Both curves are significantly different ($p=0.0382$, $F_{(1,10)}=5.697$, two-way ANOVA). These results indicate that neural adaptation was significantly better in WT mice in comparison with *Bral2*^{-/-} KO mice.

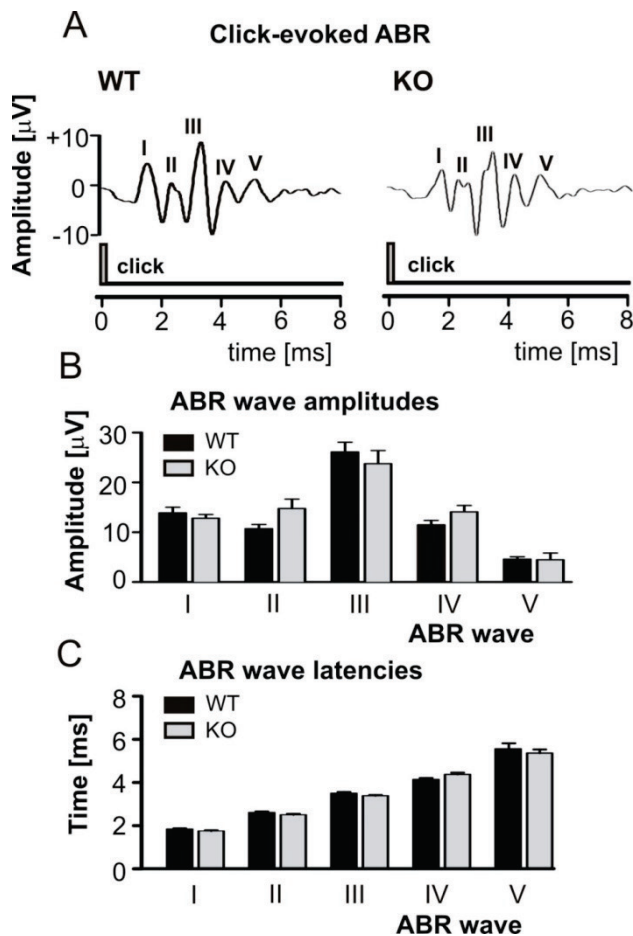


Fig. 5. (A) examples of ABR, evoked by clicks of 70 dB SPL in 2-month-old WT and KO mice. (B) peak-to-peak amplitudes of individual ABR waves in WT and KO mice. (C) peak latencies of individual ABR waves in WT and KO mice. Bars represent \pm SEM.

Temporal resolution ability

The temporal resolution ability was tested using two broad band noise stimuli separated by a gap of variable duration. The schema in Figure 7A represents examples of ABR recording using a gap duration of 2 ms, 5 ms and 25 ms. When gap duration was short, the ABR amplitude as a response to second stimulus (2nd stim.) was very small. The longer gap, the larger the ABR amplitudes to second stimulus were observed. The dependence of the relative

ABR amplitudes to the second stimulus (2nd stim.) with respect to 1st stimulus amplitudes (taken as 100 %) on gap duration, evaluated in WT and KO mice, is demonstrated in Figure 7B. The 2nd stimulus amplitude in WT mice reached the 100 % level of 1st stimulus amplitude at gap duration of 20-25 ms, whereas in KO mice the 2nd stimulus function did not reach the 1st stimulus amplitude at any gap duration. Both curves are significantly different ($p=0.0213$, $F_{(1,11)}=7.204$, two-way ANOVA). These results indicate that the temporal resolution ability was significantly better in WT mice in comparison with *Bral2*^{-/-} KO mice.

Discussion

The results demonstrated significant differences in the hearing function between young adult WT and KO mice. Auditory behavior activity (ASR amplitude, PPI of ASR efficiency) was similar in very young WT and KO mouse pups aged 2 weeks, but differences occurred in subjects older than one month. The differences in hearing function comprise a worse hearing threshold at high frequencies, weaker neural adaptation and poorer temporal resolution ability in 2-month-old KO mice in comparison with age-matched WT mice.

Development of behavioral activity

In the mouse auditory brainstem PNNs are formed during the developmental period, but the adult configuration is not achieved before the end of the first postnatal month (Brückner *et al.* 2000, Lurie *et al.* 1997, Myers *et al.* 2012). In the auditory cortex, the development of PNNs continues up to the 6th postnatal week (Friauf 2000, Koppe *et al.* 1997). Thus, the PNNs are formed after the onset of acoustically-evoked signal processing (i.e. after the postnatal day 12), and might be involved in completing the critical developmental period of hearing (Blatchley *et al.* 1987, Geal-Dor *et al.* 1993, Sonntag *et al.* 2009).

On the basis of these morphological data, we investigated whether the development of PNN formation is also accompanied by changes in behavioral responses to sound using ASR recording. The ASR – a transient motor response to an intense unexpected auditory stimulus – is a primitive behavior observed in all mammals. The primary acoustic startle circuit consists of three serially connected structures: cochlear root neurons, neurons in the caudal pontine reticular nucleus, and motoneurons in the medulla and spinal cord (Davis 1984, Koch 1999, Gomez-Nieto *et al.* 2014). In spite of its relative simplicity, the startle reflex shows several forms

of behavioral plasticity including habituation and sensitization, and reveals remarkable sensitivity to a variety of experimental manipulations (Ison and

Hammond 1971, Young and Fechter 1983, Rybalko *et al.* 2011, Rybalko *et al.* 2012, Rybalko *et al.* 2015, Popelář *et al.* 2013).

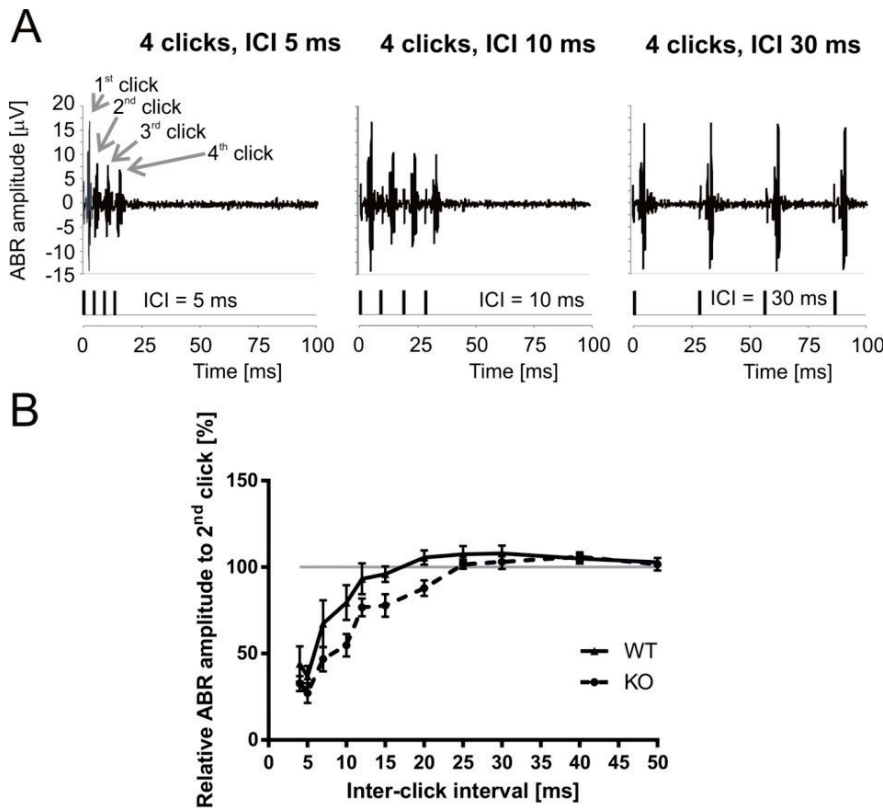


Fig. 6. (A) examples of ABR recordings to stimulation with series of 4 clicks with inter-click intervals (ICI) of 5 ms, 10 ms and 30 ms. (B) the dependence of the relative ABR wave III amplitudes to the 2nd click with respect to the ABR wave III amplitudes to the 1st click (taken as 100 %) on ICI, evaluated in WT and KO mice. Bars represent \pm SEM.

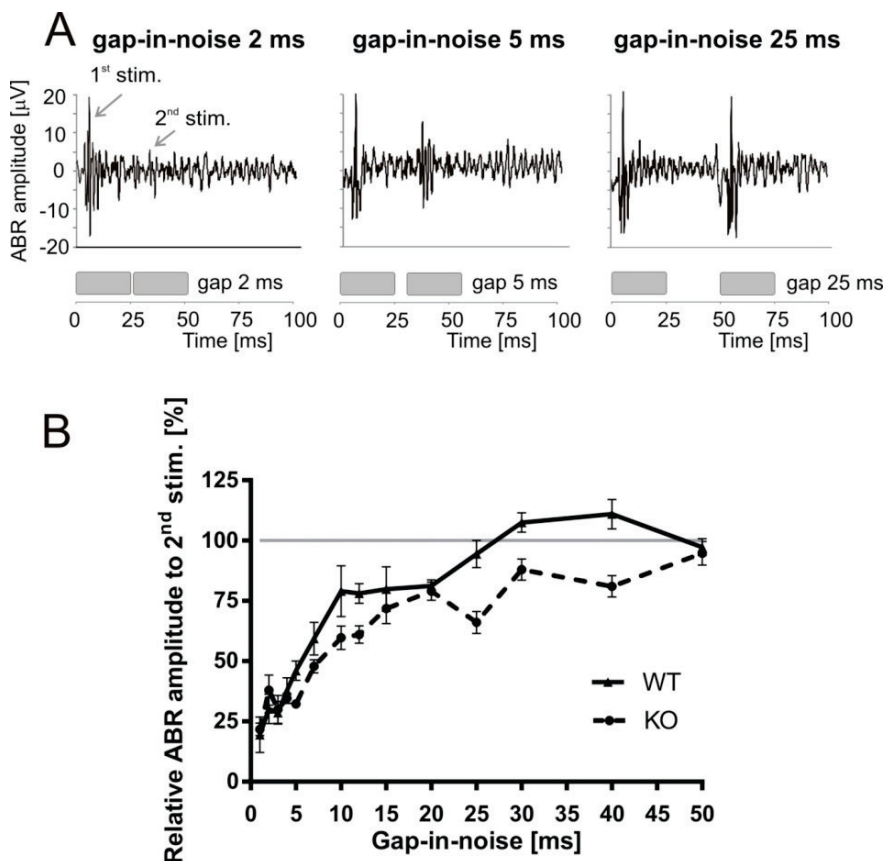


Fig. 7. (A) examples of ABR recordings to stimulation using two broad band noise stimuli separated by a gap with duration of 2, 5 and 25 ms, (B) the dependence of the relative ABR wave III amplitudes to the 2nd stimulus with respect to the ABR wave III amplitudes evoked by 1st stimulus (taken as 100 %) on gap duration, evaluated in WT and KO mice. Bars represent \pm SEM.

Modulation of the ASR by prepulse inhibition (the PPI of ASR), i.e. the inhibition of the ASR induced by the presentation of an acoustic stimulus shortly preceding the startling sound, is frequently used to investigate the auditory function. The PPI phenomenon is considered an example of sensori-motor gating, which reflects inhibitory processes that regulate sensory input to the brain. Results of animal studies have suggested that the effect of auditory prepulse stimulus on the startle circuit involves primarily the cochlear nucleus, the inferior and superior colliculus and the pedunculopontine tegmental nucleus (Koch 1999, Swerdlow *et al.* 2001). The PPI can be modulated by the auditory cortex, thalamus, amygdala, hippocampus, striatum, ventral pallidum, and globus pallidum (Swerdlow *et al.* 2001). It was shown that evaluating the PPI of ASR is a simple yet efficient method for estimating supra-threshold auditory sensitivity (Fitch *et al.* 2008, Young and Fechter 1983, Carlson and Willott 1996, Ison and Allen 2003, Popelář *et al.* 2013). We demonstrated in our experiments that the ASR amplitude and the efficiency of the PPI of ASR measured in 2-week-old WT and KO mice are almost identical. While the parameters of the PPI of ASR in KO mice did not change during the later postnatal period (from one month to two months), the efficiency of the PPI of ASR measured in one-month-old WT mice ameliorated significantly. This result corresponds with the hypothesis that the formation of PNNs in auditory structures is completed by the end of the first postnatal month. Further improvement of startle activity can correspond with the delayed closure of PNN development in the auditory cortex and subcortical structures (Friauf 2000, Koppe *et al.* 1997). In KO mice the PNNs are not formatted properly and some parameters of hearing function remained undeveloped. Because mainly inhibitory GABAergic neurons are surrounded by PNNs (Härtig *et al.* 1992), the weaker efficiency of the PPI of the ASR in KO mice can be associated with the altered function of GABAergic neurons in auditory structures.

The ASR amplitudes in KO mice increased during the two-month postnatal period, but remained significantly smaller in comparison with age-matched WT mice. Previous studies demonstrated that Bral2 protein is present in the auditory brainstem and cerebellum (Bekku *et al.* 2003, Bekku *et al.* 2012, Carulli *et al.* 2006). Since the ASR represents a sensory-motor reflex, and because the activity of motoneurons as the effectors of the ASR pathways can be modulated by cerebellum (Siegert and Klingberg 1971), the smaller

ASR amplitudes in KO mice can be at least partly caused by altered modulatory signal from the cerebellum due to disorganized PNNs in this structure.

ABR threshold, temporal resolution ability

Results of previous studies suggested that PNNs serve as a buffer system for cations such as potassium or sodium close to the neuron membrane (Härtig *et al.* 1999). Such a function could be of importance for neurons that fire with high spike rates. These neurons can process high-frequency signals or enable the processing of signals requiring higher temporal resolution. The structural malformations of PNNs in KO mice in our experiments are reflected in higher hearing thresholds at high frequencies in comparison with WT mice. Similar results, i.e. substantial differences in the shape of excitatory response areas of medial nucleus of the trapezoid body principal neurons in brevicin-deficient mice and a prominent reduction of hearing thresholds at high frequencies, were reported by Blosa *et al.* (2015).

Our study demonstrated that temporal resolution ability and neural adaptation were significantly weaker in 2-month-old KO mice in comparison with age-matched WT mice. These results confirm the hypothesis that the ability of fast spiking is seriously reduced in neurons with altered PNNs (Härtig *et al.* 1999).

The stability of PNNs is important not only for the proper function of individual neurons, but also for the properties of the extracellular space. Previous studies using the real-time iontophoretic method showed that qualitative and quantitative changes in extracellular matrix might evoke changes in the diffusion properties of the extracellular space (Vargová *et al.*, in preparation). The extracellular space volume fraction α ($\alpha = \text{extracellular space volume} / \text{total tissue volume}$) and the geometrical factor tortuosity λ ($\lambda^2 = \text{free} / \text{apparent diffusion coefficient}$) were measured in coronal brain slices obtained from the sensorimotor cortex and the ventral posteromedial thalamic nucleus of Bral2-deficient mice. In young adult mice, no significant differences in the extracellular space diffusion parameters between Bral2 positive and negative mice were found in either the cortex or the thalamus. In the thalamus of aged Bral2-deficient mice the extracellular space volume fraction α significantly decreased in comparison to the younger Bral2-deficient animals as well as to age-matched controls. A decrease in the extracellular space volume fraction α in the thalamus of aged Bral2-deficient mice may result from an age-related

disruption of the PNNs, that may affect the movement of neuroactive substances in the central nervous system in aged animals (Vargová *et al.*, in preparation).

Taken together, our study demonstrated that the absence of PNNs in Bral2-deficient mice results in significant changes in hearing function in comparison with WT mice. While ASR amplitude and the efficiency of the PPI of ASR are similar in very young, 2-week-old WT and KO mice, these behavioral parameters changed in WT mice after the end of the developmental period when normal PNNs are formed, but remained unchanged in KO mice with disrupted PNNs formation. Due to the altered function of mainly fast spiking neurons, 2-month-old KO mice have a higher hearing threshold at high frequencies, weaker neural adaptation and poorer temporal resolution ability in comparison with age-matched WT mice.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

ABR – auditory brainstem response, AP – action potential, ASR – acoustic startle reflex, ICI – inter-click interval, KO – knockout Bral2^{-/-} mice, PNNs – perineuronal nets, PPI – prepulse inhibition, WT – wild type Bral2^{+/+} mice.

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