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Breeding of $Ca_v2.3$ deficient mice reveals Mendelian inheritance in contrast to complex inheritance in $Ca_v3.2$ null mutant breeding

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High voltage-activated $Ca_v2.3$ R-type Ca^{2+} channels and low voltage-activated $Ca_v3.2$ T-type Ca^{2+} channels were reported to be involved in numerous physiological and pathophysiological processes. Many of these findings are based on studies in $Ca_v2.3$ and $Ca_v3.2$ deficient mice. Recently, it has been proposed that inbreeding of $Ca_v2.3$ and $Ca_v3.2$ deficient mice exhibits significant deviation from Mendelian inheritance and might be an indication for potential prenatal lethality in these lines. In our study, we analyzed 926 offspring from $Ca_v3.2$ breedings and 1142 offspring from $Ca_v2.3$ breedings. Our results demonstrate that breeding of $Ca_v2.3$ deficient mice shows typical Mendelian inheritance and that there is no indication of prenatal lethality. In contrast, $Ca_v3.2$ breeding exhibits a complex inheritance pattern. It might be speculated that the differences in inheritance, particularly for $Ca_v2.3$ breeding, are related to other factors, such as genetic specificities of the mutant lines, compensatory mechanisms and altered sperm activity.

Abbreviations

HT	Heterozygous
HVA	High voltage-activated
KO	Knock-out
LTP	Long-term potentiation
L-type	"Long-lasting" type Ca^{2+} channel
LVA	Low voltage-activated
PCR	Polymerase chain reaction
R-type	"Resistant" type Ca^{2+} channel
SEM	Standard error of the mean
T-type	"Transient" type Ca^{2+} channel
VGCC	Voltage-gated Ca^{2+} channel
WT	Wild-type

Voltage-gated Ca^{2+} channels (VGCCs) play an essential role in various physiological and pathophysiological processes, such as excitation–contraction coupling, excitation-secretion coupling, neurotransmitter release, regulation of gene expression, developmental processes and reproduction[1–6]. The fine tuning of intracellular/cytosolic Ca^{2+} concentrations is a prerequisite for triggering specific subcellular, cellular and supracellular responses in a complex spatiotemporal manner[1,4,7,8]. The distinct electrophysiological characteristics of VGCCs together with their complex spatiotemporal distribution guarantee this fine tuning of Ca^{2+} entry in various cell types of the organism and mediate their broad spectrum of functional implications[1,2,4,6]. Ten different pore-forming Ca_v-α_1 subunits have been cloned and they have been subdivided due to their activation threshold into seven high voltage-activated (HVA) and three low voltage-activated (LVA) channels. The HVA channels are further segregated into *long-lasting* (L-Type) $Ca_v1.1$-$Ca_v1.4$ VGCCs and Non-L-type $Ca_v2.1$-$Ca_v2.3$ channels [1,4]. The LVA group consists of $Ca_v3.1$-$Ca_v3.2$ channels. In the cellular context, the pore-forming Ca_v-α_1 subunits are often

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associated with various auxiliary subunits, such as $\alpha_2\delta$, β and γ, building up a VGCC complex. The auxiliary subunits are capable of modulating the pharmacological and electrophysiological properties of the underlying pore-forming Ca_v-α_1 subunit[9,10]. Further structural and functional modifications originate from alternative splicing processes and post-translational modifications, such as protein cleavage or interconversion phenomena due to phosphorylation/dephosphorylation[11–13]. In order to get more detailed insight into the physiological relevance of the various VGCCs, scientific groups around the world have inactivated the different Ca_v-α_1 subunits. These studies have tremendously increased our understanding on the role of VGCCs and their involvement in the etiopathogenesis of animal and human diseases[1,5,14].

Mouse lines lacking the $Ca_v2.3$ or the $Ca_v3.2$ VGCCs have first been generated and described 17–20 years ago and many physiological/pathophysiological implications of both channels were characterized in these models. $Ca_v2.3$ knockout mice, for example, exhibit a complex phenotype including, i.a., impaired pancreatic beta cell function and glucose tolerance[15–17], cardiac arrhythmia and altered autonomic regulation[18–20], reduced seizure susceptibility[21–27], dysregulation in hippocampal theta genesis and altered theta architecture[28,29], impaired presynaptic long-term potentiation (LTP)[30], distorted circadian rhythmicity and sleep[31,32], altered myelinogenesis[33], modified (neuropathic) pain perception[34–36], enhanced fear[37] and altered auditory information processing[38,39]. $Ca_v2.3$ VGCCs also serve as key factors in regulating neuronal firing in the CNS, i.e., the tonic, intermediate and burst firing modes and modulate facultative neuronal oscillatory activity in specific neuronal ensembles and networks[40–45].

The phenotype of $Ca_v3.2$ deficient mice is characterized, i.a., by alteration of mechanoreception[46] and pain response[47–50], age-induced endothelial dysfunction[51], retinal dysfunction[52], (sensory) neuronal hyperexcitability[53–55], elevated anxiety, impaired memory and reduced sensitivity to psychostimulants[56]. $Ca_v3.2$ was also reported to be involved in epileptogenesis/ictogenesis[57–59]. In addition, longitudinal body weight analysis indicated a complex developmental impairment, particularly in $Ca_v3.2^{-/-}$ mice, which could be related to the cardiovascular phenotype. The latter includes coronary arteriole constriction and focal myocardial fibrosis[60,61]. Recently, we also demonstrated that $Ca_v3.2$ deficient mice exhibit altered auditory information processing[62,63] and alterations in theta genesis and theta architecture[64,65].

It has recently been reported by Alpdogan et al. (2020)[66] that inbreeding of both $Ca_v2.3$ and $Ca_v3.2$ deficient mice exhibits non-Mendelian inheritance, e.g., for $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$ and for $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ offspring with significant reduction of $Ca_v3.2^{-/-}$ animals. For $Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$ breeding, Alpdogan et al. (2020)[66] reported a deviation from Mendelian inheritance for heterozygous (HT) male mice, but not for $Ca_v2.3^{-/-}$ animals. We have been using the same $Ca_v3.2$ null mutant line as Alpdogan et al. (2020)[66] and an alternative $Ca_v2.3$ null mutant line for several years with a total number of 926 and 1142 offspring, respectively. Based on our $Ca_v2.3$ and $Ca_v3.2$ breedings and genotyping results, we analyzed our data for potential deviations from Mendelian inheritance in both lines.

Results

$Ca_v3.2$ breeding results and characteristics of inheritance. $Ca_v3.2$ mutant mice were bred for eight years in different projects of our group (see[62–64,67]). $Ca_v3.2^{+/+}$, $Ca_v3.2^{+/-}$ and $Ca_v3.2^{-/-}$ mice were generated using three different breeding schemes, i.e., $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$, $Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$, and $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$. In total, 926 offspring from 164 litters were analyzed. For the $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$ breeding scheme (including both sexes) with 344 offspring from 58 litters, a deviation from Mendelian inheritance was detected with an increase of $Ca_v3.2^{+/-}$, and a decrease of $Ca_v3.2^{+/+}$ and $Ca_v3.2^{-/-}$ mice compared to the Mendelian distribution (Fig. 1A_I, Table 1A, Suppl. Tab. 1A). Interestingly, a sex-specific analysis of the related breeding results did not confirm this non-Mendelian inheritance in both combined sexes (Fig. 1A_{II} for ♂, Fig. 1A_{III} for ♀, Table 1, Suppl. Tab. 2 and 3).

No alterations were detected for the $Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$ scheme (with 273 offspring from 43 litters, Fig. 1B_I, Table 1A, Suppl. Tab. 1). The same held true for the sex-specific analysis (Fig. 1B_{II} for ♂, Fig. 1B_{III} for ♀, Table 1A, Suppl. Tab. 2, 3).

For the $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ breeding (with 309 offspring from 62 litters), a deviation from the Mendelian inheritance pattern was detected for both sexes as well as in the sex-specific analysis. There turned out to be an increase of $Ca_v3.2^{+/-}$ and a decrease of $Ca_v3.2^{-/-}$ mice compared to the Mendelian distribution (Fig. 1C_I, C_{II}, C_{III}, Table 1A, Suppl. Tab. 1–3). For the three different breeding schemes, a significant alteration in litter size was only observed for both sexes, but not for separate analysis of male and female offspring (Table 2A).

$Ca_v2.3$ breeding results and characteristics of inheritance. $Ca_v2.3$ mutant mice were bred for about eight years in different projects of our group (see[28,31,38,39,67]). $Ca_v2.3^{+/+}$, $Ca_v2.3^{+/-}$ and $Ca_v2.3^{-/-}$ mice were generated using three different breeding schemes, i.e., $Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$, $Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$, and $Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$. In total, 1142 offspring from 170 litters were analyzed. For the $Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$ breeding scheme (including both sexes) with 349 offspring from 55 litters, no deviation from Mendelian inheritance was detected (Fig. 2A_I, Table 1B, Suppl. Tab. 4). The same held true for the $Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$ scheme with 357 offspring from 51 litters (Fig. 2B_I, Table 1 B, Suppl. Tab. 4). Finally, for $Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$ breeding (with 436 offspring from 64 litters), again no alterations from Mendelian distribution could be observed (Fig. 2C_I, Table 1B, Suppl. Tab. 4).

Next, we carried out a sex-specific analysis of the offspring breeding results. Notably, neither in females nor in males, we observed any significant deviation from Mendelian inheritance pattern (Fig. 2A_{II}, B_{II}, C_{II} for ♂, Fig. 2A_{III}, B_{III}, C_{III} for ♀, Table 1B, Suppl. Tab. 5, 6). In summary, there is no indication of non-Mendelian inheritance in the $Ca_v2.3$ mutant line described here. For the three different breeding schemes, no significant alteration in litter size was detected for both sexes and male and female offspring (Table 2B).

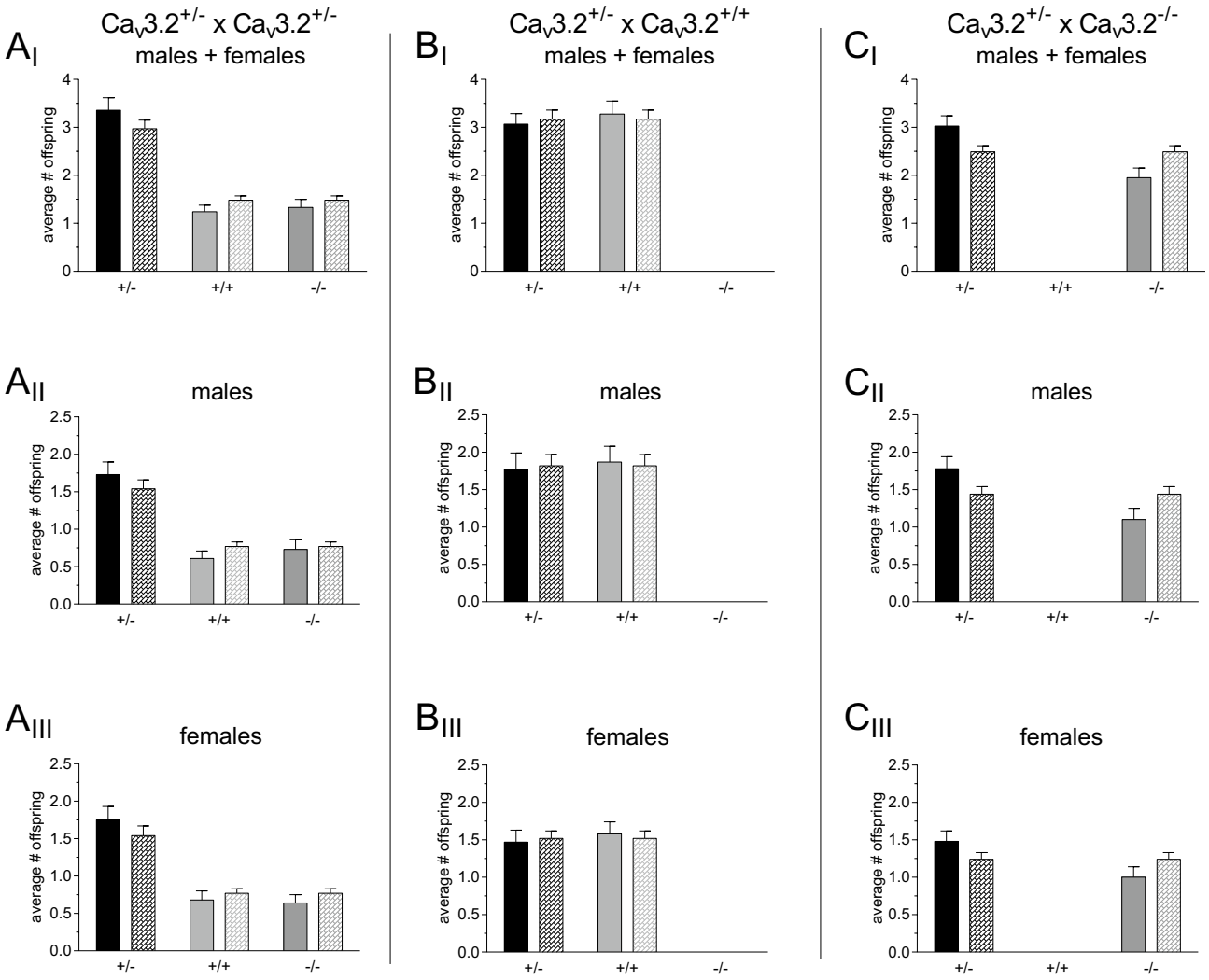

Figure 1. Real and theoretical average number of offspring from different $Ca_v3.2$ breeding schemes. (**A_I–C_I**) Breeding results for both sexes using a $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$, $Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$ and $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ breeding scheme. In addition, these data were analyzed separately for male offspring (**A_{II}–C_{II}**) and female offspring (**A_{III}–C_{III}**). Plain bars indicate real average offspring number, patterned bars indicate the theoretical average offspring number based on the Mendelian inheritance.

(A) $Ca_v3.2$ Breeding scheme	♂ + ♀	♀	♂
$Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$	*p = 0.043 (non-Mendelian) [0.6062]*	**p = 0.183 (Mendelian) [0.3591]**	**p = 0.184 (Mendelian) [0.3582]**
$Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$	**p = 0.586 (Mendelian) [0.0731]**	**p = 0.662 (Mendelian) [0.0646]**	**p = 0.737 (Mendelian) [0.0583]**
$Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$	*p < 0.001 (non-Mendelian) [0.9369]*	*p = 0.022 (non-Mendelian) [0.5239]*	*p = 0.002 (non-Mendelian) [0.7901]*
(B) $Ca_v2.3$ Breeding scheme	♂ + ♀	♀	♂
$Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$	**p = 0.076 (Mendelian) [0.5171]**	**p = 0.079 (Mendelian) [0.5096]**	**p = 0.628 (Mendelian) [0.1265]**
$Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$	**p = 0.560 (Mendelian) [0.0766]**	**p = 0.082 (Mendelian) [0.3236]**	**p = 0.374 (Mendelian) [0.1143]**
$Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$	**p = 0.292 (Mendelian) [0.1427]**	**p = 0.639 (Mendelian) [0.0669]**	**p = 0.304 (Mendelian) [0.1379]**

Table 1. Results of Chi-square testing for $Ca_v3.2$ and $Ca_v2.3$ breeding. Breeding results for $Ca_v3.2$ (see Suppl. Tab. 1–3) and $Ca_v2.3$ (see Suppl. Tab. 4–6) were analyzed using the Chi-square test to check for Mendelian inheritance. For details on the statistical procedure see Montoliu et al. (2012)[115] Power values are given in brackets.

(A) $Ca_v3.2$ Breeding scheme	♂ + ♀	♀	♂
$Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$	5.93 ± 0.36	3.07 ± 0.25	3.07 ± 0.23
$Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$	6.35 ± 0.37	3.05 ± 0.21	3.64 ± 0.29
$Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$	4.98 ± 0.25	2.48 ± 0.17	2.88 ± 0.19
	p = 0.0109	**p = 0.0839**	**p = 0.0845**
(B) $Ca_v2.3$ Breeding scheme	♂ + ♀	♀	♂
$Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$	6.35 ± 0.28	3.35 ± 0.16	3.30 ± 0.20
$Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$	7.00 ± 0.32	3.43 ± 0.24	3.79 ± 0.23
$Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$	6.81 ± 0.27	3.54 ± 0.18	3.44 ± 0.22
	p = 0.2791	**p = 0.7808**	**p = 0.2923**

Table 2. Litter sizes in $Ca_v3.2$ and $Ca_v2.3$ null mutant breeding. (A) Litter sizes for $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$, $Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$ and $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ breeding schemes. (B) Litter sizes for $Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$, $Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$ and $Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$ breeding schemes. All values are displayed as mean ± SEM.

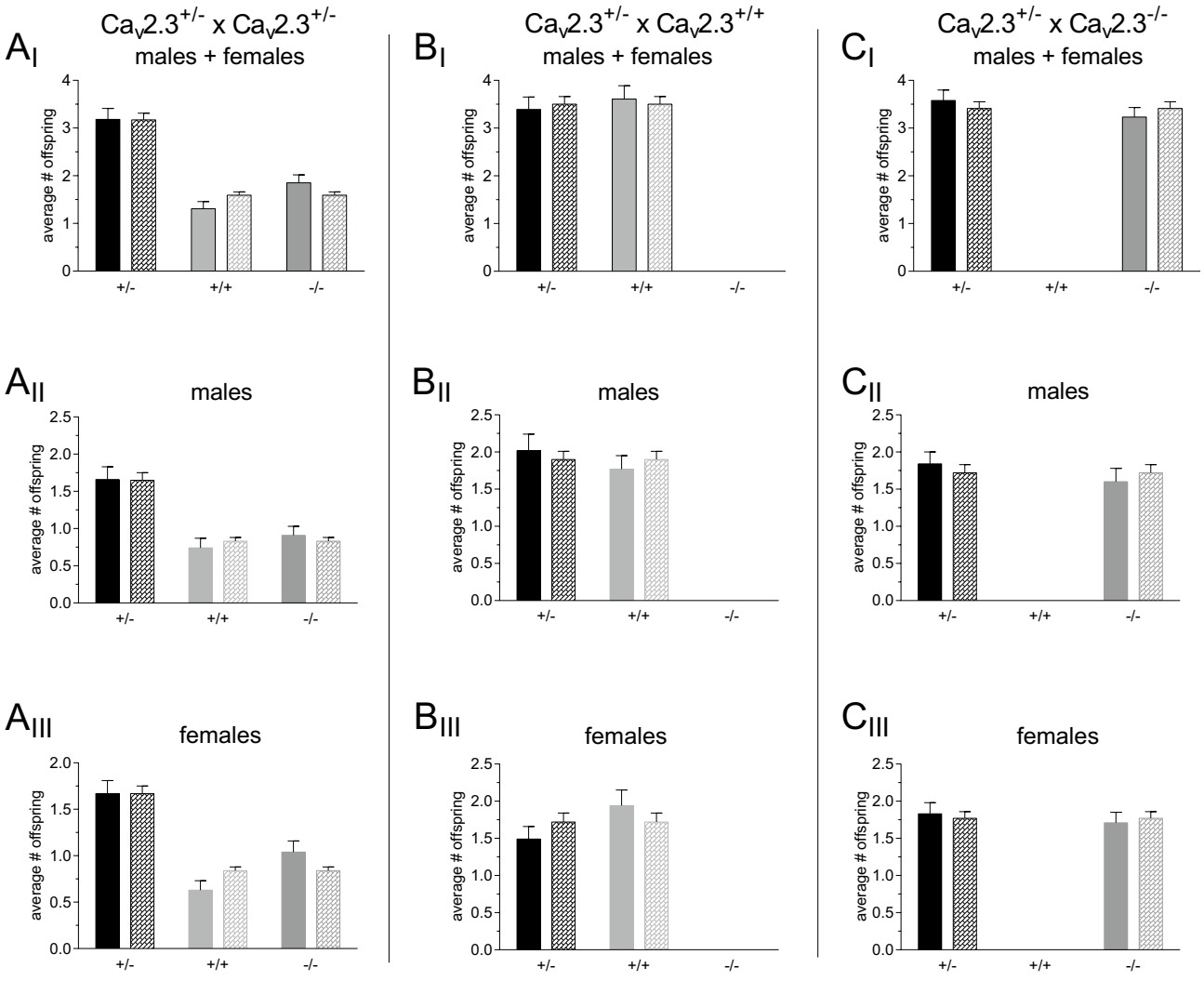

Figure 2. Real and theoretical average number of offspring from different $Ca_v2.3$ breeding schemes. (**A_I–C_I**) Breeding results for both sexes using a $Ca_v2.3^{+/-} \times Ca_v2.3^{+/-}$, $Ca_v2.3^{+/-} \times Ca_v2.3^{+/+}$ and $Ca_v2.3^{+/-} \times Ca_v2.3^{-/-}$ breeding scheme. In addition, these data were analyzed separately for male offspring (**A_{II}–C_{II}**) and female offspring (**A_{III}–C_{III}**). Plain bars indicate real average offspring number, patterned bars indicate the theoretical average offspring number based on the Mendelian inheritance.

Discussion

Our large-scale breeding studies for $Ca_v3.2$ and $Ca_v2.3$ null mutant mice have revealed a complex deviation from Mendelian inheritance for $Ca_v3.2$, but no deviation from Mendelian inheritance for $Ca_v2.3$ null mutants. Importantly, our results on $Ca_v3.2$ mutant breeding are partially confirming previous findings from Alpdogan et al. (2020)[66]. However, our findings for $Ca_v2.3$ mutant breeding are in opposite to what has been reported previously. In the following, we will discuss in detail potential reasons for the deviation from Mendelian inheritance in $Ca_v3.2^{-/-}$ breeding and for the discrepancies observed in $Ca_v2.3^{-/-}$ breeding.

Functional implications of $Ca_v3.2$ allelic loss in $Ca_v3.2$ null mutant inheritance. It has recently been suggested by Alpdogan et al. (2020)[66] that $Ca_v3.2$ and $Ca_v2.3$ deficient mouse lines do not exhibit Mendelian inheritance. Alpdogan et al. (2020) presented a plethora of reasons that might be responsible for this observation and the authors concluded that prenatal lethality might account for the suggested non-Mendelian inheritance. The $Ca_v3.2$ mutant mouse line described by Alpdogan et al. (2020)[66] is the same as we used in our studies. We observed a deviation from Mendelian inheritance for the $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ breeding results with a decrease of $Ca_v3.2^{-/-}$ mice. This observation is similar to what has been described by Alpdogan et al. (2020)[66]. In addition, a deviation from Mendelian inheritance was also detected for $Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$ breeding for both sexes, but not for male and female offspring separately. The latter is in contrast to what has been reported by Alpdogan et al. (2020)[66].

In the past, breeding studies of numerous mutant mouse lines often revealed Mendelian inheritance, following Mendel's first law, i.e., the principle of segregation, and Mendel's second law. i.e., the principle of independent assortment[68] (see also informatics.jax.org; https://www.komp.org). However, some mutant lines were also proven to exhibit deviation from Mendelian inheritance[69,70]. Notably, multiple reasons for exceptions to Mendelian inheritance have been characterized, e.g., polygenic inheritance, incomplete dominance, codominance, multiple alleles, pleiotrophy, epistasis, unstable/dynamic mutations, genomic imprinting, uniparental disomy, other epigenetic inheritance and gene-environment related interactions, and lethality[71–73].

Currently, there is no scientific evidence that any of the aforementioned genetic aspects could be responsible for the exceptions to Mendelian inheritance in $Ca_v3.2$ null mutant breeding. One aspect that justifies special attention is the functional involvement of $Ca_v3.2$ VGCCs in sperm and oocyte physiology.

In many species including mice, molecular, pharmacological and electrophysiological studies suggested that VGCCs are involved in spermatogenesis and sperm function, particularly sperm motility and the acrosome reaction[74–84]. The mammalian acrosome reaction is Ca^{2+} dependent and requires a complex spatio-temporal activation of different entities of Ca^{2+} influx, i.e., via $Ca_v3.2$ VGCCs, IP_3 receptors, and TRPC2 channels[85,86]. Early reports suggested the presence of both $Ca_v3.1$ and $Ca_v3.2$ VGCCs in sperm[87]. However, the dominant T-type Ca^{2+} currents in spermatogenic cells turned out to be related to $Ca_v3.2$, as Ca^{2+} current density in spermatogenic cells was not reduced in $Ca_v3.1^{-/-}$ mice compared to control animals[87]. Furthermore, studies in testes from immature and adult mice revealed a complex spatio-temporal transcription pattern for $Ca_v3.2$ VGCCs[88]. The $Ca_v3.2$ function in murine spermatogenesis, sperm motility, capacitation and acrosome reaction was not further evaluated for the potential consequences on breeding upon $Ca_v3.2$ ablation[74,89,90]. However, inhibition of spermatogenic T-type Ca^{2+} channels by genistein was shown to attenuate mouse sperm motility and acrosome reaction[91].

Importantly, the spatio-temporal fine tuning of Ca^{2+}-influx is also critical in maturing oocytes and eggs and proper mammalian development post fertilization[92]. The mouse egg remains arrested at metaphase of the second meiotic division until fertilization triggers sustained Ca^{2+} oscillations[92,93]. These oscillations are critical for the activation of embryonic development in mice[93–98]. Bernhardt et al. (2015) demonstrated in mouse eggs that $Ca_v3.2$ VGCCs are a prerequisite for proper accumulation of Ca^{2+} during oocyte maturation, for Ca^{2+} influx following fertilization, and for proper egg activation[92]. In $Ca_v3.2^{+/+}$ eggs, characteristic T-type Ca^{2+} currents were detected which are in accordance with previous studies[99]. As expected, T-type Ca^{2+} currents were reduced by 44% in $Ca_v3.2^{+/-}$ eggs (compared to $Ca_v3.2^{+/+}$ eggs) and not measurable in $Ca_v3.2^{-/-}$ eggs. Thus, $Ca_v3.2$ VGCCs seem to represent the only functional T-type Ca^{2+} channel in mouse eggs with severe impact on Ca^{2+} homeostasis and dynamics[92]. Importantly, the $Ca_v3.2^{-/-}$ mouse line was originally reported to be viable and fertile[60]. Recent analysis of fertility revealed that the number of pups per litter was significantly reduced in $Ca_v3.2^{-/-}$ females compared to $Ca_v3.2^{+/+}$ females[92]. These findings are in accordance with the results of our large-scale breeding studies in which a reduced litter size from $Ca_v3.2^{-/-}$ females was detected. Also, the results of Bernhardt et al. (2015) are in line with our observation of fewer homozygous mutant mice than expected in the $Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$ breeding scheme and a relative increase in $Ca_v3.2^{+/-}$ mice[92]. As $Ca_v3.2$ null mutant mice are not completely infertile, it was also suggested that additional Ca^{2+} entry mechanisms may act as a partial compensatory mechanism to sustain Ca^{2+} oscillations[92].

Current scientific data point to the fact that the favorite explanation for the observed deviation from Mendelian inheritance in $Ca_v3.2$ null mutant breeding originates from the important roles of $Ca_v3.2$ VGCCs during oocyte maturation and following fertilization[92] as well as the implications in spermatogenesis, sperm motility and acrosome reaction[74,77–79,89,90]. As genotyping in our study was carried out at the post weaning state, we do not have information about a potential decrease in null alleles at the pre and post-embryonic stage. Litter size analysis for our breeding schemes revealed alterations for offspring of both sexes, but not for separate analysis of male or female offspring (Table 2A). We cannot comment on knockout and wild-type litter sizes, as we did not breed $Ca_v3.2^{-/-} \times Ca_v3.2^{-/-}$ or $Ca_v3.2^{+/+} \times Ca_v3.2^{+/+}$. In summary, transmission ratio distortion with biased genotype distribution and reduced litter size often gives rise to either selective embryonic lethality (impaired embryonic development at the pre- or post-implantation state) or reduced oocyte production (dysgametogenesis)[73]. Whether prenatal lethality—as previously suggested by Alpdogan et al. (2020)[66]—accounts for the reduced number of $Ca_v3.2^{-/-}$ mice and reduced litter size remains to be proven in the future.

Functional implications of $Ca_v2.3$ allelic loss in $Ca_v2.3$ null mutant inheritance. As regards the breeding of $Ca_v2.3$ deficient mice, we were not able to confirm a deviation from Mendelian inheritance as reported by Alpdogan et al. (2020)[66]. Four $Ca_v2.3^{-/-}$ models have been generated so far, i.e., the "Miller $Ca_v2.3$ model/Chicago"[100], the "Tanabe $Ca_v2.3$ model/Tokyo"[36], the "Schneider $Ca_v2.3$ model/Cologne"[15] and the "Shin $Ca_v2.3$ model/Seoul"[37]. The genetic engineering specificities and backgrounds of all these models were reviewed in detail before by Weiergräber et al. (2006)[26]. Importantly, the mutant $Ca_v2.3$ line we used here in our study ("Miller $Ca_v2.3$ model") was different from the one used by Alpodogan et al. (2020) ("Schneider $Ca_v2.3$ model")[66]. What both lines have in common is that they represent constitutive knockout models breed into C57BL/6 J mice[26]. Thus, the observed discrepancies between both inheritance studies might be based on the genetic specificities and the underlying strategies of genetic engineering of the mutant $Ca_v2.3$ model described by Alpodogan et al. (2020)[66] and the $Ca_v2.3$ null mutant model that we used. The mutant $Ca_v2.3$ line which our study is based on, was the first $Ca_v2.3^{-/-}$ model to be described in literature[100] and is widely used in the scientific community[31,38,39,101]. Based on the gene inactivation strategy in this model, the potential existence of a protein remnant/fragment, i.e., a truncated form of $Ca_v2.3$ cannot be fully ruled out. However, there is no evidence that such truncated forms of $Ca_v2.3$ are expressed and thus their existence remains speculative[100]. Importantly, it has been demonstrated that neither fragments of domain I-II or domain III-IV of, e.g., $Ca_v2.2$, another HVA non L-type Ca^{2+} channel closely related to $Ca_v2.3$, can form functional channels when expressed individually, together with accessory subunits such as β_{1b} and $\alpha_{2\delta1}$[102]. Therefore, there is no molecular, biochemical or electrophysiological evidence that suggests or even proves the formation of functional $Ca_v2.3$-like channels based on potential two domain fragments in the model we used. Also, there are no indications that such potential fragments could be cytotoxic and influence the inheritance pattern. Notably, we previously checked for compensatory mechanisms in the $Ca_v2.3^{-/-}$ model ("Miller $Ca_v2.3$ model") and carried out real-time PCRs on other VGCCs[31]. We also performed micro-array analysis of brains from our $Ca_v2.3^{+/+}$ and $Ca_v2.3^{-/-}$ animals that also did not reveal significant compensatory up- or down-regulation of other genes in the $Ca_v2.3^{-/-}$ model described in Wilson et al. (2000)[100]. In summary, we do not have evidence that the genetic manipulation of the $Ca_v2.3$ null mutant line used here affects the inheritance pattern.

Importantly, the $Ca_v2.3^{-/-}$ mice described by Alpdogan et al. (2020)[66] might also generate a protein remnant, i.e., a N-terminal $Ca_v2.3$ peptide fragment[15]. The N-terminus of Ca_v2 Ca^{2+} channels is not only involved in G-protein regulation but also responsible for dominant negative (cross-) suppression of Ca_v2 channels in general[103]. It is essential to note that a reduction/elimination of $Ca_v2.3$ expression shown by Western blotting using antibodies directed against domain I or domain IV does not rule out the potential existence of such an N-terminal protein fragment in this model ("Schneider $Ca_v2.3$ model")[15]. However, the existence of such fragments and their potential devastating impact on e.g., gametogenesis (spermatogenesis/oogenesis) remains speculative as well. Given the lack of available micro-array data from this model, compensatory mechanisms that might account for the observed deviation from Mendelian inheritance in Alpdogan et al. (2020)[66] cannot be ruled out.

It should also be noted that the mouse model used in Alpdogan et al. (2020) had first been described by Sochivko et al. (2002) and Pereverzev et al. (2002). The latter publications originally stated that genotyping the offspring from heterozygous $Ca_v2.3^{+/-}$ matings exhibited a Mendelian inheritance and that the general ablation of $Ca_v2.3$ was not embryonically lethal. This suggests that other parameters, e.g. backcrossing strategies or environmental factors/changes might have interfered with their results and the obvious alterations in inheritance patterns of their $Ca_v2.3$ null mutant breeding. Alpdogan et al. (2020) did not further comment on this contradictory description of the inheritance pattern in their model.

Given the important physiological roles of $Ca_v2.3$ R-type VGCCs, e.g., in the cardiovascular system and germ cell physiology, it is tempting to hypothesize that ablation of this channels might have severe effects on prenatal development and might thus influence the inheritance pattern. In the heart for example, $Ca_v2.3$ is involved in the impulse generating and conduction system, but also the autonomic cardiac control[104]. Although a number of cardiac electrophysiological alterations have been described in $Ca_v2.3^{-/-}$ mice using multi-electrode arrays (MEA) and radiotelemetric electrocardiographic (ECG) recordings, there are no indications that these alterations directly impair the lifespan of $Ca_v2.3$ deficient mice or cause prenatal lethality[18–20,105].

Another aspect that warrants attention is the expression of $Ca_v2.3$ VGCCs in sperms. Several publications have suggested the expression of $Ca_v2.3$ in mature sperms, pachytene spermatocytes and other spermatogenic cells[106,107]. In the $Ca_v2.3$ null mutant model generated by Tanabe's group, ablation of the $Ca_v2.3$ Ca^{2+} channel resulted in reduced Ca^{2+} transients in the sperm head region and impaired sperm motility[107,108]. These findings also suggest that $Ca_v2.3$ VGCCs contribute to the control of flagellar movement, particularly the asymmetry in flagellar beat and randomized swimming patterns[108]. The latter seems to be based on $Ca_v2.3$ expression on the proximal segment of the principal piece of mouse sperm and is thus important for chemotaxic orientation[108,109]. Importantly, it turned out that the effect of $Ca_v2.3$ ablation on flagellar movement was medium-dependent, e.g., on the bicarbonate concentration. Furthermore, the motility of sperms is known to depend on the complex intravaginal/intrauterine environment[110]. We are still lacking information how $Ca_v2.3^{-/-}$ sperm act in the in vivo environment of the mouse female reproductive tract. Notably, there might be differences in this reproductive environment between the various $Ca_v2.3$ null mutant lines that affects breeding results. Interestingly, Sakata et al. (2002) reported that Ca^{2+} transient induced by KCl mediated depolarization tended to be higher in $Ca_v2.3^{-/-}$ sperm compared to $Ca_v2.3^{+/+}$ sperm. This and further findings indicate that other VGCCs might (over) compensate the lack of Ca^{2+} influx in $Ca_v2.3$ null mutant sperms[108,111–113]. Sakata et al. (2002) also did not report about an exception from Mendelian inheritance[108]. Later, Cohen et al. (2014) elaborated in detail the relevance of $Ca_v2.3$ in acrosome reaction and the authors reported subfertility (smaller offspring size), e.g., in knockout breeding compared to wild-type breeding[101]. As we never bred homozygous $Ca_v2.3$ null mutant mice ($Ca_v2.3^{-/-} \times Ca_v2.3^{-/-}$) or wild-type animals ($Ca_v2.3^{+/+} \times Ca_v2.3^{+/+}$), we cannot comment on these findings based on our own

large-scale breeding. For our breeding schemes, analysis of litter sizes did not reveal any significant alterations, neither for offspring of both sexes, nor for male and female offspring separately (Table 2B).

It is essential to note that previous phenotyping studies on $Ca_v2.3$ null mutant mice did not always reveal consistent findings. Whereas impairment of glucose tolerance and insulin release, for example, was described consistently in both the "Tanabe $Ca_v2.3$ model"[114] and the "Schneider $Ca_v2.3$ model"[15], substantial discrepancies were found for thalamocortical oscillations between the "Shin $Ca_v2.3$ model"[44] and the "Schneider $Ca_v2.3$ model"[45]. The same held true for sleep architecture and circadian rhythmicity between the "Schneider $Ca_v2.3$ model"[32] and the "Miller $Ca_v2.3$ model"[31]. Differences between the models might thus also affect the reproductive system.

Conclusions

1. Our results from large-scale breeding studies partially confirm a previous report about a deviation from Mendelian inheritance in the $Ca_v3.2$ null mutant line[66]. Whether this phenomenon is related to prenatal lethality—as suggested by Alpdogan et al. (2020)—cannot be specified here, as no scientific evidence is yet available to prove this hypothesis. It might be speculated that the described role of $Ca_v3.2$ VGCCs in spermatogenesis, oogenesis, fertilization and embryonic development is responsible for the observed exceptions to Mendelian inheritance.

2. We cannot confirm a deviation from Mendelian inheritance in $Ca_v2.3$ null mutant breeding. This discrepancy might be due to the specificities in genetic engineering in both models and related physiological consequences. Although $Ca_v2.3$ VGCCs are involved in sperm physiology as well, there is no direct scientific evidence that a lack of $Ca_v2.3$ alters classic inheritance. Importantly, we have no indication of prenatal lethality in the $Ca_v2.3$ null mutant line that we used in our study.

3. Four different $Ca_v2.3$ null mutant lines have been generated and there are examples of physiological discrepancies between these models, e.g. in the field of sleep architecture and circadian rhythmicity or in inheritance patterns as outlined in this study. Intrinsic phenomena related to the specificities of genetic engineering and compensatory mechanisms upon gene inactivation might account for such phenotypic variation. Though resource-intensive, our results suggest that physiological studies should be carried out and confirmed in more than one null mutant line if possible.

Methods

$Ca_v3.2$ mutant mouse line. Controls ($Ca_v3.2^{+/+}$), heterozygous ($Ca_v3.2^{+/-}$) and $Ca_v3.2$ deficient ($Ca_v3.2^{-/-}$) mice were generated from cryopreserved heterozygous embryos obtained via the Mutant Mouse Resource & Research Centers (MMRRC, supported by NIH). For further details, see MMRCC stock number 9979, strain name: B6.129-Cacna1htm1Kcam/Mmmh, strain of origin: C57BL/6 × 129, strain genetic background: C57BL/6[60,62,63]. The $Ca_v3.2$ mutant mice were used in different projects of our group for several years[62–65].

Professional breeding under state-of-the-art conditions was carried out in the central animal facility of the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM, Bonn, Germany) under the aegis of the German Center for Neurodegenerative Diseases (Deutsches Zentrum für Neurodegenerative Erkrankungen, DZNE, Bonn, Germany).

All animal procedures were performed according to the guidelines of the German Council on Animal Care, and all protocols were approved by the local institutional and national committee on animal care (State Agency for Nature, Environment and Consumer Protection; Landesanstalt für Natur, Umwelt und Verbraucherschutz, LANUV, Germany, AZ 87-51.04.2010.A321, AZ 84-02.04.2013.A426). The authors further certify that all animal experimentation was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24th November 1986 (86/609/ EEC) and September 22nd, 2010 (2010/63/EU). In all related projects[62–65,67], specific effort was made to minimize the number of animals used and their suffering (3R strategy).

Breeding of $Ca_v3.2^{+/+}$, $Ca_v3.2^{+/-}$ and $Ca_v3.2^{-/-}$ mice. For breeding, three different approaches were performed, i.e., mating heterozygous mice ($Ca_v3.2^{+/-} \times Ca_v3.2^{+/-}$), heterozygous with control mice ($Ca_v3.2^{+/-} \times Ca_v3.2^{+/+}$) and heterozygous with knockout mice ($Ca_v3.2^{+/-} \times Ca_v3.2^{-/-}$). For quantitative aspects, see the "Results" section.

Genotyping of $Ca_v3.2$ mutant mice. $Ca_v3.2$ mutant mice were genotyped by polymerase chain reaction (PCR) based on the protocol of the KAPA Mouse genotyping kit (Sigma Aldrich, Germany). As described previously, the following primers were used: WT-forward: 5′-ATT CAA GGG CTT CCA CAG GGT A-3′, WT-reverse/ KO-reverse: 5′-CAT CTC AGG GCC TCT GGA CCA C-3′, KO-forward: 5′-GCT AAA GCG CAT GCT CCA GAC TG -3′ (see[60,62,63]). PCRs were carried out using a C1000 thermal cycler (BioRad, Germany) with initial denaturation (94 °C for 3 min), followed by 35 cycles (denaturation, 94 °C for 15 s; annealing, 61 °C for 15 s; extension 72 °C for 15 s) and final extension (72 °C for 1 min). Finally, PCR products were separated using agarose gel electrophoresis and visualized by ChemiDoc Touch (BioRad, Germany). Examples of our genotyping of $Ca_v3.2$ mutant mice are provided in detail in[62,63]. Note that genotyping of all experimental animals was carried out twice per animal (see supplementary tables 1–3) at the post weaning state. Further molecular details on the mutant $Ca_v3.2$ line are also described by Chen et al. (2003)[60]. The reduction/absence of the $Ca_v3.2$ expression in $Ca_v3.2^{+/-}$ and $Ca_v3.2^{-/-}$ mice was further proven by our group using the Western blot approach[62,63].

$Ca_v2.3$ mutant mouse line. $Ca_v2.3^{+/-}$ embryos (kindly provided by Richard J. Miller; Department of Neurobiology Pharmacology, and Physiology; The University of Chicago; Chicago) were re-derived with C57BL/6 J mice and maintained with random intra-strain mating obtaining all genotypes, i.e., $Ca_v2.3^{+/+}$, $Ca_v2.3^{+/-}$ and

$Ca_v2.3^{-/-}$ (Wilson et al., 2000). The mutant line was originally generated by the use of homologous recombination. The S4–S6 region of domain II was replaced with a neomycin/URA3 selection cassette. A null allele of Cacna1e was obtained by removal of the pore-lining and its neighboring transmembrane regions. No $Ca_v2.3$ transcript was detected in Northern blot analysis[100] and no $Ca_v2.3$ protein was found in Western blot analysis in $Ca_v2.3$ knockouts[100]. The resultant $Ca_v2.3^{-/-}$ mice represent a constitutive knockout. The $Ca_v2.3$ mutant mice were used in different projects of our group for several years[31,38,39,67].

As for the $Ca_v3.2$ mutant line, professional breeding of $Ca_v2.3$ mutant mice was carried out under state-of-the-art conditions in the central animal facility of the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM, Bonn, Germany) under the aegis of the German Center for Neurodegenerative Diseases (Deutsches Zentrum für Neurodegenerative Erkrankungen, DZNE, Bonn, Germany).

All animal experimentation was carried out according to the guidelines of the German Council on Animal Care, and all protocols were approved by the local institutional and national committee on animal care (State Agency for Nature, Environment and Consumer Protection; Landesanstalt für Natur, Umwelt und Verbraucherschutz, LANUV; AZ 87-51.04.2010.A321, AZ 84-02.04.2013.A426). The authors further certify that all animal experimentation was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24th November 1986 (86/609/ EEC) and of 22ndSeptember 2010 (2010/63/EU). Specific effort was made to minimize the number of animals used and their suffering (3R strategy).

Breeding of $Ca_v2.3^{+/+}$, $Ca_v2.3^{+/-}$ and $Ca_v2.3^{-/-}$ mice. For breeding, three different approaches were carried out, i.e., mating heterozygous mice ($Ca_v2.3^{+/-}$ × $Ca_v2.3^{+/-}$), heterozygous with control mice ($Ca_v2.3^{+/-}$ × $Ca_v2.3^{+/+}$) and heterozygous with knockout mice ($Ca_v2.3^{+/-}$ × $Ca_v2.3^{-/-}$). For quantitative aspects, see the "Results" section.

Genotyping of $Ca_v2.3$ mutant mice. $Ca_v2.3$ mutant mice were genotyped by PCR based on the protocol of the KAPA Mouse genotyping kit (Sigma-Aldrich, Germany). The following primers were used: WT forward 5′-GGC TGC TCT CCC AGT ATA CT-3′; WT reverse/KO reverse 5′-CAG GAA GCA TCA CTG CTT AG-3′; KO forward 5′-ATT GCA GTG AGC CAA GAT TGT GCC-3′. PCR was carried out using the C1000 thermal cycler (Bio-Rad) with an initial denaturation (94 °C for 3 min) followed by 35 cycles (each cycle containing the following steps: denaturation 94 °C for 15 s, annealing 59 °C for 15 s, extension 72 °C for 15 s) and final extension (72 °C for 1 min). Subsequently, PCR products were separated via agarose gel electrophoresis and detected by ChemiDoc Touch (Bio-Rad). For details on the procedure and genotyping results see also[28,31,38,39]. Note that genotyping of all experimental mice was carried out twice per animal (see supplementary tables 4–6) at the post weaning state. Further molecular characterization of the model is provided by Wilson et al. (2000)[100]. The reduction/absence of the $Ca_v2.3$ expression in $Ca_v2.3^{+/-}$ and $Ca_v2.3^{-/-}$ mice was further proven by our group using the Western blot approach[38,39].

Statistics. As widely used in genetics, Pearson's chi-square test was used to check for Mendelian inheritance. The procedure applied here was described in detail by Montoliu et al. (2012)[115] (see Table 1). Litter size analysis was carried out using One-Way ANOVA. Statistical analysis and graphical representations were conducted using GraphPad Prism (version 6) for Windows (Graphpad Software, Inc., USA). All data were displayed as mean ± standard error of the mean (SEM).

Data availability

All relevant data are provided within this manuscript and the related supplementary information.

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