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Citation of this paper:
Typlt, Marei; Mirkowski, Magdalena; Azzopardi, Erin; Ruth, Peter; Pilz, Peter K D; and Schmid, Susanne, "Habituation of reflexive and motivated behavior in mice with deficient BK channel function." (2013). Brain and Mind Institute Researchers' Publications. 39.
https://ir.lib.uwo.ca/brainpub/39
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Habituation of reflexive and motivated behavior in mice with deficient BK channel function

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Habituation is considered the most basic form of learning. It describes the decrease of a behavioral response to a repeated non-threatening sensory stimulus and therefore provides an important sensory filtering mechanism. While some neuronal pathways mediating habituation are well described, underlying cellular/molecular mechanisms are not yet fully understood. In general, there is an agreement that short-term and long-term habituation are based on different mechanisms. Historically, a distinction has also been made between habituation of motivated versus reflexive behavior. In recent studies in invertebrates the large conductance voltage- and calcium-activated potassium (BK) channel has been implicated to be a key player in habituation by regulating synaptic transmission. Here, we tested mice deficient for the pore forming α-subunit of the BK channel for short-term and long-term habituation of the acoustic startle reflex (reflexive behavior) and of the exploratory locomotor behavior in the open field box (motivated behavior). Short-term habituation of startle was completely abolished in the BK knock-out mice, whereas neither long-term habituation of startle nor habituation of motivated behavior was affected by the BK deficiency. Our results support a highly preserved mechanism for short-term habituation of startle across species that is distinct from long-term habituation mechanisms. It also supports the notion that there are different mechanisms underlying habituation of motivated behavior versus reflexive behavior.

Keywords: BK channel, sensorimotor gating, habituation, locomotion, startle

INTRODUCTION

The brain constantly receives a vast amount of sensory information. In order to be able to extract salient information and respond appropriately, it is necessary to suppress repetitive non-informative input. One important sensory filtering mechanism responsible for suppression is habituation. Habituation describes the progressive decrease of a behavioral response to repetitive non-threatening sensory stimuli. It is considered to be the most basic form of learning and allows to ignore irrelevant stimuli in favor of relevant stimuli (Poon and Young, 2006). It is further believed to be a prerequisite for other learning forms (Rankin et al., 2009). In humans, disruption of habituation is strongly correlated with cognitive impairments. This was found in patients with mental disorders like schizophrenia (Geyer and Braff, 1982; Weber et al., 2002; Simons-Weidenmaier et al., 2006; Gover and Abrams, 2009). However, previous data have also shown that a common cause of synaptic depression, namely vesicle depletion, is unlikely to contribute substantially to the synaptic depression underlying habituation (Castellucci and Kandel, 1974; Byrne, 1982; Weber et al., 2002); the cause for synaptic depression therefore remains elusive. Recently, it has been shown that a loss-of-function mutation of the large conductance voltage- and calcium-activated potassium (BK) channel impairs short-term habituation of an escape response in Drosophila (Engel and Wu, 1998). BK channels are expressed throughout the mammalian nervous system (Knaus et al., 1996; Wanner et al., 1985; Sausuber et al., 2003).
We used mice of the F1 generation of a hybrid SV129/C57BL6. We only tested mice at ages from 3 to 5 months to avoid effects of inbreeding. The animals were litter- and/or age matched. We hypothesized that BK channel deficient mice show a disruption of short-term habituation of the startle response, corresponding to findings in Drosophila and Caenorhabditis elegans. We then tested whether BK channel knock-out mice also show disruptions in long-term habituation of startle. Finally, we analyzed whether BK channels play a role in habituation of motivated behavior, measuring exploratory behavior of BK channel knock-out mice in the locomotor box.

MATERIALS AND METHODS

ANIMALS AND ANIMAL CARE

We used mice of the F1 generation of a hybrid SV129/C57BL6 line with a deficient BK channel function bred at University of Tübingen. The BK channel function was abolished by deleting the slo1 gene (accession ID: AA349225.1) which encodes the pore forming channel protein (α-subunit, for details about generation of mice and genotyping please see supporting information in Sausbier et al., 2004). Heterozygous C57Bl6 mice were paired with heterozygous SV129 mice. Exclusively mice of the respective F1 generation, wild-type (WT), heterozygous (BKα+/−), and homozygous knock-out (BKα−/−), were tested in order to avoid effects of inbreeding. The animals were litter- and/or age matched. We only tested mice at ages from 3 to 5 months to avoid effects of aging.

All mice were generated and genotyped at the Pharmaceutical Institute, University of Tübingen, Germany. They were ear-tagged and shipped to Canada at the age of 1.5–3 months and subsequently quarantined and allowed to acclimate for 2 weeks before behavioral testing started. Mice were group housed with mixed genetic background within groups, with a 12 h light–dark cycle and with ad libitum food and water. Tail’s were marked according to their ear-tags for easy identification. Behavioral testing occurred during the light cycle. After all behavioral testing was finished, mice were sacrificed, and genotype was once more verified, comparing the ear-tags, shipping list, and tail marks.

All procedures were in accordance with the ethical guidelines of the Canadian Council on Animal Care (CCAC) and approved by the University of Western Ontario Animal Use Subcommittee.

ACOUSTIC STARTLE REFLEX

Reflexive behavior was measured using the acoustic startle reflex. 18 WT (10 males/8 females), 17 BKα−/− (10/7), and 19 BKα−/− mice (9/10) were tested as described previously (Geyer and Swerdlow, 2001; Valsamis and Schmid, 2011). Testing was conducted in sound attenuated startle boxes from MED Associates (MED-ASR-PR01, St Albans, VT, USA), where animals were placed into small enclosures mounted on a movement sensitive platform within a sound attenuated chamber. A piezoelectric transducer mounted below the platform converted vertical movements of the platform induced by startle responses of the mouse into a voltage signal. The maximum amplitude (positive peak to negative peak) of the signal was measured in a 100 ms time window after the acoustic stimulus onset, using the associated software for stimulus presentation and recordings (see Figure 1; Startle Reflex version 6.0, MED Associates, Inc.).

Before the actual testing the animals were acclimatized to the startle boxes for 5 min on three consecutive days. The duration of the test sessions was 2.5 min. During the acclimation period only the background noise (65 dB sound pressure level, SPL, white noise) was presented. On the third day, acclimation was followed by a short input/output (I/O) test to determine the appropriate gain setting for amplifying the voltage signal of the transducer for each individual animal, so that a large portion of the dynamic range of the startle system was used for measuring startle in each animal: the I/O test consisted of 12 startle stimuli with increasing intensity starting at 65 dB SPL and increasing by 5 dB SPL each trial to 120 dB SPL (20 ms duration, white noise, every 20 s). The stimulus level was presented on top of the background noise. The gain was set so that the maximum startle amplitude would cover a large portion of the dynamic range and the gain was subsequently kept constant for all recordings of a given animal. The absolute startle response amplitude was later corrected for the gain factor.

On the next five consecutive days the animals were tested using the following protocol: the animals were acclimatized to the startle box and the background noise for 5 min. Subsequently, the startle stimulus (20 ms, 105 dB SPL white noise) was presented 100 times with varying inter-trial intervals (10–20 s) on top of the background noise. Recordings started 50 ms before the stimulus was given and lasted for a total of 300 ms. To account for the muscular tremor occurring in the BKα−/− mice, we subtracted the peak-toperk transducer displacement during the phase before the startle. 

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** Acoustic startle response measurement. One acoustic startle response, measured by the Med Associates startle box, of a BKα−/− mouse (A), and of a wild-type animal (B): both recorded with a transducer signal gain of 1. The red line shows the voltage signal produced by the transducer that reflects the vertical displacement of the platform by the animals’ movement, and the short horizontal black bar represents the startle stimulus. The blue arrow indicates the peak-to-peak amplitude measured by the system. The black diamonds indicate the baseline noise generated by the knock-out mouse’s tremor that was subtracted from the total startle amplitude.
OPEN FIELD LOCOMOTOR ACTIVITY
Motivated behavior was measured using open field locomotor activity that reflects exploratory behavior. Locomotor activity was measured in 16 mice of each genotype (WT: nine males/seven females, BKα+/−: 10/6, BKα−/−: 9/7). Each animal was placed in a squared (40 cm × 40 cm) open field box (Versamax animal activity monitor, Accuscan Instruments, Columbus, OH, USA) in a dimly lit room for five consecutive days and was allowed to explore freely for 2 h. In order to assess habituation, the distance traveled during these 2 h was analyzed in 5 min blocks using the VersaMaxTM software (Accuscan Instruments).

RESULTS
HABITUATION OF REFLEXIVE BEHAVIOR
The startle reflex to a sudden acoustic stimulus significantly decreased in the WT and BKα+/− mice across testing trials within a session, but not in the BKα−/− mice (Figure 2). A repeated measurement ANOVA (genotype × gender × trials) on amplitudes normalized to the first five trials (Figure 3A) reported a main effect for trial ($F_{(2,5),22.87,571.75}=5.58$, $p<0.001$), as well as a significant difference between genotypes ($F_{(2,5)}=8.57$, $p=0.001$) and an interaction between both ($F_{(2,5),22.87,571.75}=1.74$, $p=0.018$). A post hoc test confirmed that habituation of the startle amplitudes in the BKα+/− mice were significantly different from that of their WT littermates ($p<0.001$).

In order to quantify the amount of habituation occurring, we calculated the short-term habituation scores as the ratio between the average of the last five and the first five trials. The scores of the heterozygote BKα+/− mice across testing trials were significantly different from either ($p=0.119$, $p=0.009$, respectively). The scores of the heterozygote BKα+/− mice fell between the scores of WT and BKα−/− mice and were not significantly different from either ($p=0.737$, $p=0.119$, respectively). Figure 3B shows the median habituation scores in a box-whisker plot in order to give an idea about the distribution of scores across animals.

Notably, the absolute startle amplitudes were significantly lower in the BKα−/− mice (average $148±9$ mV SE) compared to their WT litter mates ($273±30$ mV SE). A two-way ANOVA on the absolute startle amplitudes resulted in a significant genotype effect ($F_{(2,20)}=6.57$, $p=0.003$) and post hoc analysis showed that the difference was between the WT and the BKα−/− mice ($p=0.001$).
HABITUATION OF MOTIVATED BEHAVIOR

Locomotor activity as a measure for exploratory behavior was assessed in a locomotor box (Crawley and Naylor, 1997). Within the 2 h test in the open field box all animals habituated to the environment, leading to a strong decline in locomotion (Figure 5A). A repeated measurement ANOVA (genotype × gender × time) on normalized data reported an effect of day ($F_{(6,42)} = 9.56$, $p < 0.001$), but no significant effect of the genotype ($F_{(2,41)} = 2.43$, $p = 0.101$), nor a genotype × day interaction ($F_{(12,41)} = 1.236$, $p = 0.281$, Figure 5A). Accordingly, also the long-term habituation scores were not significantly different between genotypes ($F_{(2,42)} = 0.785$, $p = 0.463$, Figure 4B).

Also the habituation across days did not significantly differ between genotypes for the locomotor behavior. The respective repeated measurement ANOVA (genotype × gender × day) only showed an effect for the day ($F_{(6,42)} = 7.93$, $p < 0.001$), but not for genotype ($F_{(2,41)} = 2.72$, $p = 0.078$). There also was no genotype × day interaction ($F_{(12,41)} = 1.067$, $p = 0.39$, Figure 4A). The long-term habituation scores were also statistically not significantly different between the genotypes ($F_{(2,41)} = 2.41$, $p = 0.102$, Figure 4B).

see Figure 3C. Still, the startle amplitudes of the BKα−/− mice were well above the noise level, which was at 43 ± 1 mV SE.

Across 5 days of testing the initial startle amplitude increased in all animals suggesting that they sensitized to the stimulus between sessions rather than habituated. In order to account for the difference in baseline startle, amplitudes of the first block of each day were normalized to the first block of day 1 for each genotype. A repeated measurement ANOVA (genotype × gender × day) on normalized data reported an effect of day ($F_{(6,42)} = 9.56$, $p < 0.001$), but no significant effect of the genotype ($F_{(2,41)} = 2.43$, $p = 0.101$), nor a genotype × day interaction ($F_{(12,41)} = 1.236$, $p = 0.281$, Figure 4A). Accordingly, also the long-term habituation scores were not significantly different between genotypes ($F_{(2,42)} = 0.785$, $p = 0.463$, Figure 4B).

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see Materials and Methods (for detailed description). Data is displayed as median in a box-whisker plot, with whiskers indicating the 95th and 95th percentile. "*p < 0.001. **p = 0.005. ***p < 0.001. In (B) the habituation scores of the respective genotypes are displayed (first five versus last five responses,
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FIGURE 5 | Short-term habituation of open field locomotor activity. 
(A) Habituation of exploratory behavior was measured in a locomotor box. 
Within the 2 h test in the open field box all animals habituated to the 
environment, leading to a strong decline in locomotion. Figure shows 
average values and standard errors. 
(B) All genotypes had a similar 
habituation score of around 0.2 (displayed as median and whiskers 
indicating the 5th and 95th percentile of first five versus last five time bins, 
see Materials and Methods). 
(C) Distance traveled (median, with whiskers indicating the 5th and 95th percentile) was also not 
significantly different between the genotypes. 
WT (16 WT, nine males/seven females), 10 BKα+/- (10/6), and 16 
BKα−/- (9/7) were tested.

FIGURE 6 | Long-term habituation of open field locomotor activity. 
(A) Changes in average locomotion across days showed no 
statistically significant difference between genotypes. Error bars 
indicate standard errors. 
(B) The long-term habituation scores (median, with whiskers indicating the 5th and 95th percentile) were also not 
significantly different between the genotypes. Knock-out animals show 
a slightly higher score (less long-term habituation), which is mainly 
due to an initial sensitization on day 2, followed by a decline.

In summary, our study reveals and impact of deficient BK 
channel function on short-term habituation of the acoustic star-
tle response, but not on short-term habituation of exploratory 
behavior, nor on long-term habituation.

DISCUSSION

The present study shows a lack of short-term habituation of 
startle responses in BK channel knock-out mice, indicating a cru-
icial role for BK channels in short-term habituation of reflexive 
responses, but no alterations in long-term habituation of startle 
or in habituation of exploratory behavior.

LACK OF SHORT-TERM HABITUATION OF STARTLE IN BKα−/- MICE

Our data clearly show that the BKα−/- mice we used startled 
in response to a sound stimulus and that no habituation of this 
startle occurred. Several difficulties had to be considered 
in experimental design: it has been shown that a BK channel 
deficiency can alter locomotion (Sausbier et al., 2004) and hear-
ing (Rüttiger et al., 2004; Oliver et al., 2006; Pyott et al., 2007; 
Kurt et al., 2012) which could affect the acoustic startle mea-
sures (as well as exploratory behavior in the locomotor box). 
We therefore used a F1 hybrid mouse in present study that 
has no hearing impairment in the relevant frequency spectrum 
(Typlt et al., 2013). Still, BKα−/- mice showed a lower baseline 
startle response than their WT siblings. It is difficult to determine 
if this is due to lower body weight in BKα−/- mice, lower anxiety 
levels, or motor impairments. We accounted for the lower baseline 
startle amplitude by normalizing the data of each mouse to its star-
tle amplitude in the first trials. However, lower startle responses 
in general may influence the amount of habituation. The major 
concern is a floor effect, i.e., that startle response amplitude may 
not be sufficiently different from a general noise level and can 
therefore not be further reduced. However, the noise level in our 
experiments was still considerably lower than startle amplitudes 
of BKα−/- mice. Furthermore, we subtracted the noise caused by 
tremor in knock-out mice so that it does not influence habitua-
tion scores. It also has been shown in the same BKα−/- mice that 
startle responses are substantially reduced by prepulse inhibition 
(Typlt et al., 2013), indicating that there is still enough room for 
a substantial reduction of startle, which makes it unlikely that a 
floor effect accounts for the lack of habituation. We also looked at 
habituation exclusively in WT low startler with a baseline startle 
response comparable to BKα−/- mice. Although the number of WT 
low startler is too small to statistically analyze the data (n = 5), 
they all have habituation scores well below 1 (data not shown). We 
are therefore confident that there is a true lack of habituation in 
BKα−/- mice.

POSSIBLE ROLE OF THE BK CHANNELS IN SHORT-TERM HABITUATION 
OF STARTLE

It has been proposed that a calcium-dependent presynaptic 
depression mechanism in sensorimotor synaptic terminals within
the primary startle pathway mediate short-term habituation of startle (Weber et al., 2002; Simons-Weidenmaier et al., 2006; Gover and Abrams, 2009). BK channels that are activated by depolarization and intracellular calcium drive the membrane potential towards the potassium equilibrium potential and therefore re- and hyperpolarize the neuron (Kaczorowski et al., 1996; Vergara et al., 1998; Poulos and Johnston, 1999; Hu et al., 2001). By limiting the duration of action potentials, they regulate the general excitability of neurons (Shao et al., 1999; Nelson et al., 2003; Brenner et al., 2005), as well as limit the transmitter release at presynaptic terminals (Robitaille and Charlton, 1992; Robitaille et al., 1993; Hu et al., 2001; Raffaelli et al., 2004; Wang, 2008). They co-localize with voltage-gated calcium channels at the active synaptic zone, establishing a link between intracellular free calcium and neurotransmitter release in synaptic terminals (Robitaille et al., 1993; Yazejian et al., 1997; Saier et al., 2006). All these described properties make BK channels excellent candidates for mediating calcium-dependent synaptic depression in the startle pathway thereby causing habituation to repetitive strong stimuli.

Short-term habituation lasts for several minutes, whereas intracellular calcium is elevated in synaptic terminals only for milliseconds. So, how is synaptic depression maintained for minutes? BK channels can be phosphorylated by PKA, PKC, and CaMKII (Kaczorowski et al., 1996; Liu et al., 2007; Wang, 2008). The latter has been shown to be enriched in presynaptic terminals (Gorelick et al., 1990; Walaa et al., 1999) and to act as a strong regulator of synaptic strength and plasticity (Wang, 2008). CaMKII is activated by elevations of intracellular calcium and can auto-phosphorylate upon large calcium accumulation. Auto-phosphorylation leads to a prolonged activity of CaMKII that persists after calcium levels have returned to baseline. The prolonged activity of CaMKII leads to a prolonged phosphorylation of BK channels and therefore to a lasting decrease in synaptic efficacy (Wang, 2008). In fact, this proposed mechanism meets all requirements for a habituation mechanism, such as a presynaptic localization, calcium dependence, and the reversibility of the phosphorylation process within a timescale of several minutes.

Short-term habituation of startle in rodents has been shown to be mediated at the sensorimotor synapse in the pontine reticular formation where synaptic depression occurs upon repeated strong stimulation (Davis et al., 1982; Lingnhoß and Fraunz, 1992, 1994; Weber et al., 2002; Simons-Weidenmaier et al., 2006). Since the phosphorylation of BK channels requires a strong activation as described above, they are likely to mediate synaptic depression at this synapse, however, future electrophysiological experiments have to confirm this. It will also be intriguing to see in the future to what extend habituation of other reflexive behaviors depend on BK channel activation.

**POTENTIAL ROLE OF THE BK CHANNELS IN HABITUATION OF MOTIVATED BEHAVIOR**

BK channels can potentially control transmitter release at any kind of synapse regardless of the type of neurotransmitter released. Furthermore, BK channels are expressed throughout the nervous system, so it could be hypothesized that their activation represents a universal mechanism for habituation. In our study, however, we found an effect of a functional BK channel deficiency only for short-term habituation of startle and not for long-term habituation nor habituation of motivated behavior. In fact, habituation of motivated behavior has been previously suggested to be based on separate mechanisms to habituation of reflexive behavior (Williams et al., 1974, 1975; Brown, 1976). Moreover, the proposed BK channel mechanism is unlikely to be able to mediate motivated behavior since in contrast to reflexive behavior there is no strong eliciting input for motivated behavior which could trigger the phosphorylation of CaMKII. Thus, a different mechanism is likely to account for habituation of motivated behavior.

**POTENTIAL ROLE OF THE BK CHANNELS IN LONG-TERM HABITUATION**

Long-term habituation of startle has been shown to be located extrinsically to the primary startle pathway and involves the cortex and the cerebellar vermis (Leaton and Supple, 1986, 1991; Lopiano et al., 1990) and potentially cholinergic mechanisms (Schmid et al., 2011). It has been hypothesized to be an associative learning process. Since associative learning is affected by a lack of BK channel function (Matthews and Disterhoft, 2009; Typlt et al., 2013) we would have expected to see an effect of the BK channel deficiency on long-term habituation of startle as well. Unfortunately, neither WT- nor BK-deficient animals really showed long-term habituation of startle, which is common for many mouse strains. This makes it difficult to assess any differences between genotypes. The lack of statistically significant differences between genotypes in long-term habituation testing of both startle and locomotor behavior does therefore not completely rule out that there is a potential contribution of BK channels, for instance through their impact on associative learning.

**CONCLUSION**

The results of this study show that BK channel activation is necessary for short-term habituation of startle. It demonstrates that this mechanism underlying short-term habituation is highly preserved throughout evolution, since BK channel-dependent short-term habituation of a startle-like response has been found in *C. elegans* (C. Rankin, personal correspondence) and *Drosophila* (Engel and Wu, 1998). Additionally, genetic alterations of BK channel function has been implicated in different disorders in humans that are associated with short-term habituation deficits in startle, e.g., in schizophrenia (for review see Zhang et al., 2006), mental retardation (Higgins, 2008; Deng et al., 2013), and autism (Laumonnier et al., 2006). Furthermore, the fragile-x related protein, which is impacted in a specific form of autism in humans that is associated with a disruption of habituation has recently shown in mice to directly regulate BK channel activity (Deng et al., 2013). This highly preserved function of BK channels in habituation goes well with the notion of the importance of intact habituation for sensory filtering and higher cognitive function.

BK channels do not seem to play a role in short-term habituation of motivated behavior, and we could not find any evidence for a role in long-term habituation. This supports the idea that there is no universal habituation mechanism, but probably a variety of different mechanisms mediating habituation of different behaviors and at different time scales, as previously proposed (Williams et al., 2013).
et al., 1974, 1975; Davis, 1984; Leaton et al., 1983; Weber et al., 2002; Schmid et al., 2010).

**ACKNOWLEDGMENTS**

The study was supported by the Canadian Institute for Health Research (CIHR) and the Natural Sciences and Engineering Council (NSERC).

**AUTHOR CONTRIBUTIONS**

Susanne Schmid conceptualized the study, consulting with Peter Ruth and Peter Pilz. Peter Ruth created the knock-out mice. Peter Pilz, Marei Tüpt, and Magdalena Mirkowski conducted experiments and analyzed data. Marei Tüpt ran the tests, wrote the first draft of the manuscript, and made the figures. All authors revised the manuscript. Susanne Schmid finalized the manuscript and submitted it. Marei Tüpt and Magdalena Mirkowski have contributed equally to the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 August 2013; accepted: 29 October 2013; published online: 19 November 2013

Citation: Typlt M, Mirkowski M, Azzopardi E, Ruth P, Pilz PKD and Schmid S (2013) Mice with deficient BK channel function show impaired prepulse inhibition and spatial learning, but normal working and spatial reference memory. Front. Integr. Neurosci. 7:38. doi: 10.3389/fnint.2013.00038

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