International Journal of Neuropsychopharmacology (2010), **13**, 499–513. Copyright © CINP 2009
doi:10.1017/S1461145709990368

ARTICLE

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$Ca_V1.3$ L-type Ca^{2+} channels modulate depression-like behaviour in mice independent of deaf phenotype

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Abstract

Mounting evidence suggests that voltage-gated L-type Ca^{2+} channels can modulate affective behaviour. We therefore explored the role of $Ca_V1.3$ L-type Ca^{2+} channels in depression- and anxiety-like behaviours using $Ca_V1.3$-deficient mice ($Ca_V1.3^{-/-}$). We showed that $Ca_V1.3^{-/-}$ mice displayed less immobility in the forced swim test as well as in the tail suspension test, indicating an antidepressant-like phenotype. Locomotor activity in the home cage or a novel open-field test was not influenced. In the elevated plus maze (EPM), $Ca_V1.3^{-/-}$ mice entered the open arms more frequently and spent more time there indicating an anxiolytic-like phenotype which was, however, not supported in the stress-induced hyperthermia test. By performing parallel experiments in Claudin 14 knockout mice (*Cldn14*$^{-/-}$), which like $Ca_V1.3^{-/-}$ mice are congenitally deaf, an influence of deafness on the antidepressant-like phenotype could be ruled out. On the other hand, a similar EPM behaviour indicative of an anxiolytic phenotype was also found in the *Cldn14*$^{-/-}$ animals. Using electroretinography and visual behavioural tasks we demonstrated that at least in mice, $Ca_V1.3$ channels do not significantly contribute to visual function. However, marked morphological changes were revealed in synaptic ribbons in the outer plexiform layer of $Ca_V1.3^{-/-}$ retinas by immunohistochemistry suggesting a possible role of this channel type in structural plasticity at the ribbon synapse. Taken together, our findings indicate that $Ca_V1.3$ L-type Ca^{2+} channels modulate depression-like behaviour but are not essential for visual function. The findings raise the possibility that selective modulation of $Ca_V1.3$ channels could be a promising new therapeutic concept for the treatment of mood disorders.

Received 6 April 2009; Reviewed 8 May 2009; Revised 26 June 2009; Accepted 7 July 2009; First published online 11 August 2009

Key words: L-type Ca^{2+} channels, $Ca_V1.3$ α_1-subunits, depression, deafness, retinal function.

Introduction

Mood and anxiety disorders have a high lifetime prevalence and represent a growing health and economic problem (Andlin-Sobocki *et al*. 2005). Despite different effective approaches including drug therapy, the majority of patients continue to experience residual symptoms and some patients are refractive to therapy. An improved understanding of the neurobiology of depression and anxiety disorders is thought to ultimately lead to novel treatments, which may act on entirely different targets as currently licensed drugs (Harro, 2006; Holtzheimer & Nemeroff, 2006; Millan, 2006; Pillay & Stein, 2007).

Calcium influx through voltage-gated Ca^{2+} channels controls a variety of cellular events in electrically

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excitable cells (Catterall *et al*. 2005; Striessnig & Koschak, 2008). L-type calcium channels (LTCCs) are characterized by their high sensitivity to organic calcium channel blockers, such as dihydropyridines (DHPs), and the inhibition of LTCCs in the cardiovascular system underlies the therapeutic effects of these drugs to treat cardiovascular diseases (Basile, 2004).

$Ca_V1.2$ and $Ca_V1.3$ have a broad and overlapping expression profile in the mammalian neuronal system (Hell *et al*. 1993; Sinnegger-Brauns *et al*. 2008). Interestingly, these predominant isoforms are present in brain areas implicated in mood and anxiety (e.g. hippocampus, amygdala, prefrontal cortex) (Hell *et al*. 1993, Ludwig *et al*. 1997). Both can couple electrical activity to transcriptional regulation (Deisseroth *et al*. 2003; Moosmang *et al*. 2005; Zhang *et al*. 2006). $Ca_V1.3$ channels activate at more negative voltages enabling them to modulate neuronal firing behaviour and to serve pacemaker function in neurons (Chan *et al*. 2007; Olson *et al*. 2005). Their important role for neuronal signalling suggests that their pharmacological modulation could also affect brain function. Therefore these channels may represent potential new therapeutic targets for the treatment of central nervous system (CNS) disorders.

Convincing evidence for the involvement of different LTCC isoforms for brain function has been obtained in studies employing $Ca_V1.2$ (Moosmang *et al*. 2005), $Ca_V1.3$ knockout mice ($Ca_V1.3^{-/-}$; Platzer *et al*. 2000) and mutant mice in which high DHP sensitivity of $Ca_V1.2$ channels was eliminated allowing selective modulation of $Ca_V1.3$ channels with DHPs ($Ca_V1.2DHP^{-/-}$ mice; Sinnegger-Brauns *et al*. 2004). $Ca_V1.2$ channels are required for spatial hippocampal memory and $Ca_V1.3$ channels participate in long-term fear memory and drug-taking behaviour in mice (for review see Striessnig & Koschak, 2008). The role of LTCCs in mood and anxiety behaviour is less clear, although a number of *in-vivo* studies do suggest a role for these channels. In rodents, systemic application of high doses of DHPs induces antidepressant- (Sinnegger-Brauns *et al*. 2004 and references therein) and anxiolytic-like behaviours (El Ganouni *et al*. 1998; Matsumoto *et al*. 1994; Soubrie, 1989). In $Ca_V1.2DHP^{-/-}$ mice antidepressant-like effects of systematically administered DHPs were lost, proving involvement of $Ca_V1.2$ channels for antidepressant-like behaviour (Sinnegger-Brauns *et al*. 2004). However, it remains unclear if these CNS effects of systemically applied DHPs result from peripheral (i.e. cardiovascular) rather than direct central effects on $Ca_V1.2$ channels (Busquet *et al*. 2008; Waltereit *et al*. 2008). Using $Ca_V1.2DHP^{-/-}$ mice we also obtained indirect evidence for a role of $Ca_V1.3$ in emotional behaviour. In these mice systemic application of the DHP channel activator BAYK 8644 selectively promoted calcium entry through $Ca_V1.3$ channels (Sinnegger-Brauns *et al*. 2004). It induced depression-like behaviour with neuronal activation of several brain regions involved in emotional processing (Hetzenauer *et al*. 2006; Sinnegger-Brauns *et al*. 2004). This provides evidence for a possible role of $Ca_V1.3$ channels in mood behaviour.

Here we study the role of $Ca_V1.3$ channels for anxiety- and depression-like behaviours employing $Ca_V1.3^{-/-}$ mice. They exhibit resting bradycardia and arrhythmia (Platzer *et al*. 2000) but this phenomenon disappears during physical activity, does not compromise motor function or spontaneous activity (Clark *et al*. 2003), and is not expected to interfere with behavioural testing. However, $Ca_V1.3^{-/-}$ mice are congenitally deaf and it is so far unclear how deafness affects emotional behaviour in mice. We therefore used another mouse model suffering from congenital deafness, Claudin 14-deficient mice ($Cldn14^{-/-}$; Ben-Yosef *et al*. 2003) as a control to address this question. These mice appeared suitable for this purpose because they suffer from congenital hearing loss but do not exhibit evidence for vestibular dysfunction or any other histopathological and functional changes interfering with behavioural studies (Ben-Yosef *et al*. 2003). As $Ca_V1.3$ channels are expressed in the retina (Kamphuis & Hendriksen, 1998; Morgans, 1999) we also investigated $Ca_V1.3^{-/-}$ mice for possible disturbances in retinal morphology and visual function that could interfere with behavioural analysis.

Method

Animals

Male homozygous ($Ca_V1.3^{-/-}$) or heterozygous ($Ca_V1.3^{+/-}$) $Ca_V1.3$ knockouts were backcrossed for at least five generations into a C57BL/6N background (Platzer *et al*. 2000). Behavioural experiments were performed on $Ca_V1.3^{-/-}$, $Ca_V1.3^{+/-}$ and wild-type (WT) mice using littermates from heterozygous breeding. Claudin 14-deficient ($Cldn14^{-/-}$) mice were bred on a C57BL/6J background (Ben-Yosef *et al*. 2003). All mice used for behavioural experiments were group-housed and were tested during their passive phase. All animals were bred under identical conditions in the same animal facility, were aged 3–6 months and weighed 20–30 g at the time of experiments.

Procedures were approved by the Austrian Ethical Committee on Animal Care and Use in compliance with international laws and policies.

At least 24 h prior to behavioural testing, mice were allowed to habituate to the testing room with water and food available *ad libitum*. All tests were performed between 09:00 and 15:00 hours except for the home-cage activity which was assessed continuously over several days. Test environments were thoroughly cleaned between test sessions. To decrease the number of animals used, behavioural experiments were conducted with the same animals starting with the least invasive test. The elevated plus maze (EPM) and open-field tests were performed with a 5-d inter-test interval. Open-field test and tail suspension test (TST) had a 15-d inter-test interval during which time the Morris water maze test was performed 9 d after the open-field test. The forced swim test (FST) was subsequently performed with a 6-d inter-test interval with TST. Home-cage activity recordings and stress-induced hyperthermia were each performed in separate batches of animals. All the tests revealing significant differences were performed at the beginning of the testing schedule (EPM) or were replicated with naive animals by different experimenters leading essentially to the same results.

Home-cage activity and open-field test were performed as previously described (Singewald *et al*. 2004). The open-field test was performed to assess the locomotor activity as well as anxiety-related behaviour of the animals. The time spent and the number of entries in the central zone, the number of vertical rearings and the overall distance travelled by the mice were monitored during the test duration of 10 min.

FST

The FST was performed as described previously (Singewald *et al*. 2004). Mice were individually placed in a glass cylinder (diameter 11.5 cm, height 24 cm) containing 15 cm fresh tap water maintained at 23–24 °C. All sessions (6 min each) were recorded by a video camera positioned above the cylinders. The duration of immobility throughout the final 4 min of the test was subsequently scored using Eventlog 1.0 (EMCO Software, Iceland) by a trained observer blind to the genotype. Mice were judged immobile when they stopped any movements except those that were necessary to keep their heads above the water level.

TST

The TST was performed essentially as described previously (Steru *et al*. 1985). The illumination on the floor of the table was about 100 lx. The activity of the mice was videotaped and immobility, defined as when the animal hung passively without limb movement, was scored over a 6-min test session by a trained observer blind to the genotype of the mice. Although it is thought that C57BL/6 mice are not an ideal strain to use in the TST as they have a tendency to climb their tail (Mayorga & Lucki, 2001), this phenomenon was not observed in our experiments.

EPM

The EPM test was performed as described previously (Tschenett *et al*. 2003). The apparatus was elevated 73 cm above the floor and exposed to red light (15 lx). At the beginning of each trial, a mouse was placed onto the central area of the maze, facing a closed arm. Mice were tested for a period of 5 min and their movements on the maze were tracked and subsequently analysed by the TSE VideoMot2 system (TSE Technical & Scientific Equipment GmbH, Germany). In addition to the conventional parameters (total arm entries, number of entries and time spent in the open arms), the number of head dips and stretched-attend postures (SAPs; Blanchard *et al*. 1990; Rodgers *et al*. 1992) were assessed.

Stress-induced hyperthermia (*SIH*)

The test procedure of SIH was performed as described previously (Van der Heyden *et al*. 1997). Rectal temperature was measured in each mouse twice, i.e. at $t = 0$ min (T1) and 10 min later (T2) and this procedure was repeated three times, at 09:00, 12:00 and 15:00 hours After the first measurement of temperature the mouse was reallocated to its cage. The difference in temperature (T2 – T1) is considered as SIH. Rectal temperature was measured with an accuracy of 0.1 °C using a digital thermometer DM 852 (Ellab, Denmark) by inserting a glycerol lubricated thermistor probe into the rectum until a stable temperature was measured for 20 s.

Morris water maze visible platform test

The water maze was a circular pool 1.2 m in diameter filled with water maintained at 23–24 °C. A 10-cm diameter escape platform was raised just above the level of the water and a flag was attached to the platform. Mice were gently placed in the pool for three consecutive days to perform four consecutive trials of 60 s every day, each with 90-min inter-trial interval. Each trial started from the same position of the pool and animals had 60 s to reach the platform. A trial ended

as soon as the animal climbed on the platform and animals were left on the platform for additional 10 s. If the mouse failed to find the platform within the allocated 60 s, it was guided to the platform by the experimenter. The behaviour of the animals was monitored and recorded by the TSE VideoMot2 system (TSE Technical & Scientific Equipment GmbH) and the escape latency (expressed in seconds) was calculated.

Statistical analysis

Data are represented as mean ± S.E.M. Numbers of animals per group are given in the figure legends. Statistical analysis of home-cage activity, Morris water maze and basal temperature experiments were performed using repeated-measures ANOVA following Bonferroni's *post-hoc* test where appropriate. Statistical analysis of the open-field test, EPM, SIH, TST and FST were dependent on the number of groups compared during each test as well as on the normality of the data: unpaired *t* test or Mann–Whitney *U* test for parametric and non-parametric data, respectively, were used for comparisons between two groups. When more than two groups were compared, statistical analysis was performed using ANOVA followed by Bonferroni's *post-hoc* test (for parametric data) or Kruskal–Wallis test followed by Mann–Whitney *U* *post-hoc* testing (for non-parametric data). p levels <0.05 were considered statistically significant.

Electroretinography

Electroretinograms (ERGs) were performed according to previously described procedures (Seeliger *et al*. 2001). Briefly, at the age of 9 and 18 months, both $Ca_V1.3^{-/-}$ and WT mice were dark-adapted overnight and anaesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). The pupils were dilated and single flash ERG recordings were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Light adaptation was accomplished with a background illumination of 30 cd/m² starting 10 min before recording. Single white-flash stimulation ranged from 10^{-4} to 25 cd × s/m², divided into ten steps of 0.5 and 1 log cd × s/m². For 1, 3, 10, and 25 cd × s/m² values are calculated as average from ten responses with an inter-stimulus interval of either 5 or 17 s. Band-pass filter cut-off frequencies were 0.1 and 3000 Hz.

Immunofluorescent staining of retinal sections

Eyes of WT and $Ca_V1.3^{-/-}$ mice were enucleated and their corneas and lenses removed. The posterior pole of the eye was fixed by immersion for 1 h at room temperature in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), then washed 3 × 10 min in phosphate-buffered saline (PBS) at the same pH, before immersion in 30% sucrose (overnight at 4 °C). Cryostat sections were cut at ~18 μm, mounted onto Super-Frost glass, dried for 1–2 h at 37 °C and then kept frozen until required. For fluorescence immunocytochemistry, cryostat sections were thawed, washed in PBS, and incubated with primary antibody diluted in blocking solution for either 2 h at room temperature or overnight at 4 °C. Primary antibodies and dilutions were as follows: polyclonal rabbit anti-$Ca_V1.3$ (Chemicon International, USA; at 1:200); anti-$Ca_V1.2$ (Alomone Laboratories, Israel; aa 848–865 at 1:200); rabbit polyclonal anti-$Ca_V1.4$ was produced and kindly provided by Dr J. McRory (McRory *et al*. 2004); anti-protein kinase Cα (Sigma, USA; aa 185–199 at 1:5000); anti-calbindin D-28K (Chemicon International, at 1:1000); anti-CtBP2/Ribeye (BD Biosciences, Pharmingen, USA; aa 361–445 at 1:5000). After washing three times in PBS retinal sections were incubated for 1–2 h with appropriate secondary antibodies diluted in PBS: anti-mouse Cy3, 1:500 (Jackson Immunoresearch, USA); anti-rabbit Alexa Fluor 488, 1:1000 (Molecular Probes, USA). Sections were rinsed three times in PBS and coverslipped with Prolong Gold Antifade Mounting Medium (Invitrogen, USA) and examined with a confocal laser-scanning microscope Nikon Eclipse C-1 (Nikon, Japan). Digital images (single optical sections) were acquired using a 60× oil-immersion objective lens and EZ-C1 (Nikon) software. All images were adjusted for brightness and contrast (Adobe Photoshop 7.0, USA) such adjustments were made uniformly to all parts of the image.

Results

Locomotor activity in $Ca_V1.3$ and Claudin 14-deficient mice

To test if either $Ca_V1.3$ or Claudin 14 deficiency causes a general alteration in spontaneous motor activity that could interfere with the interpretation of behavioural tests we measured the activity of mice over 60 h in their home-cage environment (Fig. 1). $Ca_V1.3^{-/-}$ and WT mice did not differ significantly in their locomotor activity and showed indistinguishable circadian phases of activity (Fig. 1*a*). $Cldn14^{-/-}$ mice showed differences in locomotor activity from WT particularly during the dark cycle (Fig. 1*b*). To minimize potential locomotor effects on our experiments, behavioural

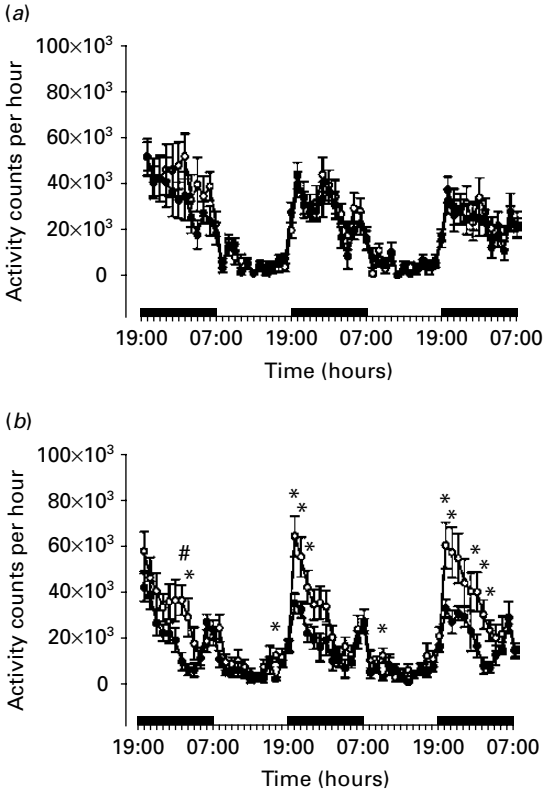

Fig. 1. Effect of $Ca_V1.3$ and Claudin14 deficiencies on home-cage activity. The hourly activity is shown during a 60-h recording period (three dark and two light cycles) for (*a*), $Ca_V1.3^{-/-}$ mice ($n=7$, ●) and their respective wild types (WTs) ($n=7$, ○) and (*b*), *Cldn14*$^{-/-}$ mice ($n=9$, ●) and their respective WTs ($n=13$, ○). The three dark cycles are indicated by the black bars. Data represent mean ± S.E.M. Statistical analysis was performed using univariate repeated-measures ANOVA (* $p<0.05$, # $p<0.01$). *Cldn14*$^{-/-}$ mice showed significantly lower activity compared to their respective WTs at different time-points mainly at the beginning of the dark cycle, while $Ca_V1.3^{-/-}$ mice did not differ from their respective WTs.

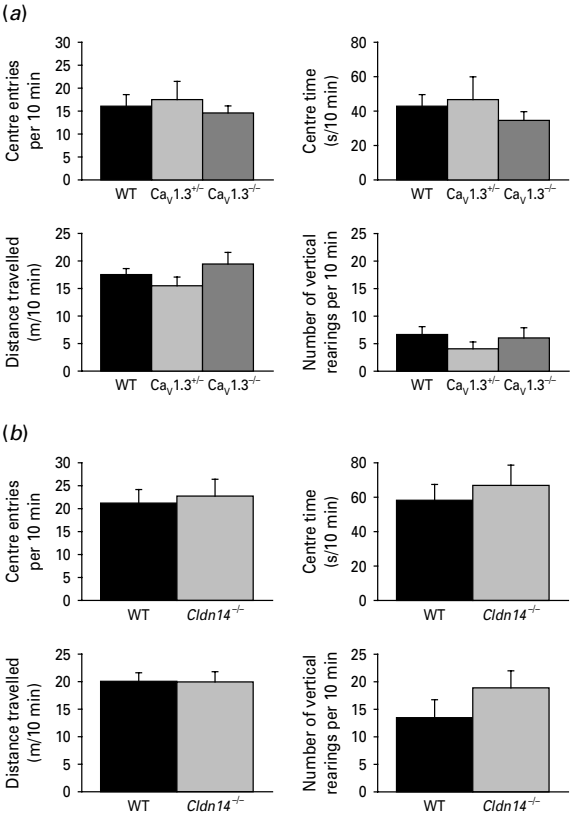

Fig. 2. Exploratory behaviour and locomotor activity in a novel open field. (*a*) The number of centre entries, the time spent in the centre zone, the total distance travelled in the open field and the number of vertical rearings are shown for $Ca_V1.3^{-/-}$ mice ($n=7$), $Ca_V1.3^{+/-}$ mice ($n=9$) and their respective wild types (WTs) ($n=11$). Data represent mean ± S.E.M. No significant differences between the lines were found (Kruskal–Wallis test). (*b*) The same parameters are represented for *Cldn14*$^{-/-}$ mice ($n=10$) and their respective WTs ($n=14$). Data represent mean ± S.E.M. No significant differences between the lines were found (unpaired *t* test).

tests with these mice were performed during the light cycle of the animals, between 09:00 and 15:00 hours. To further determine whether $Ca_V1.3$ or Claudin 14 deficiencies influence locomotor activity in a novel environment, we monitored the spontaneous activity of the animals in a novel open field during the light cycle (Fig. 2). When the distance travelled was examined, we observed that $Ca_V1.3^{+/-}$ and $Ca_V1.3^{-/-}$ were as active as the WT mice [Kruskal–Wallis test $\chi^2(2,27)=1.800$, $p=0.407$] (Fig. 2*a*). Similarly, no differences were observed between the *Cldn14*$^{-/-}$ and WT mice [unpaired *t* test: $t(22,24)=0.036$, $p=0.972$] (Fig. 2*b*). As for distance travelled, no differences were observed between the genotypes concerning vertical rearing [Kruskal–Wallis test: $\chi^2(2,27)=2.050$, $p=0.359$; unpaired *t* test: $t(22,24)=-1.185$, $p=0.249$ for Fig. 2*a*, *b*, respectively]. (For description of centre entries and time, see below.)

These results show that $Ca_V1.3$ and Claudin 14-deficient animals do not differ in their general motor activity compared to their respective WT when exposed to a novel environment.

Depression-related behaviour in $Ca_V1.3^{-/-}$ and Cldn14$^{-/-}$ *mice*

We have previously reported increased depression-like behaviour after selective stimulation of $Ca_V1.3$ LTCCs in $Ca_V1.2DHP^{-/-}$ mice (Sinnegger-Brauns *et al.*

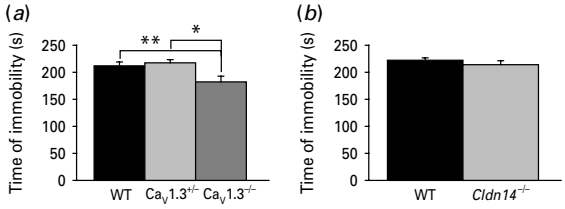

Fig. 3. Behaviour in the forced swim test. (*a*) Immobility times are shown for $Ca_V1.3^{-/-}$ mice ($n=8$), $Ca_V1.3^{+/-}$ mice ($n=9$) and their respective wild types (WTs) ($n=11$). Data represent mean $\pm$ S.E.M. Statistical analysis was performed using Kruskal–Wallis test followed by Mann–Whitney *post-hoc* testing (* $p<0.05$, ** $p<0.01$). (*b*) Immobility times are shown for *Cldn14*$^{-/-}$ mice ($n=7$) and their respective WTs ($n=6$). Data represent mean $\pm$ S.E.M. No significant differences were found (unpaired *t* test).

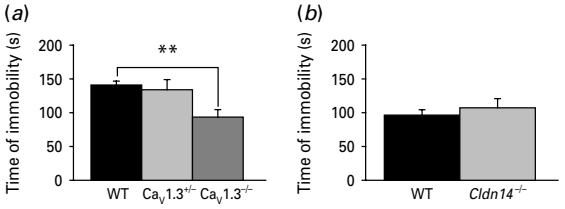

Fig. 4. Behaviour in the tail suspension test. (*a*) Immobility times are shown for $Ca_V1.3^{-/-}$ mice ($n=8$), $Ca_V1.3^{+/-}$ mice ($n=8$) and their respective wild types (WTs) ($n=10$). Data represent mean $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's *post-hoc* test (** $p<0.01$). (*b*) Immobility times are shown for *Cldn14*$^{-/-}$ mice ($n=10$) and their respective WTs ($n=9$). Data represent mean $\pm$ S.E.M. No significant differences between the lines were found (unpaired *t* test).

2004). Here we utilized $Ca_V1.3^{-/-}$, $Ca_V1.3^{+/-}$ and WT mice to provide direct evidence for a role of this channel in mood behaviour, by conducting two validated tests, the FST and the TST. In the FST $Ca_V1.3^{-/-}$ mice displayed significantly lower immobility time than WT (18% decrease) and $Ca_V1.3^{+/-}$ mice [Kruskal–Wallis test: $\chi^2(2,28)=8.126$, $p=0.017$] (Fig. 3*a*) indicative of reduced depression-like behaviour (Cryan & Mombereau, 2004) in $Ca_V1.3^{-/-}$ mice. Moreover, the reduced depression-like behaviour in $Ca_V1.3^{-/-}$ mice was even more pronounced in naive animals compared to WT mice (in seconds, immobility times evaluated were 126.78 ± 14.48 and 67.91 ± 11.77 for WT and $Ca_V1.3^{-/-}$ mice, respectively; unpaired *t* test: $t(14,16)=3.155$, $p=0.007$, data not shown]. This phenotype was confirmed using the TST in which $Ca_V1.3^{-/-}$ mice displayed a more active coping style and reduced depression-related behaviour (Fig. 4*a*). This was evident from a significant 33% decrease in immobility time as compared to WT mice [one-way ANOVA: $F(2,26)=5.776$, $p=0.009$]. No differences were found between $Ca_V1.3^{-/-}$ and $Ca_V1.3^{+/-}$ mice in this test.

To investigate whether congenital deafness alone could account for the antidepressant-like phenotype observed in the $Ca_V1.3^{-/-}$ mice, FST and TST were performed with congenitally deaf *Cldn14*$^{-/-}$ mice (Figs 3*b*, 4*b*). Both genotypes displayed immobility scores indistinguishable from WT in both tests [statistical analysis, unpaired *t* test: $t(11,13)=1.108$, $p=0.291$; unpaired *t* test: $t(17,19)=-0.674$, $p=0.509$ for Figs 3*b* and 4*b*, respectively].

It should be noted that, in the TST, the Claudin WT mice had comparatively low immobility scores making it more difficult to see potential antidepressant effects in the knockouts.

Overall, our results suggest that the antidepressant-like phenotype observed in $Ca_V1.3^{-/-}$ mice is specific for $Ca_V1.3$ deficiency and independent of deafness.

Anxiety-related behaviour in $Ca_V1.3^{-/-}$ and Cldn14$^{-/-}$ *mice*

Anxiety-related responses were assessed in different anxiety tests. In the EPM the number of entries into the open arms [Kruskal–Wallis test: $\chi^2(2,28)=7.687$, $p=0.021$] and the percentage of time spent in the open arms [Kruskal–Wallis test: $\chi^2(2,28)=6.384$, $p=0.041$] were significantly increased in $Ca_V1.3^{-/-}$ mice compared to WT mice (Fig. 5*a*). However, no significant differences in the number of entries and the percentage of time spent in the open arms between $Ca_V1.3^{-/-}$ and $Ca_V1.3^{+/-}$ mice were revealed by Mann–Whitney *U post-hoc* testing. No significant difference in the number of total entries into both arms was detected between the three groups [Kruskal–Wallis test $\chi^2(2,28)=0.351$, $p=0.839$] supporting the notion that there is no general locomotor activity difference between the lines. These results are consistent with a reduced anxiety-related phenotype of $Ca_V1.3^{-/-}$ mice. Although there was a trend for a lower number of head dips for WT mice, no statistically significant differences in head dipping and SAPs were found between the three genotypes (Kruskal–Wallis test: $\chi^2(2,28)=2.709$, $p=0.258$ and $\chi^2(2,28)=0.106$, $p=0.949$, for head dipping and SAPs, respectively).

Next we used a non-exploration based anxiety test and monitored SIH. Because basal temperature assessed at different time-points during the day can be influenced by the genotype of mice (e.g. Painsipp *et al*. 2008), we first tested if $Ca_V1.3$ deficiency affects

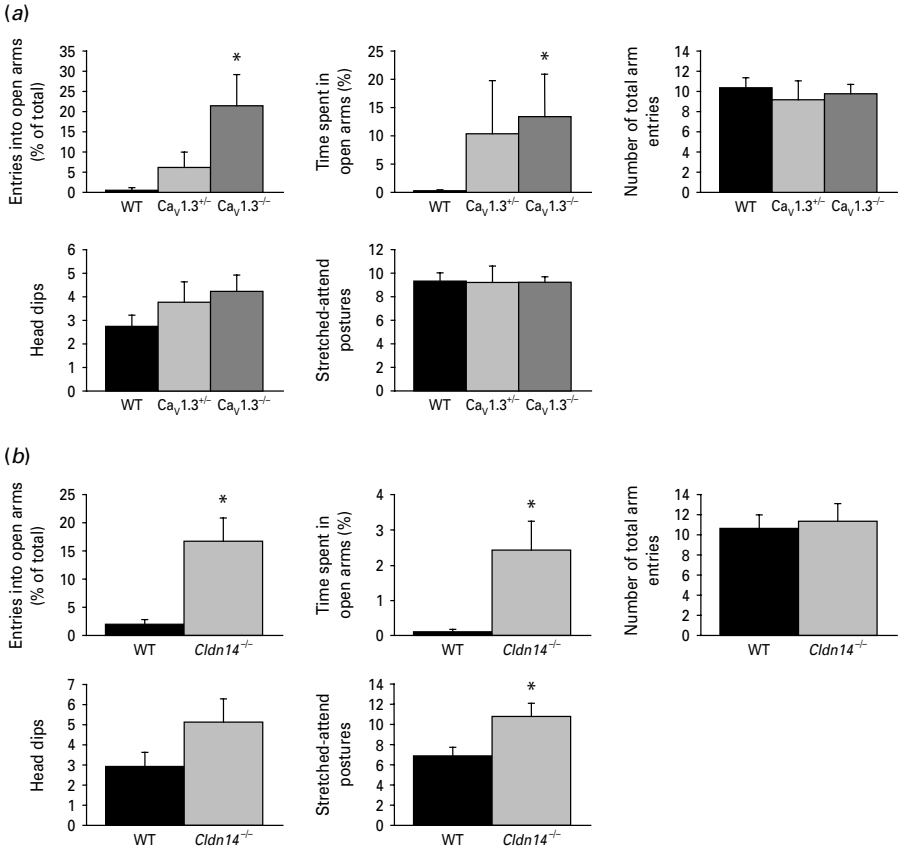

Fig. 5. Behaviour in the elevated plus maze test. (*a*) Open arm entries, time spent in the open arms, total arms entries, number of head dippings and stretched-attend postures are shown for $Ca_V1.3^{-/-}$ mice ($n=8$), $Ca_V1.3^{+/-}$ mice ($n=9$) and their respective wild types (WTs) ($n=11$). Data represent mean ± S.E.M. Statistical analysis was performed using Kruskal–Wallis test followed by Mann–Whitney *post-hoc* testing (* $p<0.05$). (*b*) The same parameters are shown for *Cldn14*$^{-/-}$ mice ($n=10$) and their respective WTs ($n=13$). Data represent mean ± S.E.M. Statistical analysis was performed using a Mann–Whitney *U* test (* $p<0.05$).

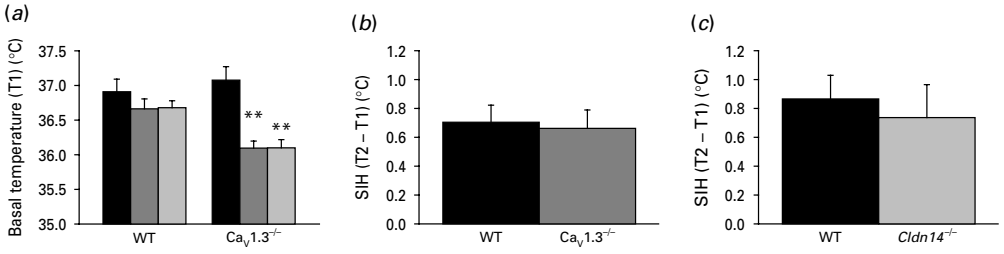

Fig. 6. Stress-induced hyperthermia (SIH). (*a*) Basal rectal temperature (T1) of $Ca_V1.3^{-/-}$ ($n=10$) and wild type (WT) mice ($n=14$) recorded at different time-points during the day (■, 09:00 hours; ■, 12:00 hours; □, 15:00 hours) are shown. Data represent mean ± S.E.M. Statistical analysis was performed using ANOVA for repeated measures followed by Bonferroni's *post-hoc* test (** $p<0.01$ WT *vs.* $Ca_V1.3^{-/-}$ mice). (*b*) SIH performed at 09:00 hours was recorded 10 min after measuring basal temperature for $Ca_V1.3^{-/-}$ mice and WTs as well as for (*c*) *Cldn14*$^{-/-}$ mice ($n=10$) and WTs ($n=14$). Data represent mean ± S.E.M. No significant differences in SIH were found between the lines (unpaired *t* test).

the body temperature of our animals. As illustrated in Fig. 6*a*, the basal temperature of WT mice measured at 09:00 hours (36.9 ± 0.2 °C) was in agreement with previous reports (Rabin *et al.* 1980), indicating that animals were not pre-stressed under our experimental conditions. Body temperature of $Ca_V1.3^{-/-}$ mice was

indistinguishable from WT at 09:00 hours but there was a clear difference between the lines later in the day, i.e. at 12:00 and 15:00 hours, where $Ca_v1.3^{-/-}$ mice displayed significantly lower body temperature compared to WTs [repeated-measures ANOVA; for genotype: $F(1,22)=4.4853$, $p=0.04572$; for day time: $F(2,44)=13.236$, $p<0.001$]. We therefore compared SIH at 09:00 hours. At the second monitoring of the temperature (T2) (Fig. 6*b*), 10 min following the mild stress of measuring T1, the resulting SIH (calculated as T2 – T1) was identical for $Ca_V1.3^{-/-}$ mice compared to WT [unpaired *t* test: $t(22,24)=0.260$, $p=0.798$].

We also evaluated centre entries and centre time in the open-field test and found no significant differences between $Ca_V1.3^{-/-}$ and their respective WTs [Kruskal–Wallis test: $\chi^2(2,27)=0.025$, $p=0.987$ and $\chi^2(2,27)=0.696$, $p=0.706$ for centre entries and centre time, respectively, Fig. 2*a*].

Together these data suggest the presence of an anxiolytic-like phenotype in $Ca_V1.3^{-/-}$ mice in the EPM but not in the other paradigms tested (SIH, open field). To determine if the result obtained in the EPM was specific for $Ca_V1.3$ LTCC deficiency or could possibly be explained by congenital deafness alone, we performed the same panel of experiments with the *Cldn14*$^{-/-}$ mice. In the EPM, *Cldn14*$^{-/-}$ mice, like $Ca_V1.3^{-/-}$ mice, showed significant increases in the number of entries into the open arms (Mann–Whitney *U* test: $Z=-2.644$, $p=0.012$) and in the percentage of time spent in the open arms (Mann–Whitney *U* test: $Z=-2.510$, $p=0.018$) compared to WT mice (Fig. 5*b*). No significant difference in the number of total entries into both arms was detected between the two groups (Mann–Whitney *U* test: $Z=-0.218$, $p=0.832$). These results, similar to those found with the $Ca_V1.3^{-/-}$ mice, are in agreement with an anxiolytic-like phenotype for the *Cldn14*$^{-/-}$ mice. A slight trend for a higher number of head dips was observed (Mann–Whitney *U* test: $Z=-1.315$, $p=0.208$) and a significant increase in the total SAPs was measured for *Cldn14*$^{-/-}$ compared to WT mice (Mann–Whitney *U* test: $Z=-2.209$, $p=0.026$). Body temperature at 09:00 hours [WT: 36.9 ± 0.1 °C; *Cldn14*$^{-/-}$: 36.9 ± 0.2 °C; unpaired *t* test: $t(22,24)=0.022$, $p=0.983$, data not shown] and SIH [Fig. 6*c*; unpaired *t* test: $t(22,24)=0.922$, $p=0.367$] were also not different between the genotypes. Additionally, in the open-field test, no significant differences in the centre entries and the centre time between *Cldn14*$^{-/-}$ mice and their respective WTs were found [unpaired *t* test $t(22,24)=-0.322$, $p=0.751$ and $t(22,24)=-0.596$, $p=0.557$ for centre entries and centre time respectively, Fig. 2*b*].

Taken together, these results suggest that the anxiolytic-like phenotype observed in the EPM test in the $Ca_V1.3^{-/-}$ mice may be explained by congenital deafness in mice.

Visual function and retinal morphology in $Ca_V1.3^{-/-}$ mice

$Ca_V1.3$ LTCCs are expressed together with $Ca_V1.4$ in the mammalian retina (Morgans, 1999; Morgans *et al*. 1998; Xu *et al*. 2002). However, the contribution of the $Ca_V1.3$ channels to retinal morphology and visual function remains unknown.

Studies in mammalian retina show that the loss of $Ca_V1.4$ channels lead to an aberrant outgrowth of rod bipolar cell dendrites and horizontal cell processes into the outer nuclear layer and to the formation of ectopic synaptic contacts with rod photoreceptors (Chang *et al*. 2006; Mansergh *et al*. 2005). To study the consequences of $Ca_V1.3$ deficiency on retinal structure, we performed parallel immunocytochemical analyses of WT and $Ca_V1.3^{-/-}$ retinas (Fig. 7). Immunoreactivity for $Ca_V1.3$ was absent from all retinal layers in $Ca_V1.3^{-/-}$ mice indicating successful deletion in this tissue (Fig 7*a*). $Ca_V1.3$ deficiency did not lead to detectable changes in basic retinal architecture, or to a loss of neurons in the nuclear layers (not shown). The distribution of $Ca_V1.4$ (Fig. 7*b*) and $Ca_V1.2$ (Fig. 7*c*) was similar in the WT and $Ca_V1.3^{-/-}$ retinas. An anti-calbindin antibody strongly labelled horizontal cell bodies and a dense plexus of horizontal cell processes in the outer plexiform layer of both WT and $Ca_V1.3^{-/-}$ retinas (Fig. 7*d*). Labelling rod bipolar cells with anti-PKCα also showed no difference in WT *vs*. knockout retina (Fig. 7*e*). In contrast to $Ca_V1.4$-deficient mice (Chang *et al*. 2006; Mansergh *et al*. 2005), we found no evidence of rod bipolar cell or horizontal cell processes sprouting into the outer nuclear layer of $Ca_V1.3$ knockout retinas.

To determine possible alterations in photoreceptor synapses to second-order neurons, we examined the expression patterns of synaptic ribbon proteins Bassoon and RIBEYE (Brandstatter *et al*. 1999; Schmitz *et al*. 2000), which are present in both rod and cone terminals, and of peanut agglutinin (PNA) that recognizes only cone photoreceptor terminals. The staining pattern of PNA was similar in both retinas and was concentrated strictly in the outer plexiform layer (not shown). In the outer plexiform layer of the $Ca_V1.3^{-/-}$ retina, labelling for CtBP2/RIBEYE appeared primarily as clusters of puncta or patches (Fig. 7*f*, inset), differing from WT retinas in which labelling exhibited a mix of horseshoe-shaped

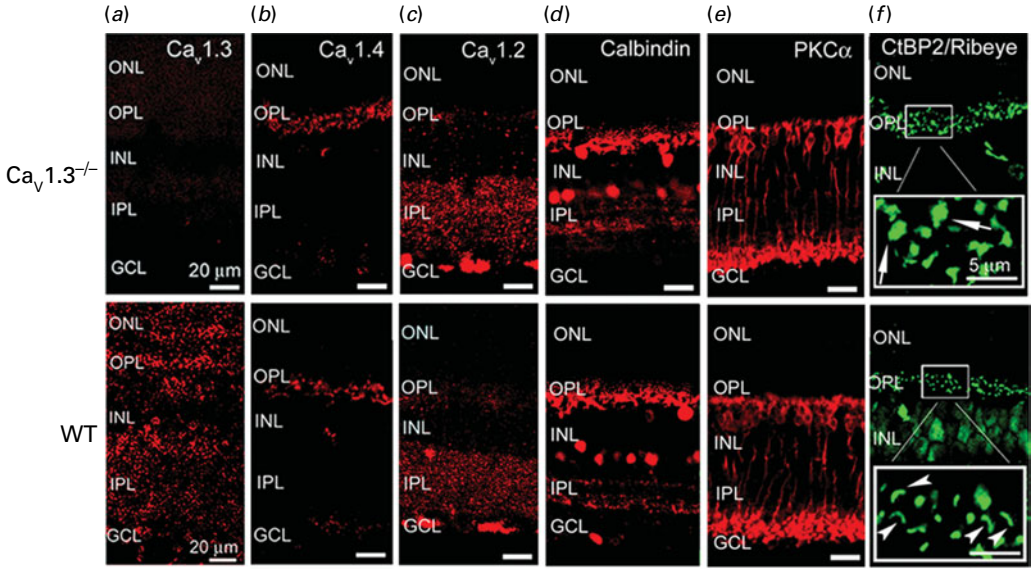

Fig. 7. Confocal fluorescence images of frozen vertical sections of $Ca_V1.3^{-/-}$ (upper panels) and wild type (WT) (lower panels) retinas immunolabelled with antibodies against the (*a*) $Ca_V1.3$, (*b*) $Ca_V1.4$, (*c*) $Ca_V1.2$, (*d*) horizontal cell marker calbindin, (*e*) rod bipolar cell marker PKCα, and (*f*) marker for ribbon synapses. The $Ca_V1.3^{-/-}$ retina showed no immunoreactivity to anti-$Ca_V1.3$ antibody indicating that this subunit of L-type channel was completely deleted. The staining patterns for $Ca_V1.4$ and $Ca_V1.2$ were similar in WT and $Ca_V1.3^{-/-}$ retinas. Unlike in retinas lacking $Ca_V1.4$ channels, no ectopic dendrites of rod bipolar cells or horizontal cell processes sprouting into the outer nuclear layer were observed in $Ca_V1.3^{-/-}$ retina (*d*, *e*). Labelling for CtBP2/RIBEYE in the outer plexiform layer of $Ca_V1.3^{-/-}$ retina appeared as clusters of puncta or patches (arrows), differing from horseshoe-like appearance (arrowheads) in WT retina. The retinal layers are indicated. Each of the shown stainings was repeated with at least three retinal preparations with similar results. Staining was performed with the antibodies as described in Methods section. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

structures (arrowheads) and discrete puncta. In contrast, the horseshoe-like appearance of the structures labelled by Bassoon in the outer plexiform layer was similar for WT and $Ca_V1.3^{-/-}$ retinas (not shown). Given the fact that RIBEYE is the major component of synaptic ribbons (Schmitz *et al*. 2000), it is reasonable to suggest possible interactions between synapse-associated proteins and $Ca_V1.3$ channels that might be implicated in structural plasticity at ribbon synapses.

To determine whether these minor morphological differences affect visual function, relevant for the assessment of behavioural phenotypes, we measured retinal function of $Ca_V1.3^{-/-}$ and WT mice of the same genetic background using electroretinography (Fig. 8). Under both scotopic (dark-adapted) and photopic (light-adapted) conditions, the amplitudes of the ERG b-wave signals were not substantially reduced (Fig. 8*a*, *c*). Therefore, there is no indication that functional vision in these mice is impaired on a retinal level. However, it must be noted, that the b-wave medians in both conditions were close to the lower limit of the 90% normal range, whereas the a-wave data was entirely within the respective range (Fig. 8*b*, *d*). Thus, it cannot be excluded on the basis of these results that the loss of $Ca_V1.3$ LTCCs does have a minor influence on signal transmission in the outer retina.

To determine if these minor changes of ERG waveforms are of functional relevance for behavioural studies we performed the visible platform test of the Morris water maze. In this task, an impairment of visual capacity leads to an increase of the escape latency (Gerlai *et al*. 2002). During the entire course of the experiment, no significant differences in the escape latencies between the three genotypes ($Ca_V1.3^{-/-}$, $Ca_V1.3^{+/-}$, WT mice) were found (Fig. 9).

Collectively these results demonstrate that $Ca_V1.3$ deficiency causes detectable changes in synaptic ribbon morphology without significantly affecting synaptic transmission from photoreceptors to bipolar cells. Although these may account for a trend for the reduction of b-wave amplitudes, a relevant decrease of visual function that could influence behavioural experiments can be ruled out.

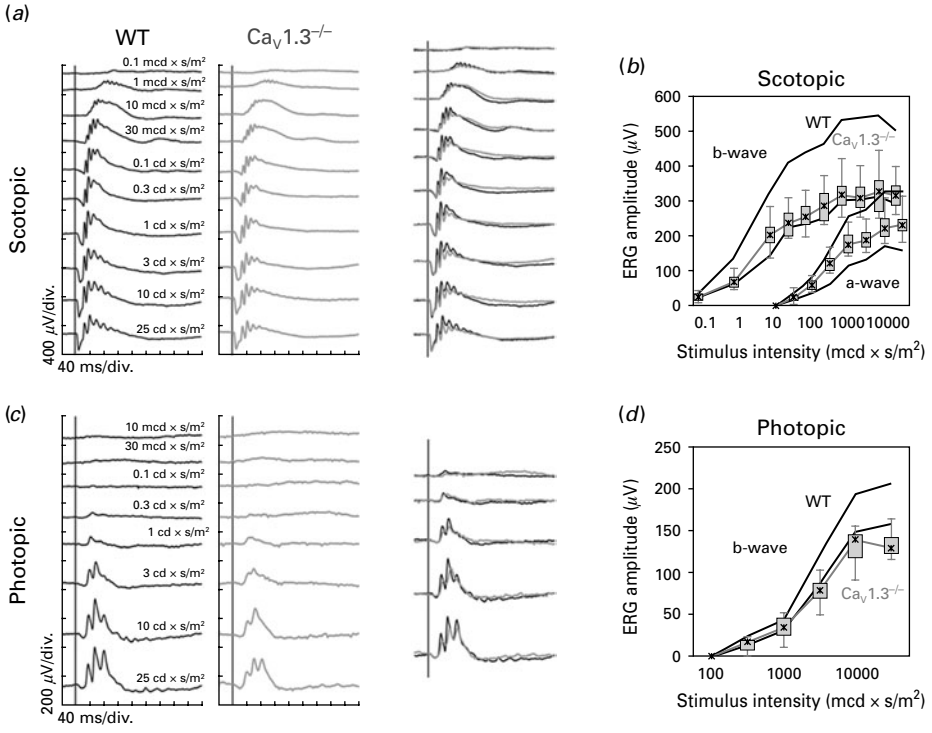

Fig. 8. Effect of $Ca_V1.3$ deficiency on retinal function under (*a*, *b*) scotopic and (*c*, *d*) photopic conditions. (*a*, *c*) Comparison of electroretinogram (ERG) curves in (*a*) dark-adapted or (*c*) light-adapted wild type (WT) and $Ca_V1.3^{-/-}$ mice. Even in the superposition series, no obvious change in light sensitivity or waveform shape was detectable. Statistical analysis was performed for the corresponding ERG a- and b-wave data (*b*, *d*). Boxes indicate the 25% and 75% quantile range, whiskers the 5% and 95% quantiles and the '×' the median of the $Ca_V1.3^{-/-}$ data. The normal range is delimited by solid lines indicating the 5% and 95% quantiles of the WT data. No evidence of impaired retinal function was found in $Ca_V1.3^{-/-}$ mice, except for a potential minor reduction in b-wave amplitude which is unlikely to cause visual impairment.

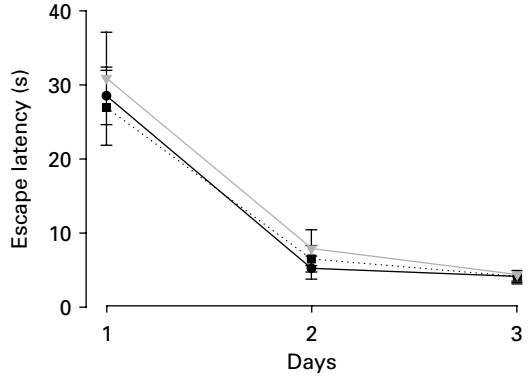

Fig. 9. Behaviour in the Morris water maze test. The latency of wild type (–●–, $n=11$), $Ca_V1.3^{+/-}$ (···■···, $n=9$) and $Ca_V1.3^{-/-}$(—▼—, $n=8$) mice searching for a visible platform in the Morris water maze task is expressed in seconds (average of four trials). Values of the first trial on the first day were 50.94 ± 5.12 s for wild types, 47.71 ± 6.71 s for $Ca_V1.3^{+/-}$ mice and 54.37 ± 5.63 s for $Ca_V1.3^{-/-}$ mice. Data represent mean ± S.E.M. No significant differences between the lines were found (repeated-measures ANOVA).

Discussion

The major findings of our study are that the absence of $Ca_V1.3$ LTCCs in mice leads to a pronounced decrease in depression-like behaviour in these animals. Using *Cldn14*$^{-/-}$ mice, which are congenitally deaf like the $Ca_V1.3$-deficient animals, we demonstrated that the antidepressant-like phenotype observed in the $Ca_V1.3^{-/-}$ mice was specific for $Ca_V1.3$ deficiency and was not related to deafness. Our data also emphasize that anxiolytic-like effects in the EPM can be found in different mouse models of congenital deafness. Moreover, we show for the first time that $Ca_V1.3$ deficiency had only a minor effect on synaptic transmission from retinal photoreceptors to bipolar cells and did not impair visual function, as revealed by ERG analysis and behavioural experiments, respectively.

Our findings support the hypothesis that selective modulation of $Ca_V1.3$ channels could serve as a novel

therapeutic principle for the treatment of mood and possibly anxiety disorders. The FST and TST are the most widely used animal tests to assess antidepressant-like efficacy of drugs and treatments in mice (Cryan *et al*. 2005; Petit-Demouliere *et al*. 2005). By using both of these tests, we provided evidence for reduced depression-like behaviour in $Ca_V1.3^{-/-}$ mice compared to WT or heterozygous knockout mice. This finding has been replicated by us with mice at different breeding stages and by two investigators. The finding that antidepressant-like effects are elicited by deletion of the $Ca_V1.3$ LTCCs is in excellent agreement with our previous observation that their acute activation (feasible in $Ca_V1.2DHP^{-/-}$ mice) induces pro-depression-like behaviour (Sinnegger-Brauns *et al*. 2004). However, and in spite of the fact that we have demonstrated a reproducible antidepressant-like behaviour in $Ca_V1.3^{-/-}$ mice using the FST and the TST, additional tests including learned helplessness, chronic mild stress and anhedonia models (for review, see Cryan *et al*. 2005) are necessary to fully confirm our results. Moreover, it will be interesting to test conventional antidepressants in $Ca_V1.3$ knockouts to reveal potential synergistic effects (see Taragano *et al*. 2005).

Anxiety-like behaviour was investigated using the EPM test, one of the most extensively used anxiety tests (for review see Carobrez & Bertoglio, 2005). Measures of anxiety and activity have usually been discerned through conventional parameters such as the number of entrances into and/or the length of time spent in the open arms. Moreover, assessment of additional ethological measures permits a more detailed behavioural analysis including factors of exploration and risk assessment (Blanchard *et al*. 1990; Rodgers *et al*. 1992). Indeed, a number of postural elements considered indicative of exploration such as, head dipping and risk assessment including SAPs have been shown to be sensitive to both increases and decreases in anxiety in rats (Shepherd *et al*. 1994) and mice (Blanchard *et al*. 1990; Kaesermann, 1986; Rodgers & Cole, 1994; Rodgers *et al*. 1992). $Ca_V1.3^{-/-}$ relative to WT mice showed a significant increase in the percentage of entries into and the time spent on the open arms indicating an anxiolytic-like phenotype (File, 2001; Lister, 1987; Pellow *et al*. 1985). Total arm entries did not differ between WT and $Ca_V1.3^{-/-}$ mice supporting the evidence from home-cage and open-field experiments that there is no significant difference in general locomotor activity that could potentially interfere with the behavioural readout of exploratory anxiety tests such as the EPM. The comparatively high level of anxiety in WT mice indicated by the low number of open arm entries, is in excellent agreement with that described in a previous report (Lamberty & Gower, 1996) and seems to be a common phenomenon seen in mice based on the C57BL/6 strain when tested in the EPM (Trullas & Skolnick, 1993). Assessment of additional parameters in the EPM test did not support the anxiolytic phenotype, since neither head dipping nor number of SAPs differed between $Ca_V1.3^{-/-}$ and WT mice. Moreover, neither the SIH nor the open-field tests revealed evidence for an anxiolytic phenotype of $Ca_V1.3^{-/-}$mice.

Since $Ca_V1.3^{-/-}$ mice are congenitally deaf, it cannot be excluded that the observed behavioural phenotypes arise from hearing loss. However, the available evidence supports either no difference or rather *enhanced* anxiety- and depression-like states in case of deafness. For example, urocortin-deficient mice show hearing impairment and increased anxiety-like behaviour in tests including the EPM (Vetter *et al*. 2002). In dogs, genetic deafness was associated with enhanced nervousness or no difference in response to fear-provoking stimuli when compared to normal dogs (Klein *et al*. 1988). Moreover, in humans, evidence that hearing-impaired persons demonstrate more symptoms of anxiety and depression than the normal hearing population has been provided (Kvam *et al*. 2007).

From a long list of mice with hearing impairment (Anagnostopoulos, 2002) we selected $Cldn14^{-/-}$ mice as another mouse model for congenital deafness. These are one of only a few strains that appeared suitable for this purpose because they suffer from profound hearing loss shortly after hearing starts by the third week of life but do not exhibit evidence for vestibular dysfunction or any other histopathological and functional changes interfering with behavioural studies (Ben-Yosef *et al*. 2003). The normal depression-like behaviour of the $Cldn14^{-/-}$ animals, as revealed by the FST and TST, allowed us to conclude that the antidepressant-like phenotype induced by $Ca_V1.3$ deficiency is not due to an indirect effect of deafness. Instead, our data provide strong support for the hypothesis that $Ca_V1.3$ channels modulate the activity of neuronal circuits involved in the control of mood behaviour in mice, in agreement with our previous findings in $Ca_V1.2DHP^{-/-}$ mice (Hetzenauer *et al*. 2006; Sinnegger-Brauns *et al*. 2004). The importance of including $Cldn14^{-/-}$ mice in our comparison is emphasized by our finding that the anxiolytic-like phenotype observed in $Ca_V1.3^{-/-}$ animals in the EPM test was similarly observed in $Cldn14^{-/-}$ mice and can therefore not be unequivocally attributed to altered $Ca_V1.3$ function. Instead, this observation leads to the

interesting hypothesis that deafness could influence the anxiolytic-like state of mice assessed in the EPM but not in the other anxiety tests used. However, looking at the risk assessment behaviour of the *Cldn14*$^{-/-}$ mice, it is interesting to note that the frequencies of SAPs which should be decreased in the case of an anxiolytic-like phenotype (Shepherd *et al*. 1994) are actually significantly increased. The reason for this dissociation is not clear but has also been observed in other knockout animal models (Yamada *et al*. 2002). Furthermore, some authors have reported that SAPs can be unaffected by the effects of anxiolytic drugs (Moser, 1989).

Our finding of antidepressant- and possibly anxiolytic-like phenotypes in a $Ca_V1.3$-deficient mouse leads to the hypothesis that $Ca_V1.3$ channels could be a potential novel therapeutic target for the treatment of anxiety and mood disorders. However, it should be noted that because of the essential role of $Ca_V1.3$ channels for hearing (Platzer *et al*. 2000) a hearing impairment could be an unwanted side-effect of such treatment, even if current DHP treatments for cardiovascular disease at therapeutic doses do not produce this effect in patients. The biophysical properties of $Ca_V1.3$-mediated L-type currents in cochlear inner hair cells (IHC) differ from those of $Ca_V1.3$-mediated currents in heart and brain (Striessnig, 2007; Striessnig & Koschak, 2008, for review) by their much slower inactivation properties during a depolarizing pulse. Based on experiences with DHP calcium channel blockers it therefore appears feasible to develop state-dependent modulators with functional selectivity for more rapidly inactivating neuronal $Ca_V1.3$ channels not affecting auditory function.

We and others recently obtained evidence that high doses of systemically applied DHPs may induce an aversive state (probably explained by a stress response) which causes indirect effects on brain function that are difficult to separate from direct CNS effects of these drugs (Busquet *et al*. 2008; Waltereit *et al*. 2008). This precluded additional experiments in which $Ca_v1.2$-channel activity was inhibited by systemic administration of DHPs (e.g. nifedipine) to our $Ca_V1.3$-deficient mice to reveal a possible role of $Ca_V1.2$ channels for anxiety and depression.

The behavioural changes reported in this and previous studies, cannot be explained by altered heart rate (it is normal during physical activity), changes in brain histology, decreased sensorimotor function or altered spontaneous locomotor activity (Clark *et al*. 2003; Platzer *et al*. 2000; and shown here). The ERG data presented here also exclude the possibility that the behavioural phenotype is due to compromised visual function. In addition, they provide important insight into the role of different LTCC isoforms for retinal function. In humans, loss of $Ca_V1.4$ function causes congenital stationary night blindness type-2 (CSNB2), emphasizing the role of this channel for signalling from photoreceptors to second-order neurons (Doering *et al*. 2007; Striessnig & Koschak, 2008). An additional role of $Ca_V1.3$ for transmitter release from photoreceptor terminals has been proposed (Barnes & Kelly, 2002; Kamphuis & Hendriksen, 1998; Morgans *et al*. 2005) but our experiments show that $Ca_V1.3$ is not required for normal vision. Although a minor effect of $Ca_V1.3$ deficiency on the b-wave/a-wave relationship (a 'scent' of a negative ERG) cannot be excluded, a relevant decrease of visual function on a retinal level large enough to influence the behavioural experiments was ruled out. We also detected no difference in immunofluorescent labelling pattern for $Ca_V1.4$ and $Ca_V1.2$ channels in WT *vs*. $Ca_V1.3^{-/-}$ retina, indicating that up-regulation of these isoforms is unlikely. However, given the circadian regulation of expression (Ko *et al*. 2007), and selective internalization of $Ca_V1.3$ (but not $Ca_V1.2$) channels by cytoskeletal reorganization (Cristofanilli *et al*. 2007), it is reasonable to suppose that $Ca_V1.3$ channels play an important but still undetermined role in retinal physiology and pathology.

Acknowledgements

We thank Anjali M. Rajadhyaksha for supplying $Ca_V1.3^{-/-}$ retinas. Supported by the Austrian Science Fund (P-17159 and W11 to J.S., NFN-S-102 to N.S.), the University of Innsbruck and by NIH grant EY 12497 (to A.A.), and the German Research Council (DFG grants Se837/5-2 and 6-1 to M.W.S.).

Statement of Interest

None.

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