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Meis1 effects on motor phenotypes and the sensorimotor system in mice

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SUMMARY STATEMENT

Loss of Meis1 results in motor restlessness in mice; a phenotype resembling human restless legs syndrome, as well as altered sensorimotor gating and improved social discrimination memory.

ABSTRACT

MEIS1 is a developmental transcription factor linked to restless legs syndrome (RLS) in genome-wide association studies. RLS is a movement disorder leading to severe sleep reduction and with significant impact on the quality-of-life of patients. In genome-wide association studies, *MEIS1* has consistently been the gene with the highest effect size and functional studies suggest a disease-relevant downregulation. Therefore, haploinsufficiency of Meis1 could be the most potential system for modeling RLS in animals. We used heterozygous *Meis1* knock-out mice to study the effects of *Meis1* haploinsufficiency on mouse behavioral and neurological phenotypes, and to relate the findings to human RLS. We exposed the *Meis1*-deficient mice to assays of motor, sensorimotor and cognitive ability and assessed the effect of a dopaminergic receptor 2/3 agonist commonly used in the treatment of RLS. The mutant mice showed a pattern of circadian hyperactivity, compatible with human RLS. Moreover, we discovered a replicable prepulse inhibition (PPI) deficit in the Meis1-deficient animals. In addition, these mice were hyposensitive to the PPI-reducing effect of the dopaminergic receptor agonist, highlighting a role of Meis1 in the dopaminergic system. Other reported phenotypes include enhanced social recognition at an older age that was not related to alterations in adult olfactory bulb neurogenesis previously shown to be implicated in this behavior. In conclusion, the *Meis1*-deficient mice fulfill some of the hallmarks of an

RLS animal model, and revealed the role of Meis1 in sensorimotor gating and in the dopaminergic systems modulating it.

INTRODUCTION

MEIS1 encodes a TALE homeobox transcription factor, known to play a role in hematopoiesis and vascular patterning (Azcoitia et al., 2005; Carramolino et al., 2010; Hisa et al., 2004), as well as in the development of the proximodistal limb axes (Mercader et al., 1999), the nervous system (Barber et al., 2013; Spieler et al., 2014) and the development of various organs, such as the heart (Mahmoud et al., 2013), eyes (Marcos et al., 2015) and pancreas (Zhang et al., 2006). *MEIS1* cooperates with other transcription factors to perturb myeloid differentiation in leukemogenesis (Nakamura et al., 1996), and in genetic association studies, it has been linked with restless legs syndrome (RLS) (Winkelmann et al., 2007; Winkelmann et al., 2011), periodic leg movements during sleep (Moore et al., 2014; Stefansson et al., 2007), symptoms of insomnia (Lane et al., 2016), PR interval (Butler et al., 2012) and waist-to-hip ratio (Shungin et al., 2015). Structurally, Meis1 consists of a Pbx interaction motif and a TALE homeodomain near the C-terminus, responsible for DNA interaction. It has two major functional splice variants, Meis1a and Meis1b, which differ in the C-terminus of the protein (Huang et al., 2005), predicted to regulate the expression of differing sets of genes and direct neural differentiation to separate directions (Maeda et al., 2001). Meis1 binds transcription start sites and genomic enhancers alone and in interaction with *PBX* and *HOX* proteins, regulating the expression of genes involved in the processes of axon guidance, protein phosphorylation and cell differentiation, among others (Marcos et al., 2015; Penkov et al., 2013).

RLS is a sensorimotor movement disorder causing severe sleep loss and impaired quality-of-life (Trenkwalder and Paulus, 2010). The hallmark symptoms include an urge to move the legs, relieved by voluntarily moving the legs. The symptoms are predominantly present in the evening and night time and at rest. Epidemiological evidence shows that RLS is

more common in females compared to males (Berger and Kurth, 2007), but the sex difference could be explained by the number of previous pregnancies (Pantaleo et al., 2010). In the context of RLS, *MEIS1* contributes to the heritability of the disease through both common and rare genetic variation. In genome-wide association studies (GWAS), Meis1 has consistently been the gene with the largest effect size with an odds ratio up to 1.74 (Winkelmann et al., 2007; Winkelmann et al., 2011). In large-scale sequencing and family studies, putative causal loss-of-function rare variants have been identified (Schulte et al., 2014). A *MEIS1* intronic haplotype linked to RLS risk is associated with decreased expression of MEIS1 (Xiong et al., 2009). Therefore, Meis1 haploinsufficiency could be considered the most potential animal model system for human RLS. Meis1 knock-out mice, where Meis1 has been inactivated by knocking in a modified ERT2 domain ($Meis1^{tm1Mtor}$), die during the development due to a fetal hematopoietic failure (Azcoitia et al., 2005). Heterozygous animals, however, show a hyperactive phenotype potentially compatible with components of human RLS (Spieler et al., 2014). In addition, the mutant animals show low fertility, low body mass at birth and various eye-related phenotypes (Azcoitia et al., 2005; Marcos et al., 2015).

In a bid to provide a more comprehensive understanding of the effect of *Meis1* haploinsufficiency on the central nervous system, we exposed these heterozygous *Meis1*-deficient mice to a series of motor, sensorimotor and cognitive behavioral and neurological tests. To further disentangle what role this gene plays in the brain and how it could be involved in RLS, we also determined the effect of a commonly used RLS therapeutic dopaminergic receptor 2/3 (D2/3R) agonist pramipexole as well as performing concomitant semi-quantitative measures of dopaminergic neuron number, iron metabolism, believed to

play an important role in the pathogenesis of RLS, and adult neurogenesis, the birth and assimilation of new neurons..

RESULTS

Meis1 haploinsufficiency leads to sex-dependent motor alterations at middle age

We have shown previously (Spieler et al., 2014) that Meis1 haploinsufficiency leads to increased locomotor activity in the open field in both male and female young adult mutant mice at the age of 9 weeks. Furthermore, these animals were hyperactive as indexed by a tendency to increased locomotor activity over a 21 hour period in the homecage environment of the PhenoMaster cages (Spieler et al,. 2014). In the current study, we determined whether the severities of these effects are maintained with age. Thus, we assessed the same locomotor activity parameters in middle aged mice (9-11 animals per genotype and sex) from the age of 38-40 weeks.

At this older age, Meis1 haploinsufficiency leads to locomotor alterations that dichotomise based on sex. This was manifest as a significant locomotor increase in the male mutant mice in the open field not evident in the females (2-way ANOVA, sex x genotype interaction effect: $F(1,43) = 5.64$, $p = 0.022$, Fig. 2C). Locomotor activity alterations in response to the open field could reflect an anxiety reaction to the novelty of the arena even though this was not evident in the anxiety-related index of centre time in the open field (2-way ANOVA, sex x genotype interaction effect: $F(1,43) = 0.16$, $p = 0.69$, genotype effect: $F(1,43) = 0.12$, $p = 0.72$, sex effect: $F(1,43) = 0.83$, $p = 0.37$). Thus, we sought to establish if this hyperactivity in the male mutant mice could be confirmed with longer term observation in the PhenoMaster cages where the mice are permitted to habituate to the surroundings. It is then possible to see if the locomotor differences are also present when exploration is the principal behavior exhibited by the mice during their active phase and if it is subject to circadian variation,

expected from an RLS animal model. With this analysis, we again saw that Meis1 haploinsufficiency caused increased locomotor activity in middle-aged males and this was evident over several one-hour time bins, most importantly in the beginning of the inactive phase (1-way ANOVA, genotype effect $F(1,21) = 4.92$, $p = 0.038$) (Fig. 2B). By dividing the 21-hour period into sub-phases it transpired that the male mutant mice were hyperactive on first transfer to the PhenoMaster cages during the habituation phase (first 3 hours). Control levels of activity were then attained until after lights off when the male mutant mice again show higher activity during the active phase. The activity levels in the control male mice continued to decrease until lights on. Nevertheless, the male mutant mice showed another peak in activity at the start of the lights-on phase when the controls were at rest. The effects of Meis1 haploinsufficiency in the female mice on homecage locomotor activity was less pronounced (Fig. 2A). During the last hour of the habituation phase, the female mutant mice showed increased activity compared to controls without major differences throughout the remainder of the testing period.

As an additional form of locomotor activity analysis, we measured voluntary wheel running within the homecage (Fig. 2D and 2E). Each mouse was given access to a running wheel, voluntary activity was recorded continuously and the data was averaged over 21 days to yield a 24 h activity profile. Meis1 haploinsufficiency in the male mice did not produce differences in the amount or pattern of voluntary running wheel activity (Fig. 2E). Nevertheless, the female mutant mice, while not exhibiting marked deviations from the control profile, showed a specific increase in activity during the first hour of the lights-on phase when the control mice are at rest (Fig. 2D).

As a measure of motor coordination and balance we also assessed latency to fall from the rotarod. There was a small tendency for the male mutant mice to show increased latencies to fall from the rod without clear differences in the females (2-way ANOVA, sex x genotype

interaction effect: F(1,39) = 1.431, p = 0.239, genotype effect: F(1,39) = 0.133, p = 0.294, sex effect: F(1,39) = 0.145, p = 705).

Together these data, with our previously published findings, indicate that Meis1 haploinsufficiency causes sex, age and situation-dependent effects on motor restlessness in mice.

Meis1 deficiency causes sensorimotor gating deficits without effects on thermal sensitivity

An alteration in thermal sensitivity has been shown in human patients with secondary RLS and intense unpleasant feelings in the legs characterize the disease (Bachmann et al., 2010). It was thus germane to assess sensory perception consequent to *Meis1* haploinsufficiency. As a measure of thermal sensitivity, we assessed the effect of *Meis1* haploinsufficiency in the hotplate test in mice at the younger age of 11 weeks. There were no significant nociceptive differences detected between the genotype groups in licking or shaking behavior, or response times (Table 1). At the age of ten weeks, the animals were also tested for acoustic startle reactivity (ASR) and prepulse inhibition (PPI) of the acoustic startle reflex. ASR was decreased in male heterozygous mutant mice with the opposite effect in female mutants compared to controls (Repeated measures ANOVA: genotype x sex x dB interaction effect, p < 0.001) (Supplemental Figure). PPI was decreased in mutant mice of both sexes at 69 (2-way ANOVA, genotype effect: F(1,56) = 7.132, p = 0.01, sex effect: F(1,56) = 0.577, p = 0.45, sex x genotype interaction effect: F(1,56) = 0.504, p = 0.48) and 73 dB (2-way ANOVA, genotype effect: F(1,56) = 5,04, p = 0.03, sex effect: F(1,56) = 0.206, p = 0.65, sex x genotype interaction effect: F(1,56) = 0.767, p = 0.38), as well as globally across all prepulse intensities (2-way ANOVA, genotype effect: F(1,56) = 5.79, p = 0.019, sex effect: F(1,56) = 0.228, p = 0.63, sex x genotype interaction effect: F(1,56) = 1.233, p = 0.27, Figure

3A). It was possible to replicate the effect of *Meis1* deficiency on PPI in an independent cohort of mice at the age of 9 weeks where again a decrease in PPI was observed (69 dB (2-way ANOVA, genotype effect: $F(1,60) = 10.575$, $p = 0.002$, sex effect: $F(1,60) = 10.88$, $p = 0.0016$, sex x genotype interaction effect: $F(1,60) = 0.009$, $p = 0.93$), 73 dB (2-way ANOVA, genotype effect: $F(1,60) = 12.227$, $p = 0.001$, sex effect: $F(1,60) = 5.74$, $p = 0.020$, sex x genotype interaction effect: $F(1,60) = 0.024$, $p = 0.88$) and 81 dB (2-way ANOVA, genotype effect: $F(1,60) = 7.198$, $p=0.009$, sex effect: $F(1,60) = 9.47$, $p = 0.0031$, sex x genotype interaction effect: $F(1,60) = 0.004$, $p = 0.95$) as well as globally (2-way ANOVA, genotype effect: $F(1,60) = 13.723$, $p<0.001$, sex effect: $F(1,60) = 9.14$, $p = 0.0037$, sex x genotype interaction effect: $F(1,60) = 0.313$, $p = 0.58$) in both sexes (Figure 3B)). The effect on ASR was not observed in this cohort (the opposite effect was found) and was thus not replicable. The assessment of ASR and PPI was not possible in the middle-aged cohort due to natural age-related sensorineural hearing loss, associated with the genetic background used. The hearing loss was not specific to either genotype group, but was seen in all mice. There were no clear differences in auditory brainstem responses (ABR) in the mice of cohort 1 at 17 weeks (Table 1).

***Meis1* deficiency confers insensitivity to D2/3R agonist effects on sensorimotor gating**

Given this robust and replicable effect of Meis1 haploinsufficiency on sensorimotor gating ability, we wanted to determine the effect of a dopaminergic receptor agonist used in the treatment of RLS on this phenotype. Thus, the effect of pramipexole, a preferential D2/3R agonist, at two doses (1 and 3 mg/kg bodyweight i.p.) was assessed on PPI with saline vehicle control. In the vehicle treated mice, the mutant animals showed a higher reactivity to acoustic startle than the wildtypes at higher sound intensities. PPI was decreased in the mutant mice after vehicle injection, replicating again the findings of the first two cohorts. The

1 mg/kg dose of pramipexole had an effect at high sound intensities in the mutant group, making their startle response even stronger. In the control group, the startle response was only altered at lower sound intensities. The 3 mg/kg dose of pramipexole, on the other hand, increased the startle response of the wildtype mice at higher stimulus intensities but had no effect on the mutant group. The results are shown in Supplemental Figure.

PPI was affected by pramipexole in a more consistent manner. In the wildtype group, PPI was significantly reduced by both high and low doses of pramipexole. The PPI in these animals was reduced to the level of the vehicle-treated mutant mice. In contrast, in the heterozygous mutants the PPI was not significantly altered by pramipexole (Figure 3C).

To investigate whether this insensitivity to the D2/3R agonist pramipexole was influenced by the number of dopaminergic neurons that could lead to altered D2/3R signaling, using optical fractionator estimates we quantified the number of TH-positive cells in the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA). We did not observe clear differences in the total number of TH+ cells in either the SNpc (2-way ANOVA, genotype effect: $F(1,12) = 0.401$, $p = 0.538$, Fig. 4C) or the VTA (2-way ANOVA, genotype effect: $F(1,12) = 0.291$, $p = 0.599$, Fig. 4F) between genotypes to indicate that neuron number differences lead to the altered signaling. Furthermore, we assessed iron homeostasis by measuring the plasma levels of iron, ferritin (iron storage) and transferrin (iron binding) (Table 2) as iron has been suggested to play a major role in RLS pathogenesis in humans (Earley et al., 2014). There were no clear differences between the groups in any of the three measures except for a tendency to a decrease in the iron storage protein ferritin in the female Meis1 deficient mice (2-way ANOVA, genotype x sex interaction effect: $F(1,56) = 3.32$, $p = 0.074$).

Meis1 haploinsufficiency alters social memory

The olfactory social recognition index was increased in both female and male mutant mice compared to wildtypes (Fig 5A, 2-way ANOVA, genotype effect: $F(1,41) = 9.48$, $p = 0.004$, sex effect: $F(1,41) = 0.58$, $p = 0.45$, genotype x sex interaction effect: $F(1,41) = 1.45$, $p = 0.24$), suggesting better social discrimination memory. There were no genotype-related differences in social investigation time during the sample phase of the test (Data not shown). The social recognition index showed no correlation with distance travelled in the open field ($r = -0.050$, $p = 0.749$), with time spent in the middle of the arena ($r = -0.166$, $p = 0.282$) or with total activity in the running wheel ($r = -0.073$, $p = 0.679$). None of the parameters showed significant correlation when the sexes were tested separately (data not shown). We have shown previously that decreased social memory can be due to decreased adult neurogenesis in mice (Garrett et al., 2015). Thus, to determine if the behavioral alterations were associated with modifications of the adult neurogenesis in *Meis1*-deficient mice, a stereological approach was used to quantify the number of DCX+ cells in the granular cell layer of the olfactory bulb (Fig 5B). DCX expression is an immature neuron marker in the adult brain that has also been shown to reflect overall levels of neurogenesis and DCX+ cell quantification can be used as an alternative to BrdU pulse/chase analysis (Couillard-Despres et al., 2005). Optical fractionator estimates showed that there was no significant effect of genotype on the number of DCX+ cells in the granular cell layer of the olfactory bulbs in these mice (Unpaired t-test: $t(10) = 0.401$, $p = 0.697$).

DISCUSSION

Haploinsufficiency of Meis1 is a potential model system for simulating RLS in mice. Here we show that this Meis1 deficit causes a sex- and context-dependent RLS-related phenotype at middle age; impaired sensorimotor gating ability that was refractory to the D2/3R agonist

pramipexole as well as increased social memory. These behavioral abnormalities occurred without differences in thermal sensitivity, plasma iron concentrations or dopaminergic neuron number in the SNpc/VTA. Thus, while Meis1 haploinsufficiency causes the RLS-related phenotype of motor restlessness in mice, the detailed nature of these effects is complex. Furthermore, it is characteristically distinct from other genetic RLS models (e.g. BTBD9 knock-out) highlighting the heterogeneous nature of the effects of these different risk genotypes in this disease.

Motor restlessness in these *Meis1*-haploinsufficient mice was detected previously in young adult male and female mice (Spieler et al., 2014). RLS is, however, more prevalent at an older age in humans (Berger et al., 2004) and therefore we also tested middle-aged mice. Human RLS occurs in a circadian fashion where the patients are typically affected in the evening hours, and therefore a circadian hyperactivity pattern specific to the beginning of the inactive period should be expected from an RLS animal model. So while we have shown here that the overall locomotor effects in the open field and PhenoMaster cages deviate per the sex of the animal in middle age both the male *and* female *Meis1*-haploinsufficient mice still show such an RLS-like phenotype: specific increases in activity during the initial stage of the rest phase. In the males, this occurs in the PhenoMaster cages and in the females, in their voluntary wheel running activity pattern. In males, however, hyperactivity is also observed during the active period, making the effect less specific to the RLS-relevant time bins. *Meis1*-haploinsufficiency thus leads to RLS-like circadian motor restlessness in middle age that, in the case of the females, requires amplification with an additional environmental stimulus. The latter could represent the urge to move, a key feature of human RLS. This outcome further reveals that RLS associated with Meis1 risk haplotypes is likely to produce sexually dimorphic motor effects in middle age.

In terms of sensory function, the *Meis1* deficiency did not alter thermal sensitivity in response to the hotplate, which is incongruous with the effects in the BTBD9 mouse RLS model (Deandrade et al., 2012). Nevertheless, augmented thermal sensitivity is a feature of patients with secondary RLS whereas patients with idiopathic RLS experience different types of sensitivity (Bachmann et al., 2010). We also report here the first evidence to indicate that a Meis1 deficit leads to a reproducibly decreased PPI response. This deficit occurs in the absence of auditory brainstem response alterations suggesting that the PPI phenotype is not secondary to impaired hearing in the mice. PPI of the acoustic startle reflex is a commonly used rodent readout for sensorimotor gating and integration. Impairment of PPI has been observed in neurological disorders (Kohl et al., 2013), and it is most frequently used as a readout in schizophrenia research (Swerdlow and Light, 2015). PPI was not altered in the single study conducted in RLS patients so far (Leon-Sarmiento et al., 2015). However, the patients were not stratified by their *MEIS1* genotype in that study and it is possible that PPI is a phenotype specific to RLS associated with Meis1 risk haplotypes. This prospect is also supported by the lack of prepulse inhibition differences in homozygous BTBD9 knockout mice (Btbd9$^{tm1b(EUCOMM)Wtsi}$); data that we have submitted and is available on the International Mouse Phenotyping Consortium (IMPC) database (http://www.mousephenotype.org; search Btbd9 for procedure: acoustic startle and prepulse inhibition). Thus, PPI should be studied in RLS patients who also are carriers of the *MEIS1* risk allele.

In our treatment experiment, pramipexole, the D2/3R agonist, did not rescue the PPI phenotype in *Meis1*-deficient mice. On the contrary, it reduced the PPI in wildtype mice to the level of the mutants. The effect of pramipexole on wildtype mice contradicts an earlier study, reporting no pramipexole effect on PPI in C57BL/6 mice (Chang et al., 2010).

However, the *Meis1*-deficient mice were bred on a C57BL/6JOlaHsd substrain background and this different outcome could be explained by the high strain-sensitivity of the dopaminergic effect on PPI (Swerdlow et al., 2004). The lack of effect on the mutant animals might indicate that the *Meis1*-deficient mice are hyposensitive to pramipexole, which may signify the involvement of Meis1 in the dopaminergic system. It has been demonstrated in neuropathological studies that there is a higher level of TH, the dopaminergic synthetic enzyme, in the substantia nigra of RLS sufferers and fewer D2 receptors in the putamen correlated with RLS severity (Connor et al., 2009). Thus, given that PPI is regulated by dopaminergic systems of the nucleus accumbens and striatum (Swerdlow et al., 2001) and to test whether it may be a causative factor in the insensitivity to pramipexole in these mice, we quantified the number of TH-positive neurons in the SNpc and VTA. Nevertheless, our results did not indicate significant differences in the numbers of dopaminergic neurons in these areas. It remains possible that the expression of dopamine receptors is reduced in *Meis1*-deficient mice and this will require further study. There were also no differences in plasma iron homeostasis. This again distinguishes the Meis1-haploinsufficient mice from the BTBD9 mice, where the latter also shows decreased serum iron levels (Deandrade et al., 2012). Nevertheless, the BTBD9 mice did not show differences in brain tissue iron levels. Thus, it would be propitious to quantify striatal iron levels in these *Meis1*-deficient mice.

We have shown here, for the first time, a non-sensorimotor cognitive phenotype associated with *Meis1* deficiency. The *Meis1*-deficient mice had improved social memory as indexed by enhanced social recognition ability. There is limited conflicting evidence available concerning the cognitive ability of RLS patients with reports of deficits (Kim et al., 2014), enhanced ability (Moon et al., 2014) and no alterations in different types of memory (Driver-Dunckley et al., 2009; Fulda et al., 2010). These inconsistencies could relate to the

type of memory assessed, the age and sex of the patient, the type of RLS (idiopathic vs. secondary), medication status and, as previously alluded, patients in these studies have not been stratified according to risk haplotypes. Moon and colleagues (Moon et al., 2014) have shown that drug-naïve idiopathic RLS patients, predominantly at middle age, exhibit enhanced verbal fluency and word memory. They conjectured that this increased ability may be an adaptation to sleep disturbance manifesting as heightened alertness with an underlying mechanism unknown. We have demonstrated previously involvement of adult neurogenesis in social discrimination memory in the DCXCreER^{T2}; DTA model where loss of DCX-positive cells and hence decreases in adult neurogenesis reversibly impaired social recognition memory (Garrett et al., 2015). Therefore, given the impaired social recognition memory, we hypothesized that abnormal neurogenesis in the olfactory bulbs of *Meis1*-deficient mice may underlie this deficit. Our quantification of DCX-positive cells in the olfactory bulb did not however support this hypothesis and no differences were observed. This indicates that alternative processes likely mediate the enhanced social memory consequent to Meis1 deficiency. Recent evidence showing zonal Meis1 expression in the main olfactory epithelium could signify one potential avenue for further investigation in this regard (Parrilla et al., 2016). Furthermore, in light of this new evidence, a more detailed analysis of cognitive ability in RLS patients with the Meis1 risk haplotype is warranted.

In summary, the *Meis1*-deficient mice show an RLS-like motor restlessness phenotype, a sensorimotor gating deficiency refractory to a D2/3R agonist and some heretofore unknown *Meis1* deficiency-related effects. Other proposed RLS-related genetic mouse models such as BTBD9 (Deandrade et al., 2012) and PTPRD (Drgonova et al., 2015) exhibit degrees of motor restlessness. There are features of *Meis1*-deficient mice however that are distinct and could be explained by the genetic architecture of the disease. For example, when a motor

phenotype (periodic leg movements during sleep) is used to assess RLS in human genetic studies, the *BTBD9* locus has the highest effect size (Moore et al., 2014; Stefansson et al., 2007). When the sensory RLS symptoms are assessed, the *MEIS1* locus is the top hit (Winkelmann et al., 2007; Winkelmann et al., 2011). This indicates that the specific disease characteristics in patients will depend on the combination of risk alleles at different genetic risk loci and this is supported by the current data. RLS is a genetically heterogeneous complex trait with high prevalence but large phenotype variability and both idiopathic and secondary forms contribute to prevalence statistics. Thus, fine mapping and further genetic studies in RLS patients may reveal differential effects of the various risk loci as observed in these mice and in other studies. In future investigations, an RLS-related mutation within *Meis1* may provide a more disease-relevant disruption of Meis1 function in mice and therefore a better RLS phenotype (Allen et al., 2017).

MATERIALS AND METHODS

Animals and breeding

The Meis1^{tm1Mtor} mice used in the experiments were created in Madrid, Spain (Azcoitia et al., 2005) and have been bred on C57BL/6JOlaHsd (Envigo, Horst, The Netherlands) background. In this strain, *Meis1* has been inactivated by knocking in a modified ERT2 domain. The result is predicted to encode an inactive protein product. The mouse line has been bred in Munich, Germany backcrossing to wildtype C57BL/6JOlaHsd every generation. Mouse husbandry was performed according to the FELASA recommendations (Nicklas et al., 2002) (http://www.felasa.eu) in a controlled specific pathogen free (SPF) hygiene standard environment. The mice had access to *ad libitum* standard feed and water always. All tests

were carried out with approval for the ethical treatment of animals by the responsible authority of the Regierung von Oberbayern (Government of Upper Bavaria).

Analysis of Meis1^{tm1Mtor} mice

Four different mouse cohorts were bred for the experiments described here and the corresponding tests and order performed are shown in Fig. 1. In all experiments, heterozygous animals (Meis1+/-) were used, homozygotes being embryonic lethal (Azcoitia et al., 2005). The mice from the different cohorts were tested together with their wildtype (Meis1+/+) littermates as control animals for behavioral/neurological measures as follows: **cohort 1** (15 male +/+, 15 male +/-, 15 female +/+ and 15 female +/-) in open field (at 9 weeks; published in Spieler et al., 2014); prepulse inhibition (at 10 weeks); hotplate (at 11 weeks); locomotor activity in PhenoMaster cages (at 12 weeks; published in Spieler et al., 2014), plasma iron level analysis (at 16 weeks) and auditory brainstem response (ABR) (at 17 weeks); **cohort 2** (10 male +/-, 13 male +/+, 9 female +/-, 12 female +/+) were tested in: open field (at 38-40 weeks); prepulse inhibition (at 39-41 weeks), rotarod (at 44-46 weeks), locomotor activity in the PhenoMaster cages (at 41-43 weeks); locomotor activity in running wheels (at 48-50 weeks) and social discrimination (at 51-53 weeks); **cohort 3 (**15 male +/+, 15 male +/-, 15 female +/+ and 15 female +/-**)** were tested for prepulse inhibition (at 9 weeks) and a subset of these animals were perfused with paraformaldehyde and used for brain tissue stereological analysis of dopaminergic neuron number (tyrosine hydroxylase+ cells) and adult neurogenesis (doublecortin+ cells). The mice (30 animals per sex per genotype) from **cohort 4** were tested for sensitivity to pramipexole in prepulse inhibition (at 10 weeks for 1mg/kg and at 11 weeks for 3mg/kg). Mice from the first cohort also underwent the standardized phenotype screening at the German Mouse Clinic (www.mouseclinic.de). The test battery included additional screening of the mice in the areas of dysmorphology, clinical

chemistry, energy metabolism, cardiovascular studies, eye function, immunology, allergy, steroid metabolism, lung function and pathology. The screens were performed as described in previous publications (Fuchs et al., 2009; Gailus-Durner et al., 2005; Gailus-Durner et al., 2009) and the results of this analysis can be found on the GMC phenomap (http://www.mouseclinic.de).

Open Field

The Open Field analysis was carried out as we described previously [19,20,22]. It consisted of a transparent and infra-red light permeable acrylic test arena with a smooth floor (internal measurements: 45.5 x 45.5 x 39.5 cm). Illumination levels were set at approx. 150 lux in the corners and 200 lux in the middle of the test arena. Data were recorded and analysed using the ActiMot system (TSE, Bad Homburg, Germany).

Pre-pulse inhibition (PPI) of the acoustic startle response (ASR)

Mice were housed with food and water available ad libitum under standard laboratory conditions. Animals were separated based on sex, but not genotype. PPI was assessed using a startle apparatus setup (Med Associates Inc., VT, USA) including four identical sound-attenuating cubicles. The protocols were written using the Med Associates "Advanced Startle" software. Experiments were carried out between 08:30h and 17:00h. Background noise was 65 dB, and startle pulses were bursts of white noise (40 msec). A session was initiated with a 5-min-acclimation period followed by five presentations of leader startle pulses (110 dB) that were excluded from statistical analysis. Trial types for the PPI included four different prepulse intensities (67, 69, 73, 81 dB); each prepulse preceded the startle pulse (110 dB) by a 50 msec inter-stimulus interval. Each trial type was presented 10 times in random order, organized in 10 blocks, each trial type occurring once per block. Inter-trial

intervals varied from 20-30 sec. This protocol is based on the protocol used in IMPRESS from the International Mouse Phenotyping Consortium (IMPC, see www.mousephenotype.org/impress), adapted to the specifications of our startle equipment.

Drug preparation

Pramipexole (pramipexole dihydrochloride, Sigma Aldrich, St. Louis, Missouri, United States) was prepared in saline and injected at two different doses (1 and 3 mg/kg). For the PPI test with drug treatment, Meis1 and control mice were assigned to receive either pramipexole (1 and 3 mg/kg) or saline vehicle and this was balanced for genotype, startle chamber and treatment. The mice were tested for PPI one hour after administration of the treatment and the intraperitoneal injection volume was 10ml/kg. The experimenter was not blinded during the PPI testing as the test is automated and there was no possibility for observer bias.

Auditory Brainstem response

Auditory Brainstem Response (ABR) measurements were performed using a RP2.1 workstation (Tucker-Davis Technologies, TDT, USA) as previously described1. In short, animals were anaesthetized and electrodes were placed on the vertex (reference) and ventrolateral to the ears (active and ground) of the animals. ABR potentials, as responses to broadband clicks or pure-tone frequencies, were recorded. Frequencies ranged from 6 to 30 kHz with a 6 kHz stepping, and sound pressure levels ranged from 0 to 85 dB with 5 dB steps. Hearing thresholds were determined for each frequency as the lowest SPL producing a measurable ABR pattern response.

Hot plate

In nociception, mice were screened using a hot-plate assay. A mouse was placed on a 28 cm diameter metal surface maintained at 52+0.2°C surrounded by a 20 cm high Plexiglass wall (TSE, Bad Homburg, Germany). Mice remained for 30 seconds on the plate or until they performed one of three behaviors regarded as indicative of nociception: hind paw licking, hind paw shake/flutter or jumping. The latency of the first sign of pain was compared.

Social Discrimination

The Social Discrimination procedure consisted of two 4-min exposures of stimulus animals (ovariectomized 129Sv females) to the test animal in a fresh cage to which the test animal had been moved 2 h prior to testing. During the first exposure, one stimulus animal was exposed to the test animal. After a retention interval of 2h, this stimulus animal was re-exposed to the test animal together with an additional, previously not presented stimulus animal. The duration of investigatory behavior of the test animal towards the stimulus animals was recorded by a trained observer with a hand-held computer. A social recognition index was calculated as time spent investigating the unfamiliar stimulus mouse / time spent investigating both the familiar and unfamiliar stimulus mouse.

Rotarod

The rotarod (Bioseb, Chaville, France) was used to measure fore limb and hind limb motor coordination, balance and motor learning ability. The machine was set up in an environment with minimal stimuli such as noise and movement. The rotarod device is equipped with a computer controlled motor-driven rotating rod. The unit consists of a rotating spindle and five individual lanes, one for each mouse. In general, the mouse is placed perpendicular to the

axis of rotation, with head facing the direction of the rotation. All mice were placed on the rotarod at an accelerating speed from 4 to 40 rpm for 300 sec with 15 min between each trial. In motor coordination testing, mice were given four trials at the accelerating speed at one day. The mean latency to fall off the Rotarod during the trials was recorded and used in subsequent analysis. Before the start of the first trial, mice were weighed.

Locomotor activity in PhenoMaster cages

For the evaluation of home cage locomotor activity, single mice were kept in respirometry cages (PhenoMaster System, TSE Systems, Germany) [24]. The measurement started at 1 pm (CET) after a two hour adaptation to the cages and continued until 10 am the next morning. The set-up allowed the analysis of locomotor activity of individual mice every 20 minutes resulting in 63 readings per individual and trial. Two infrared light beam frames allowed the monitoring of physical activity (lower frame: distance travelled per 20 minutes, upper frame: number of rearings per 20 minutes).

Voluntary Wheel Running

All mice were singly housed and provided with an angled running wheel (diameter 15.50 cm) complete with a wire-less controlled activity counter (Wheel Manager software, Med Associates Inc., VT, USA) for 21 days.

Plasma iron level analysis

Blood samples were collected in the morning at 14 or 15 weeks of age by retrobulbar puncture under isoflurane anesthesia. Determination of clinical chemistry parameters in heparinized plasma samples was done using an AU480 (Beckman-Coulter, Krefeld, Germany) and reagents kits provided by Beckman-Coulter, as described previously (Rathkolb

et al., 2013). Hematological analyses were performed with an ABCanimal blood counter (Scil animal care company, Viernheim, Germany) on whole blood samples collected in EDTA-coated tubes.

Tissue preparation

Animals were deeply anaesthetized using CO_2 and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer. Brains were dissected from the skulls, post-fixed overnight in 4% PFA at 4°C and then transferred to a 30% (w/v) sucrose solution until saturated. Brains were then sectioned on a dry ice-cooled block with a sliding microtome (Leica, Bensheim) into 40 µm-thick coronal free-floating sections and stored at -20°C in a cryoprotectant solution containing 25% ethylene glycol and 25% glycerine in phosphate buffer. A one-in-six series of sections was taken for analysis from the brains of a subset of animals from each group.

Immunostaining

For immunostaining of Doublecortin (DCX) and tyrosine hydroxylase (TH), an Avidin-Biotin Complex ABC method like that employed previously (Garrett et al., 2012) was used. For the DCX staining, a primary rabbit polyclonal anti-DCX antibody (1:500, Abcam, Cat. No: ab18723) was used on this occasion with a biotinylated goat anti-rabbit IgG (1:300; Jackson Immunoresearch Laboratories Inc.) and 3, 3'-diaminobenzidine (DAB) as the chromogen. For the TH staining, a primary rabbit anti-TH antibody (1:1000, Pel-Freeze, Cat. No: P40101) was used with a biotinylated goat anti-rabbit IgG (1:300; Jackson Immunoresearch Laboratories Inc.) and 3, 3'-diaminobenzidine (DAB) as the chromogen.

Quantification of TH- and DCX-positive neurons

Estimation of the total number of TH+ and DCX+ cells was determined using a method based on the stereological optical fractionator principle and the semiautomatic StereoInvestigator system (MicroBrightField Inc., Williston, VT, USA). For TH+ cell estimation, the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) were traced in every 6th section. For DCX+ cell estimation, the olfactory bulb granular cell layer (OB) was traced in every 6th section. Immuno-positive cells were quantified by systematic random sampling using the following settings: TH+ cells in the SNpc and VTA: a scan grid size of 200 x 200 µm and a counting frame size of 70 x 70 µm; DCX+ cells in the OB: a scan grid size of 200 x 200 µm and a counting frame of 100 x 100 µm. Cells that intersected the uppermost focal plane or the lateral exclusion borders of the counting frame were not quantified. These settings were sufficient to keep the Gunderson's coefficient of error (m = 1) less than 0.1 for each animal.

Statistics

Tests for genotype effects were done either by using t-test, Wilcoxon rank sum test, linear models, or ANOVA. The choice of the method depended on the assumed distribution of the parameter and the nature of the question posed to the data. Correlations in the aged cohort were evaluated using Pearson correlation coefficient. A p-value of 0.05 was used as level of significance without correction for multiple testing. The analyses were performed using the programming language R (version 3.0.2).

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COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

AVS contributed to conceptualization, methodology, formal analysis, writing (original draft) and visualization. LG contributed to conceptualization, methodology, supervision, visualization, formal analysis and writing (original draft). BS contributed to conceptualization, resources, writing (review and editing) and project administration. FG and KMN contributed to conceptualization, methodology and writing (review and editing). JR, LB, BR, IR, GMCC, MK, TK, EW, AZ, VGD, HF, MHdA contributed to conceptualization, methodology and supervision of experiments at the German Mouse Clinic. MT contributed to resources and writing (review and editing). WW and SMH contributed to conceptualization, resources, supervision, formal analysis, writing (original draft) and funding acquisition. JW contributed to conceptualization, resources, writing (original draft), supervision, project administration and funding acquisition.

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Figures

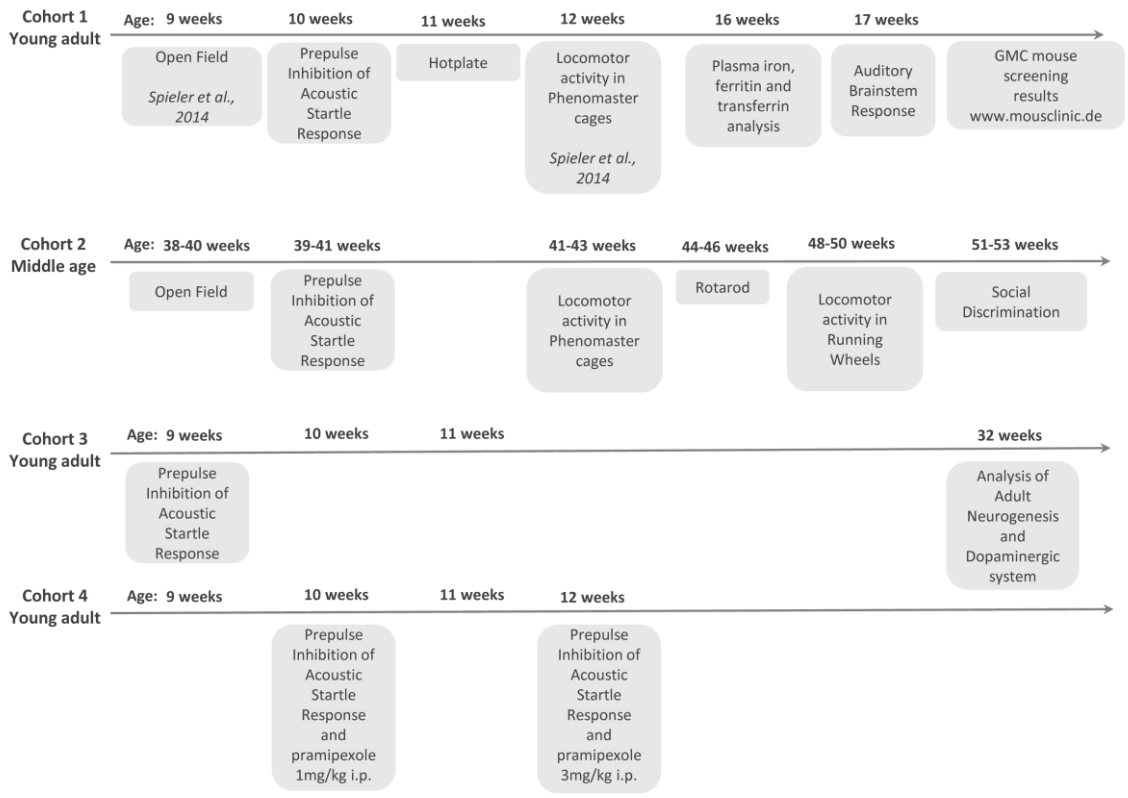


Figure 1. *An overview of the four cohorts of mice (three young adult and one middle aged) used and the age at which each of the tests were performed. Results from additional GMC screening can be found at the GMC phenomap (www.mouseclinic.de).*

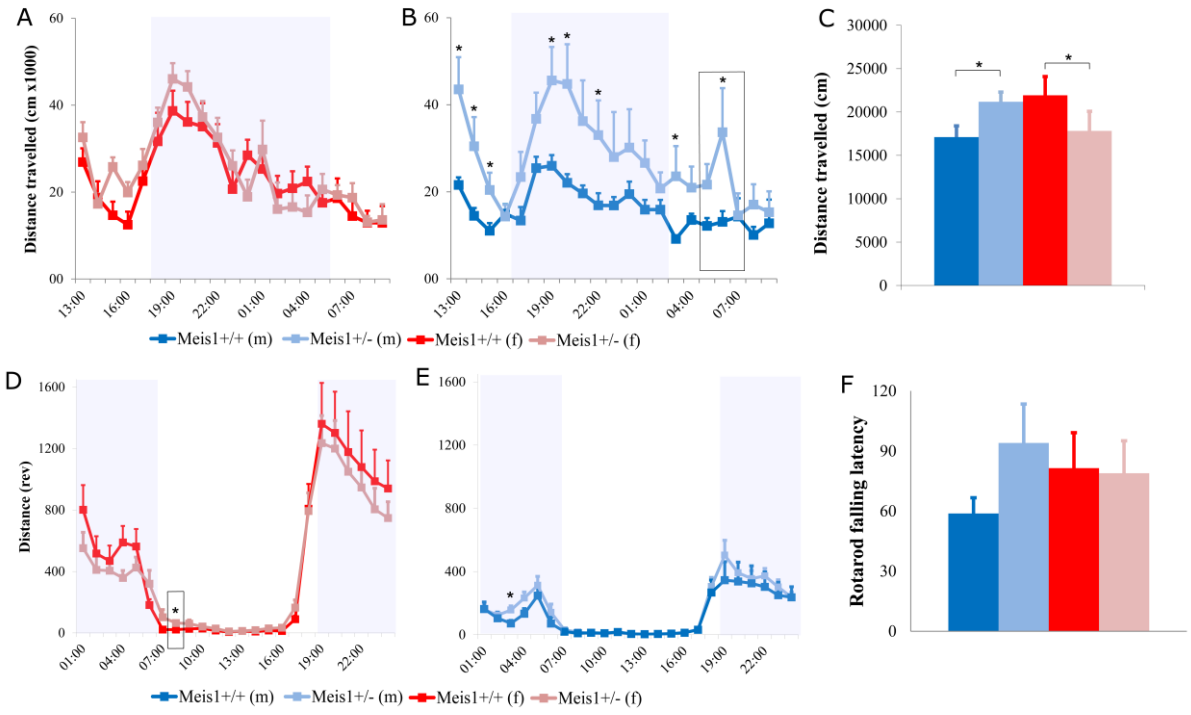


Figure 2. *Measures of locomotor activity in the PhenoMaster cages (females **A**, males **B**), open field (**C**), voluntary running wheels (females **D**, males **E**) and motor coordination and balance in the rotarod (**F**). *p < 0.05 vs. +/+ controls. Boxes indicate an increase in activity just after lights on in the rest phase seen in female +/- mice in voluntary running wheels (**D**) and in male +/- mice in homecage activity (**B**). The cohort included 21 female (9 +/- and 12 +/+) and 23 male (10 +/- and 13 +/+) mice. Results are plotted as mean ± SEM.*

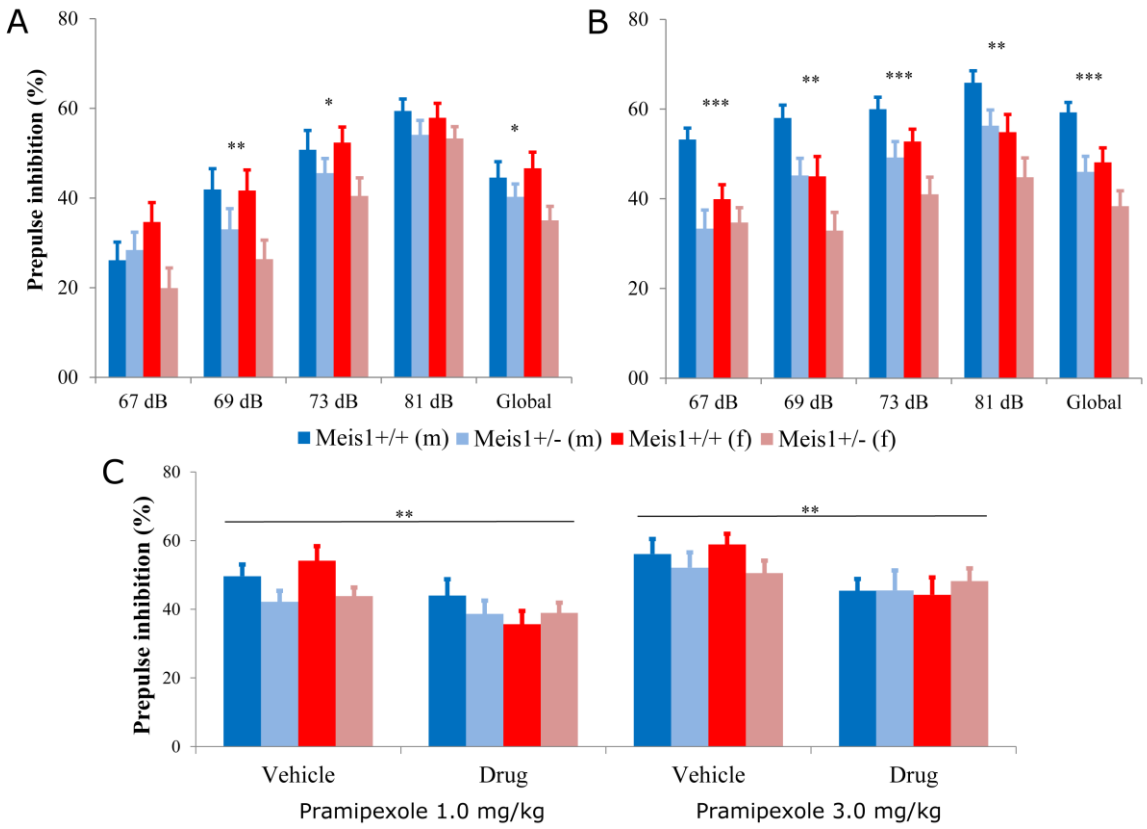


Figure 3. *The prepulse inhibition results from the first screening cohort (**A**, n=15 per sex per genotype) and the replication cohort (**B**, n=15 per sex per genotype). The results show that the Meis1 knock-out genotype effect on PPI was replicable when compared to wildtype littermates. Results are plotted as mean ± SEM. Genotype effects * p<0.05, ** p<0.005, *** p<0.001 calculated by two-way ANOVA with sex as a factor, including interaction term. The global effect of pramipexole 1.0 mg/kg (left) and 3.0 mg/kg (right) on the PPI of wildtype and heterozygous animals is shown (**C**). Results are plotted as mean ± SEM. Genotype effects * p<0.05, ** p<0.005, *** p<0.001 calculated by two-way ANOVA with sex as a factor, including interaction term.*

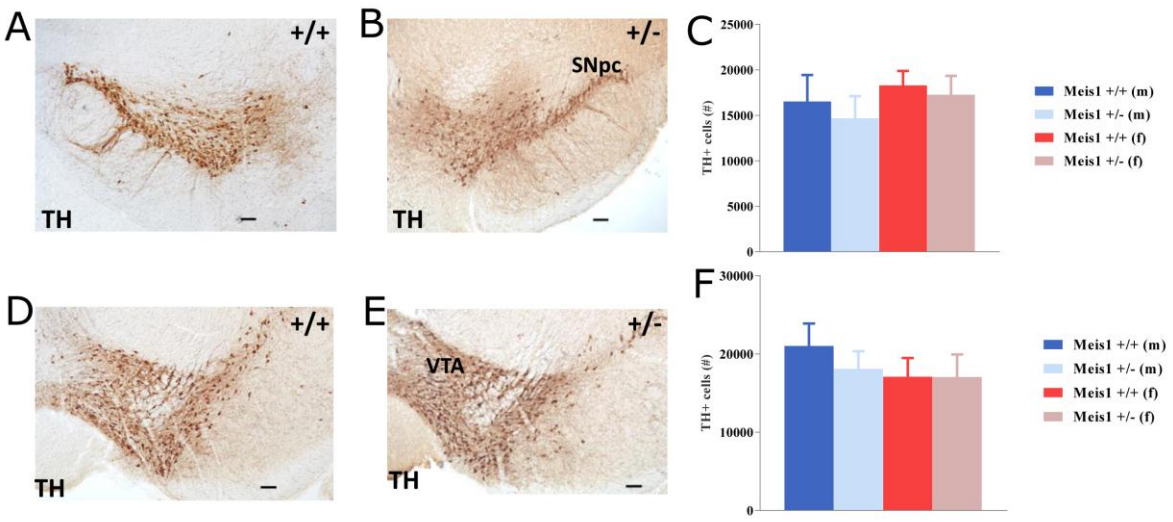


Figure 4. *Tyrosine hydroxylase (TH)+ neurons in the substantia nigra pars compacta (SNpc,* ***A*** *and* ***B****) and ventral tegmental area (VTA, D and E) of +/+ and +/- mice. Scale bar = 100µm. Results are plotted as mean ± SEM. No differences were found between the groups in either the SNpc (****C****) or VTA (****F****). Groups are n = 3 male +/+, n =4 male +/-, n = 4 female +/+, n = 5 female +/-.*

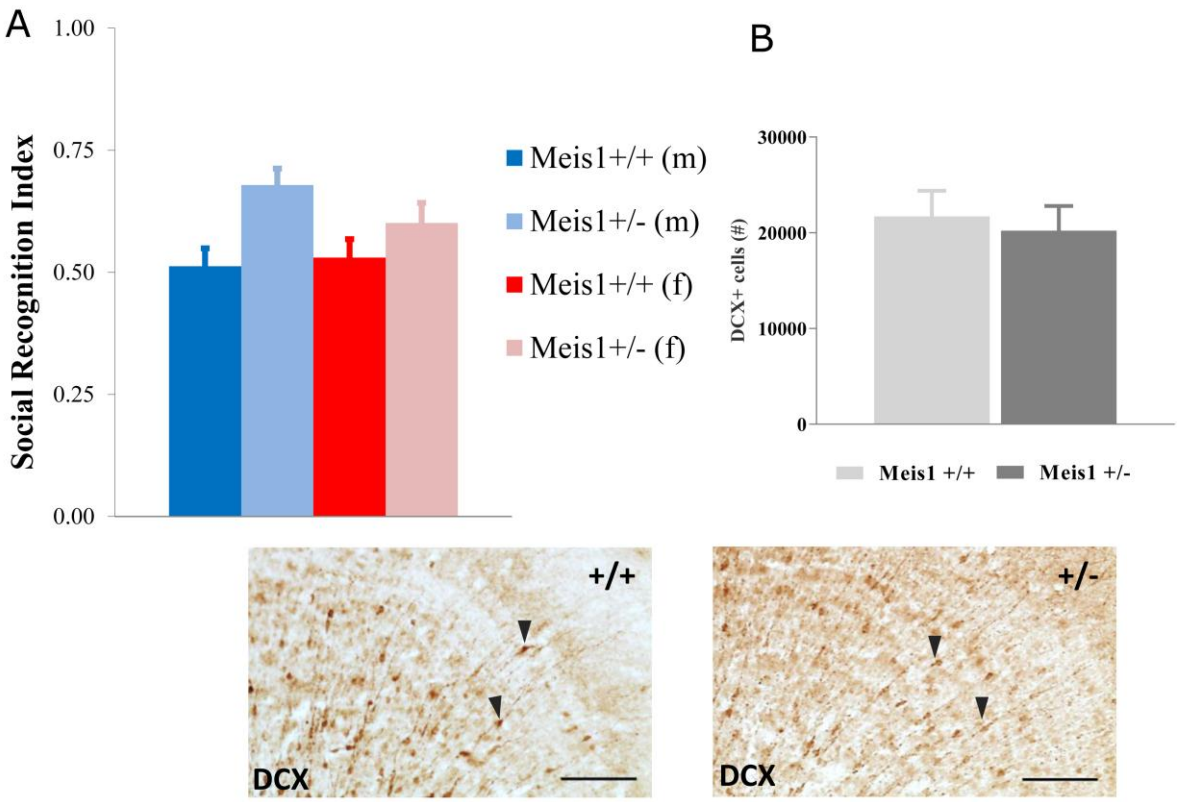


Figure 5. *(**A**). Social recognition test revealed an increased social recognition index in the mutant animals compared to wildtypes. The cohort included 21 female (9 heterozygous and 12 wildtype) and 23 male (10 heterozygous and 13 wildtype) mice. **p < 0.01, 2-way ANOVA genotype effect: F(1,41) = 9.48, p = 0.004, Meis1 +/+ vs. Meis1 +/-. No clear differences were detected in the level of adult neurogenesis in the olfactory bulb granular cell layer (OB) of the mutant mice as indexed by the number of doublecortin (DCX)+ cells in this region (**B**). Data from males and females were pooled for this analysis (n = 6 +/+, n = 6 +/-). Results are plotted as mean ± SEM. Representative photomicrographs are also depicted showing DCX+ cells (black arrows) in the OB of wildtype (+/+) and heterozygous mutant (+/-) mice. Scale bar = 100µm.*

Tables

	Female		Male		
	Meis1+/+	Meis1+/-	Meis1+/+	Meis1+/-	p-value
Nociception					
First response time (s)	10.53 ± 3.11	10.84 ± 3.29	29.41 ± 3.05	27.42 ± 2.5	0.402
First response type: licking (n)	8	10	4	11	
First response type: shaking (n)	7	5	11	4	
Second response time (s)	14.25 ± 3.72	14.33 ± 3.71	13.37 ± 2.47	13.72 ± 1.85	0.787
Second response type: licking (n)	7	5	11	4	
Second response type: shaking (n)	8	10	4	11	
ABR					
Click ABR	35 [35, 35]	42 [35,45]	30 [25, 35]	30 [25,35]	0.285
Threshold at 6 kHz (dB)	40 [40, 44]	48 [45, 50]	40 [35, 40]	35 [35, 40]	0.104
Threshold at 12 kHz (dB)	28 [25, 34]	30 [26, 34]	20 [20, 25]	15 [15, 20]	0.612
Threshold at 18 kHz (dB)	35 [30, 40]	45 [35, 49]	30 [25, 35]	25 [20, 30]	0.908
Threshold at 24 kHz (dB)	55 [46, 59]	52 [46, 55]	40 [32, 40]	35 [30, 45]	0.876
Threshold at 30 kHz (dB)	62 [48, 70]	65 [56, 75]	50 [45, 55]	55 [45, 60]	0.115

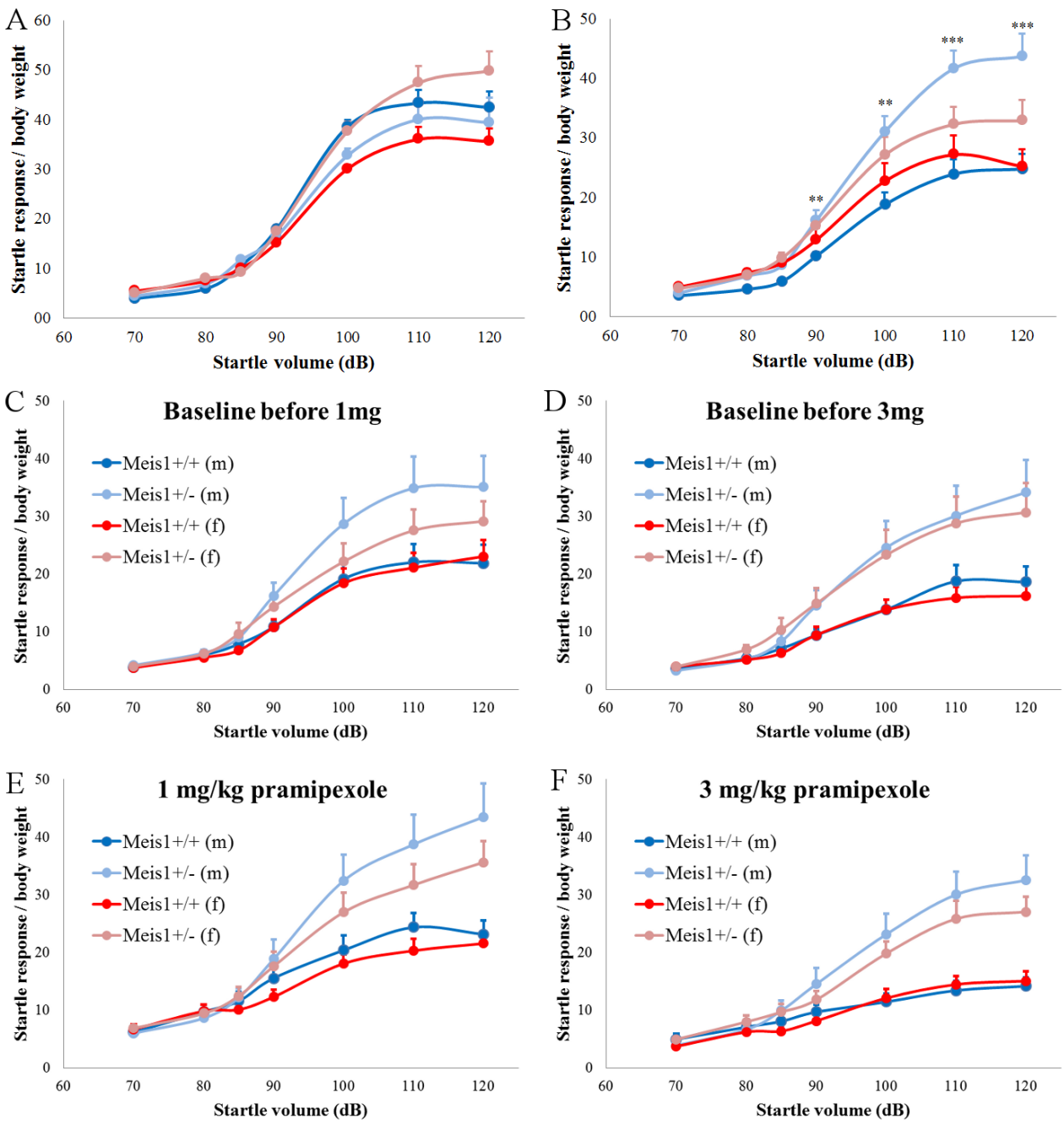
Table 1. *The results of the nociceptive phenotype screening (hot plate test) in our first mouse cohort (n=15 per sex per genotype). The results are presented as mean ± standard deviation and p-values are calculated using two-way ANOVA with sex and genotype as factors with interaction term. The auditory brainstem responses (ABR) results are presented as median [25%, 75%], n=15 per sex per genotype. The p-values are calculated with Wilcoxon rank sum test (ABR). All p-values represent the genotype effect.*

	Female		Male		Linear model p-value		
	Meis1+/+	Meis1+/-	Meis1+/+	Meis1+/-	genotype	sex	genotype:sex
n	15	15	15	15			
Iron [mumol/l]	27.4 ± 4.6	26.8 ± 6.5	17.4 ± 2.5	17.6 ± 2.4	0.875	< 0.001	0.721
Ferritin [pmol/l]	326.6 ± 62.6	284.5 ± 47.1	239.4 ± 36.3	240.9 ± 33.6	0.096	< 0.001	0.074
Transferrin [mumol/l]	18.97 ± 0.53	19.01 ± 0.58	18.72 ± 0.31	19.02 ± 0.3	0.158	0.293	0.275

Table 2. *The results of the analysis of levels of iron, ferritin and transferrin in the plasma from +/+ and +/- mice (n=15 per sex per genotype).*

No clear differences were detected between the groups for each parameter measured. The results are presented as mean ± standard deviation.

SUPPLEMENTAL FIGURE



Supplemental figure 1. *The acoustic startle response results from the first screening cohort (**A**, n=15 per sex per genotype) and the replication cohort (**B**, n=15 per sex per genotype). The results show that the Meis1 knock-out genotype effect on ASR was not replicable when compared to wildtype littermates. Results are plotted as mean +SEM. Genotype effects * p<0.05, ** p<0.005, *** p<0.001 calculated by two-way ANOVA with sex as a factor, including interaction term. The effect of pramipexole 1.0 mg/kg (left) and 3.0 mg/kg*

*(right) on the ASR of wildtype and heterozygous animals is shown (**E, F**) with corresponding baseline measurements (**C, D**). Results are plotted as mean +SEM. Genotype effects * p<0.05, ** p<0.005, *** p<0.001 calculated by two-way ANOVA with sex as a factor, including interaction term.*