Morc1 knockout evokes a depression-like phenotype in mice

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Abstract

Morc1 gene has recently been identified by a DNA methylation and genome-wide association study as a candidate gene for major depressive disorder related to early life stress in rodents, primates and humans. So far, no transgenic animal model has been established to validate these findings on a behavioral level. In the present study, we examined the effects of a *Morc1* loss of function mutation in female C57BL/6N mice on behavioral correlates of mood disorders like the Forced Swim Test, the Learned Helplessness Paradigm, O-Maze and Dark-Light-Box. We could show that *Morc1*$^{-/-}$ mice display increased depressive-like behavior whereas no behavioral abnormalities regarding locomotor activity or anxiety-like behavior were detectable. The baseline CORT plasma levels did not differ significantly between *Morc1*$^{-/-}$ mice and their wildtype littermates, yet – surprisingly - total BDNF mRNA-levels in the hippocampus were up-regulated in *Morc1*$^{-/-}$ animals. Although further work would be clarifying, *Morc1*$^{-/-}$ mice seem to be a promising epigenetically validated mouse model for depression associated with early life stress.

Keywords: depression; *Morc1*; transgenic mice; early life stress; epigenetics; BDNF.

1 Introduction

Clinical studies show that early life stress has profound and persistent effects on brain functions and is one of the major risk factors for developing a depressive disorder later in life [1], [2], [3]. The fundamental role of epigenetics mediating this process became clear in the groundbreaking animal study of Weaver et al. [4]: life-long epigenetic modifications of the glucocorticoid receptor gene in the rat hippocampus induced by early life stress were followed by a stable enhanced HPA response to stress. In the meantime, several studies identified effects of early life stress on DNA methylation for further genes in rodents [5], [6], [7], [8], [9]. For some of these genes, like BDNF, the serotonin transporter and the glucocorticoid receptor, altered DNA methylation was also revealed in humans after experiencing early life stress [10], [11], [12]. All of the above-cited studies focused on a candidate gene approach. Transgenic mice models with altered expression of the examined genes only partially show a consistent depressive-like phenotype [13], [14], [15], [16], [17]. Two more recent studies used a genome-wide methylation analysis and reported a wide range of epigenetic alterations as a result of early life stress [18], [19], but none of them led to a behaviorally validated mouse model for depression.

In a novel systematic translational genome-wide epigenetic approach, we recently succeeded in detecting an epigenetic marker of early life stress that is present in blood cell progenitors at birth in humans and monkeys, and also detectable in the prefrontal cortex of adult rats: Microrchidia (MORC) 1 – alias MORC family CW-type zinc finger 1 [20]. Moreover, we were able to verify an association between *Morc1* and major depressive disorder in a gene-set based analysis of an already available genome-wide association study [21].

MORC was first described in mammals by Watson et al. [22], who found that it is mainly expressed in male germ cells where it regulates mammalian germ cell development and meiosis. Male *Morc1*$^{-/-}$ mice are infertile due to a disrupted spermatogenesis, whereas male

110 *Morc1*$^{+/-}$ and female *Morc1*$^{-/-}$ mice do not show deficits in their reproductive system, *Morc1*
111 knockout mice were seen exclusively as a model for male fertility defects. The human MORC
112 protein consists of 984 amino acids and is 66% identical to the mouse MORC. Mutations in
113 humans might also cause male infertility and be involved in testicular germ cell tumors [23].
114 More recently Pastor et al. [24] reported that the male infertility in *Morc1* mutant mice is
115 caused by defects of DNA methylation of specific classes of transposons, resulting in failed
116 transposon silencing at these sites. Further analysis revealed that the MORC family is not only
117 decisive for male reproduction, but is also involved in the pathophysiology of numerous
118 forms of cancer: *Morc1* e.g. is frequently expressed in multiple myeloma [25] and mutated in
119 estrogen receptor-positive lobular breast cancer [26]. Additionally, *Morc1* has been related to
120 diabetes traits in a genome-wide complex trait analysis [27], which could play a role in the
121 association of major depressive disorder with type 2 diabetes mellitus. In plants, *Morc1*
122 influences immunological processes by different gene silencing mechanisms and
123 heterochromatin condensation [28], [29], [30]. Recent evidence suggests that *Morc1* plays a
124 more general biological role as part of a highly conserved nuclear protein superfamily that
125 serves as epigenetic regulators in diverse nuclear processes that are not yet fully understood
126 [31], [32], [33]. Hence *Morc1* appears to be primarily involved in gene silencing and changes
127 of the chromatin structure [34], [35]. We were the first to report a different methylation of
128 *Morc1* in brain tissue – the prefrontal cortex of adult rats – and thus strongly support the
129 hypothesis of an epigenetic influence of this gene in the brain [20].
130
131 Animal models are still valuable tools in preclinical research, yet no rodent model of *Morc1*
132 regarding depressive-like behavior has been established. Thus, in the present study we aimed
133 to implement a transgenic mouse model to validate the role of *Morc1* expression in affective
134 disorders. For this purpose we used *Morc*$^{Tg(Tyr)1Az/J}$ mice, in which exons 2-4 of *Morc1* gene
135 on chromosome 16 had been deleted with the help of a transgenic insert [36]. We

136 characterized MORC$^{Tg(Tyr)1Az/J}$ mice behaviorally in a test battery for locomotion and
137 exploratory, anxiety-like and despair behavior. Plasma corticosterone levels were analyzed as
138 a possible indicator of a depression-like HPA-system dysfunction. Furthermore, we
139 determined total BDNF mRNA-levels known to be decreased in hippocampus in depressive
140 patients and animal models of depression [37], [38], [39] as well as in closely connected and
141 presumably affected structures, such as prefrontal cortex and amygdala [40], [41]. Due to
142 difficult breeding and an increased mortality of male *Morc1*$^{-/-}$ mice, we only used female
143 animals for our experiments, which is also the sex more predisposed to develop depression in
144 humans.
145

2 Materials and Methods

2.1 Animals

Morc$^{Tg(Tyr)1Az/J}$ mice had been generated by introducing a transgenic construct containing a tyrosinase gene under the control of an RNA polymerase II 1 promoter into FVB/N fertilized mouse eggs. Sequences adjacent to the transgenic insert are disrupted resulting in the deletion of *Morc1* exons 2-4 [36]. Heterozygous *Morc1*$^{+/-}$ on the original FVB/N background were purchased from the Jackson Labs (Bar Harbour, Maine, USA) and crossed with C57BL/6N (Charles River, Sulzfeld, Germany) mice to obtain an F1 generation. Heterozygous F1 offspring were then inter-crossed to generate an F2 generation, which was used for behavioral and molecular analyses. Male *Morc1*$^{-/-}$ mice were infertile and had smaller testes than wildtypes, whereas male *Morc1*$^{+/-}$ mice and female *Morc1*$^{-/-}$ mice showed no reproductive deficits. Animals were genotyped by PCR as recommended by the Jackson Labs (Bar Harbour, Maine, USA). Female *Morc1*$^{-/-}$ mice and their wild-type littermates were housed individually 2 weeks before the first behavioural test started in macrolon type II cages with nesting material under a reversed day-night cycle (lights on from 19.00-07:00 hrs with 12h dark and 12-h light phase) and supplied with food and water *ad libitum*. All procedures complied with the regulations covering animal experimentation within the EU (European Communities Council Directive 2010/63/EU). They were conducted in accordance with the institutions' animal care and use guidelines and approved by the national and local authorities (Regierungspräsidium Karlsruhe).

2.2 Behavioral tests

At the age of 3, 5 and 6 months, 11 female *Morc1*$^{-/-}$ mice and 12 of their wild-type littermates were tested behaviorally in the dark phase, i.e., in the animals` active phase. Mice were acclimatized to the experimental room for at least 30 minutes prior to each test and tested by an investigator, who was blinded regarding their genotype. The order of the tests followed

earlier recommendations ranking the tests from least stressful to more stressful. Mice were sacrificed 2-3 weeks after the last experiment at an age of 21 - 25 weeks.

2.2.1 Novel Cage Test

Explorative behaviour was measured by counting the number of rearings within the first 5 minutes after placing the animal into a fresh standard type II macrolon cage as described earlier [42].

2.2.2 Open Field Test

To evaluate locomotor and exploratory behavior, mice were individually placed into an open arena measuring 50x50 cm^2 under dimmed light conditions (25 Lux). Activity monitoring was conducted 15 min via a Video camera (Sony CCD IRIS). The resulting data were analyzed using the image processing system EthoVison 1.96 (Noldus Information Technology, Wageningen, the Netherlands) as described earlier [43].

2.2.3 Dark-Light-Box

Anxiety-related behavior was tested in the Dark-Light Box consisting of two plastic chambers connected by a small tunnel. Mice were placed into the dark chamber, which was covered by a lid and measured 20x15 cm^2. Latency to first exit, number of exits and total time in the aversive light compartment (30x15 cm^2, illuminated with 600 Lux) was recorded for 5 min as described earlier [44].

2.2.4 O-Maze

The Elevated Zero-Maze analyzes anxiety-related behavior by assessing avoidance of the aversive unsheltered compartment of the arena. A grey plastic annular runway (width 6 cm, outer diameter 46 cm, 50 cm above ground level, illuminated with 25 Lux) was covered with black cardboard paper to prevent animals slipping off the maze. Two opposing sectors were protected by inner and outer walls with a height of 10 cm. Animals were placed in one of the

protected sectors and latency to first exit, number of exits and total time spent in the open compartments was measured for 5 minutes as described earlier [45].

2.2.5 Forced Swim Test

The Forced Swim Test was applied to measure depressive-like behavior. For this purpose mice were placed into a glass cylinder (23 cm height, 13 cm diameter) filled with water (22°C) up to a height of 8 cm. Within a period of 6 min the onset and the percentage of floating was determined as described earlier [46]. 24 hours later the animals were tested again under the same conditions as before.

2.2.6 Hot Plate Test

To exclude altered pain sensitivity as a confounding factor for Learned Helplessness, the Hotplate Test (ATLab, Vendargues, France) was applied. Temperature was set at 53 °C (±0.3 °C) and a 45s cut-off was determined to prevent injury. Latency to first reaction, i.e. licking hind paws or jumping, was assessed as described earlier [47].

2.2.7 Learned Helplessness

In the Learned Helplessness Paradigm mice were placed in a transparent plexiglas shock chamber (18×18×30cm³) with a stainless steel grid floor (Coulborn Instruments, Düsseldorf, Germany) through which they received 360 unpredictable and unavoidable footshocks (0.150mA) on 2 consecutive days. The footshocks applied varied regarding shock-duration (1–3s) and interval-episodes (1–15s) and lasted approximately 52 min in total. 24 hours after the second shock procedure, learned helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf, Germany) as described earlier [48]. Spontaneous initial shuttles from one compartment to the other were counted during the first 2min. Performance during 30 shuttle escape trials each starting with a

light stimulus of 5s, announcing a subsequent footshock (intensity 0.15mA) of maximum 10s duration was analyzed. Inter-trial interval was 30s and total testing time about 20min.

2.3 CORT levels

All animals were sacrificed at the age of 21-25 weeks by decapitation between 8.00 and 11.00 hrs in the morning, and trunk blood was collected within 30s after the animal's removal from the cage. Baseline plasma corticosterone levels – without applying any acute stress or intervention before taking the samples – were determined using commercially available radioimmunoassay kits (ICN Biomedicals, Eschwege, Germany) as described earlier [49].

2.4 RNA Preparation and Gene Expression Analysis by Quantitative Real-Time PCR

After decapitation of the animals, the brains were immediately extracted from the skull. The hippocampus, prefrontal cortex and amygdala were rapidly dissected from the whole brain, frozen on dry ice and stored for later analyses.

Total RNA was isolated by single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories) according to manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time PCR (RT-PCR) to assess total BDNF mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRTPCR instrument (CFX384 real time system; Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4). Primers and probes sequences of total BDNF (forward primer: AAGTCTGCATTACATTCCTCGA, reverse primer: GTTTTCTGAAAGAGGGACAGTTTAT and probe: TGTGGTTTGTTGCCGTTGCCAAG) and 36B4 (forward primer: AGATGCAGCAGATCCGCAT, reverse primer:

GTTCTTGCCCATCAGCACC and probe: CGCTCCGAGGGAAGGCCG) were purchased from Eurofins Genomics (Vimodrone, Italy), while probe and primer sequences for *Morc1* (Assay ID: Mm00501711_m1) were purchased from Life Technologies (Monza, Italy) and are available on request.

2.5 Statistical analyses

Intergroup comparisons were calculated by one-sided t-tests assuming that knockout-mice display higher levels of depressive and anxiety-like behavior as well as higher CORT-levels, less body weight, less locomotor and exploratory behavior and decreased total BDNF mRNA-levels in hippocampus, prefrontal cortex and amygdala than wild-types. A one-way repeated measurements ANOVA was used to analyze the open field test. Calculations regarding mRNA levels were run with fold change values. The correlation between Learned Helplessness Escape Latency and Failures was analyzed applying spearman rho correlation. A p-value ≤ 0.05 was seen as the level of statistical significance in all tests. The statistical analyses were performed using the SPSS 21.0 software package for Windows.

3 Results

3.1 Morc1 knockout mice display regular locomotor and exploratory behavior

Morc1$^{-/-}$ mice showed normal vertical locomotor activity (rearing) in the Novel Cage Test (T(21)=0.874; p=0.196; data not shown). The horizontal locomotor activity and exploratory behavior as measured by the Open Field Test also did not differ significantly from control animals: e.g. the total distance moved by *Morc1*$^{-/-}$ mice was similar to those of the control group (time*genotype: $F_{1,21}$=1.125, p=0.301; between subject-factor genotype $F_{1,21}$=0.032; p=0.859). Both genotypes moved significantly less in the second half of the test, showing that habituation had taken place as expected (Time: $F_{1,21}$=8.792; p=0.007; data not shown).

3.2 Morc1 knockout mice show unaltered anxiety-related behavior

Morc1$^{-/-}$ mice did not display increased anxiety-like behavior as monitored in the Dark-Light Box (latency: T(21)=1.023; p=0.159; end exploration: T(14.011)=0.914; p=0.188; exits: T(21)=-1.289; p=0.106; light time: T(21)=-0.074; p=0.471; data not shown). In the Elevated O-Maze, again *Morc1*$^{-/-}$ mice exhibited similar latencies to enter the aversive compartment as their littermate controls (T(21)=0.314; p=0.3785; data not shown). They also did not exit (T(21)=-0.128; p=0.4495; data not shown) or fully cross (T(21)=0.095; p=0.4625; data not shown) the maze less than controls and spent about the same amount of time in the open arms of the maze (T(21)=-0.862; p=0.199; data not shown).

3.3 Morc1 knockout mice demonstrate increased depressive-like behavior

Depressive-like behavior as measured by the Forced Swim Test showed that *Morc1*$^{-/-}$ mice display a significant lower latency to float (T(21) =2.346; P=0.015) on day 1 than wild-types (s. Fig 1A). Additionally, on day 1 immobility times were increased in *Morc1*$^{-/-}$ mice, which resulted in a statistical tendency from minute 4 to 6 of the test (minute 0 to 2: T18.051=-0.819;

p=0.216; minute 2 to 4: T21=-0,567; p=0.289; minute 4 to 6: T21=-1.343; p=0.097; s. figure 1B).

24 hours later (on day 2), the difference in the latency to start floating was not detectable any more (T21=0.113; p=0.456; s. fig. 1C.) Furthermore, *Morc1*$^{-/-}$ mice showed a significant increase in immobility on day 2 from minute 2-4 compared to wild-types (T(21)=-2.009; p=0.029) and a statistical tendency also from minute 4-6 (T21=-1.431; p=0.084) as depicted in fig. 1D. From minute 0 to 2 there was no significant difference in immobility time between the two groups (T21=-0.508; p=0.308).

In the Learned Helplessness Paradigm of depression, *Morc1*$^{-/-}$ mice displayed significantly more failures to escape in comparison to littermate controls (T(12.573)=-1.844; p=0.045) (s. Fig 2A). As a tendency, *Morc1*$^{-/-}$ mice had a higher latency to escape than controls (T (16.093)=-1.539; p=0.072) as shown in fig. 2B. A clear correlation between escape latency and number of failures with the *Morc1*$^{-/-}$ mice showing the highest values (spearman rho=0.961, p<0.000) is depicted in fig. 2 C.

3.4 Morc1 knockout mice reveal regular CORT levels, but increased total BDNF in hippocampus

Morc1$^{-/-}$ mice showed similar plasma corticosterone levels as their control littermates (T(21)=-0.941; p=0.1785; s. fig. 3A). In the hippocampus however, total BDNF mRNA-levels of *Morc1*$^{-/-}$ mice were significantly increased compared to those of wild-types (T(21)= -3.538; p=0.001; s. Fig. 3B). As a tendency, the total BDNF mRNA-levels in the prefrontal cortex (T(14.968)=1.428; p=0.087; data not shown) and in the amygdala (T(18)=1,337; p=0.099; data not shown) were down-regulated.

As expected, *Morc1* mRNA was not expressed in *Morc1*$^{-/-}$ mice, whereas we found its full mRNA expression in the wildtype animals in hippocampus, prefrontal cortex and amygdala (data not shown).

4 Discussion

In the present study, we characterized for the first time *Morc1*$^{-/-}$ mice in a test battery for emotional behavior. *Morc1*$^{-/-}$ mice floated earlier in the Forced Swim Test and showed more immobility in general. In the Learned Helplessness Paradigm, they exhibited a higher latency to escape and more escape failures, two parameters that are seen as classical indicators for increased learned helplessness in rodents [50]. *Morc1*$^{-/-}$ mice showed a significant increase of immobility in the Forced Swim Test only on day 2 and just as a statistical tendency on day 1. Although day 1 is regarded as more relevant for mice than day 2, the consistently increased means of immobility on both days taken together with the results of the Learned Helplessness Test, allow to postulate a clear increase in depressive-like behavior of *Morc1*$^{-/-}$ mice.

Anxiety is often concomitant to depression in psychiatric patients. Up to now, the question if *Morc1* is also involved in anxiety disorders has not been examined in clinical trials. In our animal model we did not find any evidence of increased anxiety in *Morc1*$^{-/-}$ mice in the Dark-Light Box Test and Elevated O-Maze, indicating that *Morc1* may specifically be involved in depression but not anxiety. We were able to exclude reduced locomotion and exploratory behavior as possible confounding factors in our study, as *Morc1*$^{-/-}$ mice did not show any difference to their wild-type littermates in the Novel Cage and Open Field Test.

All in all, this new transgenic mutation seems to represent a promising model to further investigate the depressive phenotype and its underlying neurochemical, genetic and epigenetic pathophysiology.

Besides a deficient spermatogenesis, small testical size and aberrant eye pigmentation, Watson et al. [22] described no phenotypic abnormalities in male *Morc1*-mutant mice. In our breeding, we found increased mortality in male *Morc1*$^{-/-}$ mice, and the small sample size of males in our cohort constrained us to focus only on females for our behavioral testing. Although we bred only for two generations, a random effect cannot be definitely dismissed,

yet this increased mortality might be another confirmation of *Morc1* – as already stated above – not only being involved in spermatogenesis but serving a more general biological function [31], [32], [33].

This leads to the more general question why *Morc1* might be involved in spermatogenesis as well as in mood disorders – two biological processes seemingly independent at first glance. Soumillon et al. [51] come to the conclusion that a considerable part of genes expressed in testes do not have testes-specific functions. Shen et al. [52] could demonstrate that testes-specific genes usually have a fast evolutionary rate and therefore are more likely to gain new functions. In their phylogenetic analysis they illustrate that some open reading frames were first expressed in testis and later in evolution got expressed in other tissues. The authors assume that testis may play a role in producing new genes and even in supporting testes-specific genes in gaining new functions for other organs/tissues. Blendy et al. [53] for example demonstrated that the *Crem* gene is involved in spermatogenesis. Aguado et al. [54] found the same gene being involved in hippocampal synaptogenesis. Wang et al. [55] proved another gene - *hsf-2* – to be involved in sperm production as well as central nervous development.

As women are more often affected by major depressive disorder than men [56], [57], we saw the restriction to female $^{-/-}$ mice in the present study as a possibility for validly modeling depression. Nevertheless, sex differences with respect to the development of depressive-like behaviors in mice with targeted mutagenesis have also been reported for other genes such as BDNF [58], [59], [60]. Therefore another study on male *Morc1*$^{-/-}$ mice and their conceivable depressive-like behavior seems appealing. A possible sex effect in our animal model could also shed more light on the differing pathophysiology of depression in women and men. Moreover, additional behavioral tests concerning e.g. social behavior and cognition that are

382 often correlated to depressive-like behavior, are warranted to further characterize the novel
383 *Morc1* mouse model of depression.
384
385 We did not detect any difference regarding baseline corticosterone levels in *Morc1*$^{-/-}$ mice
386 suggesting that the HPA-axis in these animals is not disrupted under resting conditions. This
387 does not exclude the possibility that the hormonal responsiveness is affected under
388 challenging conditions.
389
390 We also investigated BDNF expression that represents a prototype marker of neuronal
391 plasticity, which has often been associated with a depressive phenotype, its expression being
392 reduced in the brain of depressed subjects as well as in different animal models of depression
393 [61], [62] – summarized in the so-called neurotrophin hypothesis of depression. Contrary to
394 these previously found changes, the hippocampal expression of BDNF was upregulated in
395 *Morc1*$^{-/-}$ mice, whereas a trend toward a reduction was found in the prefrontal cortex. Despite
396 the complex interactions between glucocorticoids and BDNF, this finding might be narrowed
397 down to the undisturbed HPA-Axis in our animals, as BDNF is mostly suppressed by
398 glucocorticoids [63], [64].
399 An alternative explanation for this unexpected result may be sex differences. Interestingly, the
400 decrease of BDNF in depressed patients was found to be more pronounced in men than in
401 women [65]. Hayley et al. [66] demonstrated that in female depressed patients who committed
402 suicide BDNF protein levels were reduced in the frontopolar prefrontal cortex, but not in the
403 hippocampus. Conversely, males displayed significantly decreased BDNF protein levels only
404 in the hippocampus yet not in the prefrontal cortex. Jaworska et al. [67] showed a significant
405 decrease of BDNF in the hippocampus of male, but not of female gerbils after early life stress.
406 As we used only female mice for our experiment, this sex-specific regulation could be the
407 reason for the lack of a BDNF down-regulation in our study. Furthermore, there is growing

evidence that estradiol can induce BDNF expression and vice versa that estradiol effects in the hippocampus are mediated by BDNF [68], [69], [70], [71].

In a recent study, Calabrese et al. [72] demonstrated that BDNF in serotonin transporter knock-out rats, which had been exposed to early life stress was down-regulated in the ventral hippocampus and the ventromedial prefrontal cortex, but was significantly increased in the dorsal hippocampus and the dorsomedial prefrontal cortex. A similar region-specific process might underlie the detected increase of BDNF in hippocampus in the present study with the amount of BDNF in the dorsal parts outweighing the downregulation in the ventral parts. We did not differentiate between the dorsal and ventral regions of hippocampus and prefrontal cortex, but this issue would be very interesting to address in future experiments.

However, all these aspects fail to entirely explain the highly significant increase of BDNF we found in hippocampus of *Morc1*$^{-/-}$ mice. In contrast, the trend of reduced BDNF in prefrontal cortex and amygdala of *Morc1*$^{-/-}$ mice is more in line with previous findings showing either no change of BNDF in these two regions [73] or a decrease in depressive-like animals [74] and depressive humans [75].

Last, increased BDNF levels in the hippocampus might also be the consequence of compensatory mechanisms set in motion in *Morc1* mutants. Thus, Faure et al. [76], Marais et al. [77] and Daniels et al. [78] found a significant increase of BDNF in (dorsal) rat hippocampus after maternal separation during early life. Hellweg et al. [79] also found a significant increase of BDNF in bulbectomized depressive-like mice. According to the authors this finding in addition to the fact that bulbectomized animals display different behavioral abnormalities than glucocorticoid receptor compromised mice as well as a serotonergic dysfunction possibly defines a new endophenotype of depression. Although

Morc1$^{-/-}$ mice show the same depressive-like phenotype with deficiencies in the FST and LH paradigm as described by the animals used for the neurotrophin hypothesis of depression, their serotonergic function has not been evaluated yet. Maybe *Morc1*$^{-/-}$ mice constitute an intermediate endophenotype situated between the two previously described animal models. Nonetheless, as BDNF-levels in the brain have never been analyzed before in *Morc1*$^{-/-}$ mice, the regulation of BDNF in regard to *Morc1* and depression remains a matter of pure speculation. Systematic investigation of this question is required.

One of the most intriguing issues to address in the future will be the role of epigenetic modulation by *Morc1* in the brain. We were the first to detect a different methylation of *Morc1* in the prefrontal cortex of the rat brain [20]. In our cross-species and cross-tissues approach, we could prove the particular relevance of *Morc1* methylation after early life stress. However, to date the specific cerebral subtypes that are affected by *Morc1* and the concrete epigenetic function of *Morc1* in the brain is still completely unknown.
A further implication of this finding is the question if the methylation pattern will be replicable in the mouse brain or in other regions of the brain like e.g. the hippocampus. As epigenetic processes are always highly sex-specific, it would also be most interesting to examine in which way the epigenetic mark will be expressed differently in male individuals.

One limitation of the presented study is certainly that only females have been used so that the model might be a sex-specific. Additionally, as we analyzed BDNF and CORT levels after applying stressful behavioral tests to the animals, we cannot exclude that e.g. the FST or the LH paradigm has had some influence on these parameters. It is thinkable that the increase of BDNF in the hippocampus of *Morc1*$^{-/-}$ mice is due to some compensatory mechanism induced by the higher amount of electric shocks these animals received in the LH paradigm due to their bad performance in this behavioral test.

Our study results are further limited by the fact that we only used *Morc1* homozygous knockout mice as heterozygous knockouts are usually regarded as a better model for the human condition. We are actually planning to also behaviorally analyze *Morc1* heterozygous - and male - mice after backcrossing our animals for a few more generations into the c57/bl6 background.

In conclusion, although further work still has to be accomplished, the animal model of *Morc1*$^{-/-}$ mice will be useful in future studies on the role of *Morc1* in the pathophysiology and therapy of depression.

5 Conflict of interest

The authors declare no conflict of interest.

6 Acknowledgements

This work was supported by an Era-Net Neuron grant to PG, MAR, CF and MD, by grants from the Italian Ministry of University and Research to MAR (PRIN – grant number 20107MSMA4_002), from Fondazione CARIPLO to MAR (grant number 2012-0503) and in part by a grant from the Deutsche Forschungsgemeinschaft to PG (SFB636/B3). AL was supported by a Fondazione Umberto Veronesi Fellowship.

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