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Lack of anticipatory behavior in *Gpr88* knockout mice revealed by automatized home cage phenotyping

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Abstract

Mouse models are widely used to understand genetic bases of behavior. Traditional testing typically requires multiple experimental settings, captures only snapshots of behavior, and involves human intervention. The recent development of automated home cage monitoring offers an alternative method to study mouse behavior in their familiar and social environment, and over weeks. Here we used the IntelliCage system to test this approach for mouse phenotyping, and studied mice lacking the *Gpr88* that have been extensively studied using standard testing.

We monitored mouse behavior over 22 days in 4 different phases. In the free adaptation phase, *Gpr88*^{-/-} mice showed delayed habituation to the home cage, and increased frequency of same corner returns behavior in their alternation pattern. In the following nose-poke adaptation phase, non-habituation continued, however mutant mice acquired nose-poke conditioning similarly to controls. In the place learning and reversal phase, *Gpr88*^{-/-} mice developed preference for the water/sucrose corner with some delay, but did not differ from controls for reversal. Finally, in a fixed schedule-drinking phase, control animals showed higher activity during the hour preceding water accessibility, and reduced activity after access to water was terminated. Mutant mice did not show this behavior, revealing lack of anticipatory behavior.

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Our data therefore confirm hyperactivity, non-habituation and altered exploratory behaviors that were reported previously. Learning deficits described in other settings were barely detectable, and a novel phenotype was discovered. Home cage monitoring therefore extends previous findings and reveals yet another facet of GPR88 function that deserves further investigation.

Keywords

Intelligence System; GPR88; Long-term Phenotyping; Automated; Female Mice; Non-habituation; Hyperactivity; Perseveration; Learning; Anticipatory Behavior

Introduction

Psychiatric disorders are complex multi-factorial-dependent disorders (Karl and Arnold, 2014; Kim and Leventhal, 2015). Their diagnosis, treatment and recovery are long lasting processes and sensitive to environmental factors. Rodents have been used since decades to model behavioral dysfunctions related to mental disorders, and mouse models in particular are instrumental to understand genetic bases of psychiatric illnesses (Leung and Jia, 2016). Unfortunately, most standard behavioral testing modalities, which are used to investigate mouse models in psychiatry, take only snapshots of their behavior (Barnes, 1979; Crawley and Goodwin, 1980; Hall, 1932; Pellow et al., 1985). Further, in order to describe the effect of genetic background, mutation or drug on behavior, a battery of tests is required to tap into different aspects of behavior such as motor, sensory, cognitive and circadian functions (Rogers et al., 1999). The succession of tests in those batteries involves several major confounders such as repetitive human handling, testing during mice's rest period, in a new environment and often single-housing the animals. Those external stressors in turn influence the rodent behavioral responses, and should be carefully taken into account as they are source of variations that may lead to misinterpretations (Crabbe et al., 1999; Turner and Burne, 2013; Wahlsten, 2010; Würbel, 2002).

A solution to reduce confounding factor effects is to observe mouse behavior in their home cage. The recent development of automated home cage monitoring systems allows repetitive, objective, and consistent measurement of mice behavior, over days or even weeks, rather than minutes. Plus, continuous recording allows investigation of multi-dimensional aspects of behavior, in a freely moving animal, from basal activity and everyday life pattern, to challenged behavior (de Visser et al., 2006; Endo et al., 2012; Maroteaux et al., 2012). Under such conditions, animal motivation is intrinsic; the animal is not forced to react to a novel environment and handling does not bias animal responses. Over the last decade long-term home cage monitoring has been developed by several companies with different monitoring techniques (de Visser et al., 2006; Galsworthy et al., 2005). To investigate mice in a social and environmentally familiar situation (Galsworthy et al., 2005), and reduce the influence of external factors, we chose to undertake the characterization of *Gpr88* deficient mice using the IntelliCage system. This automated home cage monitors group-housed mice implanted with radio frequency identification chips and allows investigating multi-dimensional aspects of mice behavior (habituation, baseline and challenged behavior). Behavioral phenotypes have already been reported for *Gpr88* deficient mice using standard

behavioral testing (Logue et al., 2009; Meirsman et al., 2016a, 2016b; Quintana et al., 2012). The goal of this study was to determine whether longitudinal IntelliCage-based investigations would confirm previous findings and uncover novel aspects of GPR88 function.

GPR88 is an orphan G protein-coupled receptor, classically described as striatal-enriched receptor (Ghate et al., 2007; Logue et al., 2009; Massart et al., 2009; Mizushima et al., 2000; Van Waes et al., 2011), with detectable expression also in the cortex and central amygdala (Becker *et al.*, 2008, Befort *et al.*, 2008). Behavioral analysis of *Gpr88* deficient mice was therefore developed using standard behavioral testing paradigms known to engage areas of highest GPR88 density. Related to striatal function, repeated exposure of *Gpr88* deficient mice to a novel environment, or housing in a uncomfortable situation, triggered non-habituation hyperactivity (Meirsman et al., 2016a; Quintana et al., 2012). Mutant mice also showed difficulties in ending behavioral sequences, including foraging time and circling, impaired procedural learning on the rotarod (Meirsman et al., 2016a) and altered hippocampus/striatal-dependent behaviors in the dual solution cross maze task (Meirsman et al., 2016a). Possibly related to receptor expression in other brain areas, those mice finally exhibited sensorimotor gating alteration with decreased pre-pulse inhibition (Logue et al., 2009), as well as low levels of anxiety (Meirsman et al., 2016a). Standard behavioral testing, therefore, detected multiple and complex phenotypes in these mice, providing an attractive knockout model for subsequent analysis. Here we tested female mice lacking *Gpr88* in the group-housed and stress-reduced conditions of the IntelliCage system. Animals were monitored during several weeks and challenged throughout five behavioral phases including habituation, nose-poke adaptation, place and reversal learning and fixed schedule drinking. Together our data confirm the non-habituation phenotype, as well as altered exploratory behaviors that were described previously (Meirsman et al., 2016a), and also reveal a yet unreported phenotype that involves the lack of anticipation.

Material and methods

Animals

Total *Gpr88*^{-/-} knockout mice were produced as previously described (Meirsman et al., 2016a). Briefly, *Gpr88*-floxed mice were crossed with CMV-Cre mice expressing Cre recombinase under the cytomegalovirus promoter. This led to germ-line deletion of *Gpr88* exon 2 under a mixed background (13.96% C57B1/6N; 60.94% C57B1/6J; 0.05% FVB/N; 25% 129/SvPas; 0.05% SJL/J). Mutant mice were then fully backcrossed on the hybrid 50% C57B1/6J-129/50% SvPas background, and the Cre transgene was no longer maintained once excision had occurred on both alleles. Current breeding involves heterozygous matings, and animals used in the experiments are wild-type and homozygous littermates. All mice were bred at Institut Clinique de la Souris-Institut de Génétique et Biologie Moléculaire et Cellulaire, France. Animals were group-housed under 12 h light/dark cycle. Female *Gpr88*^{-/-} (n = 16) and controls *Gpr88*^{+/+} (n = 16). Mice were 8-10 weeks old at the time of the experiments. Temperature and humidity were controlled and food and water were available ad libitum. All animal procedures in this report were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and

approved by both the Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg (CREMEAS, 2003-10-08-[1]-58) and the local ethical comity (Comité d’Ethique en Expérimentation Animale IGBMC-ICS).

IntelliCage study

The IntelliCage apparatus (NewBehavior AG, Zurich, Switzerland, www.newbehavior.com) consists of a polycarbonate cage (20.5 cm high $\times$ 58 $\times$ 40 cm at top, 55 $\times$ 37.5 cm at bottom) with a conditioning chamber in each of the 4 corners (Fig. 1B). Each chamber allows access to two water bottles for drinking one each side, by means of closable round opening. Sensors at these opening allow registering nose-pokes (Fig. 1C). The conditioning corners are accessible via a ring containing a transponder reader antenna and presence is confirmed by temperature-differential sensor. A visit is defined by antenna reading and the presence of signal. A nose-poke is counted each time the mouse inserted its nose in the round opening, whether the door opened or not. Licks are registered by a lickometer, each time a mouse touches the drinking spout. The apparatus is controlled by the IntelliCage software 2.1, described previously (Krackow et al., 2010; Voikar et al., 2010). The study was done with female mice as they have a greater compatibility in a social home cage setting and the long-term monitoring will most likely cancel most of the fluctuation due to their 5-days long estrous cycle (Kobayashi et al., 2013).

General procedures

One week before the start of the experiment, mice were implanted subcutaneously with Radio frequency identification transponders (Planet ID GmbH, Essen, Germany) under isoflurane inhalation anesthesia.

Description of the 22 days protocol (Figure 1):

Free adaptation phase—Mice were separated in groups of 8 animals with identical genotype and placed in 4 IntelliCages. During the 1st four days, all four corners and door were opened, giving access to 2 water bottles per corner. Food was accessible *ad libitum*. The alternation pattern was defined on four consecutive visits as follow: 1) spontaneous corner alternations (SCA) were counted if three first corner visits out of a four were different, 2) alternate corner returns (ACR) were counted when a mouse visited the same corner two times, with a different corner in-between and 3) Same corner returns (SCR) were counted when a mouse visited the same corner two times in a row. As mice could visit the corners at their own rhythm, we started the analysis for spontaneous alternations using a number of visits threshold of 100 (not a time threshold). This number was chosen to be high enough to establish a percentage and also no too large to remain in the acclimation phase for the mouse (one mouse was removed as it took over two days to reach 100 visits). The last mouse reached 100 visits 11h after the beginning of the free adaptation phase. The analysis was then done on all the corner visits of the free adaptation phase.

Nose-poke adaptation—During the three following days, all doors were closed and could be opened once per visit in response to a minimum of successive 5s nose-poke giving access to the water spouts.

Sucrose preference and reversal—During the 3 following days, water was replaced by a 8% sucrose solution in one bottle of each corner. To prevent learning by imitation, cage mates were divided in 4 pairs, each pair of mice had a designated corner (correct corner) in which they could access water or sucrose in response to a 5s nose-poke. The correct corner was set as the opposite corner (in the diagonal) for the three following days in the reversal phase.

Fixed schedule drinking—Sucrose solution was replaced by water (2 water bottles per corner, as in the first two phases). During the 9 following days, water access was restricted to two one-hour time periods during the light phase (11 am and 4 pm). Mice still had to nose-poke in order to open access to the spout, but any nose-poke outside those 2 hours did not give access to the spout. We studied this phase during dark and light phase to observe the influence on the daily rhythm, and we also focused on the one-hour period before, during and after the access to water in order to analyze the anticipatory and persistent behavior of mice.

Statistical analysis

In a first step, data were checked for outliers (> 3 times the standard deviation from the strain mean) for each phase of the experiment. One mouse was removed for outlying in the number of visits and nose-pokes in the free adaptation phase. Another one was not drinking anymore in the nose-poke adaptation phase and was removed. A third animal was outlying in time spent in the corner during fixed schedule drinking and was also removed. Once removed in one phase, outlier mice were automatically removed for the following phases.

In a second step, the two cages housing *Gpr88*^{+/+} mice and the two cages housing *Gpr88*^{-/-} mice were compared to each other for total number of visits and nose-pokes in the first in habituation and nose-poke adaptation phases. The two cages housing *Gpr88*^{+/+} mice were indistinguishable, as were the two cages housing *Gpr88*^{-/-} mice. Thus, mice were regrouped genotype-wise for the analysis.

Data were analyzed using IBM SPSS statistic 20 (IBM, Armonk, NY, USA) to run two or three factors repeated-measures (RM) ANOVA. Whenever sphericity was violated a Greenhouse-Geisser correction was applied. For post-hoc and planned pairwise comparison, we used a Sidak's multiple comparison correction was applied when significant ANOVA results between factors were revealed. For mean comparison, one-way ANOVA were performed when normality and equality of the variance were met, otherwise a non-parametric Mann-Whitney U-test was applied. An error probability level of $p < 0.05$ was accepted as statistically significant. All values are represented by means $\pm$ S.E.M.

Results

We monitored spontaneous activity of group-housed *Gpr88*^{-/-} female, and their controls, using IntelliCages with saw dust-covered floor to reduce external stressors. The experimental design allowed animal monitoring under basal conditions (free adaptation), during conditioning (nose-poke adaptation; place learning then reversal learning) and in challenging conditions (fixed schedule drinking) in that order. Activity was recorded for

each mouse using RFID tracking to register individual visits of the conditioning corners. The five phases of the protocol lasted 22 days in total, and are detailed in Figure 1.

Free adaptation phase – *Gpr88*^{-/-} mice show delayed habituation and altered exploratory behavior

Prior to any challenge, it is essential to characterize responses to the novel environment, here the IntelliCage, and baseline activity of the animals. Plus, GPR88 deficiency affects striatal function impairing locomotor activity (Do et al., 2012; Meirsman et al., 2016a; Quintana et al., 2012). During the free adaptation phase, mice could freely explore the new cage for 4 days and had access to all eight water bottles. Overall, diurnal activity was similar for the two groups. Peaks of activity were observed during the active dark periods and deeps in the activity were obvious during the resting light periods (Figure 2A). Analysis was then further performed separately for dark and light periods. In Figure S1A, cumulative raw data are shown and the number of licks shows that *Gpr88*^{-/-} mice took more time to start drinking from the water bottles.

Dark period—In Figure 2B, two-ways RM-ANOVA revealed an effect of Time on the number of visits ($p < 0.001$) but not on the number of nose-pokes and licks during the active phase. Genotype had an effect on the number of nose-pokes ($p = 0.023$) but not on the number of visits and licks. Interaction between Time and Genotype had an effect on all three parameters ($p_{\text{visits}} = 0.003$, $p_{\text{nose-pokes}} = 0.002$ and $p_{\text{licks}} = 0.02$). Pairwise comparison tests yielded a significant decrease in the number of visits (Dark1 (D1) vs. D4, $p < 0.001$), nose-pokes (D1 vs. D4, $p = 0.017$) and licks (D1 to D3, $p = 0.032$) for *Gpr88*^{+/+} mice, showing a clear adaptation to the new environment. In contrast, *Gpr88*^{-/-} mice began with a lower number of visits on D1 ($p < 0.01$) and a similar number of nose-pokes was observed for the two groups. Further, *Gpr88*^{-/-} mice did not decrease their number of visits and nose-pokes over time, and therefore showed a higher number of visits ($p = 0.026$) and nose-pokes ($p = 0.012$) on D4 compared to *Gpr88*^{+/+} mice. Moreover, *Gpr88*^{-/-} mice showed no significant difference in the number of licks between D1 and 3 and a significant increase in D4 ($p = 0.043$). Together, data from the dark period of the free adaptation phase reveal a clear difference between genotypes in adapting to the new environment, with *Gpr88*^{+/+} but not *Gpr88*^{-/-} mice showing habituation to the environment after four days.

Light period—In Figure 2C, two-ways RM-ANOVA revealed an effect of Time on the number of visits ($p < 0.001$), nose-pokes ($p < 0.001$) and licks ($p = 0.044$) but no effect of Genotype. Interaction between Time and Genotype showed an effect in the number of nose-pokes ($p = 0.004$) and licks ($p = 0.008$) but not in the number of visits. Pairwise comparison tests revealed a stable number of nose-pokes and licks throughout the free adaptation phase for *Gpr88*^{+/+} mice, and a significant increase of nose-pokes (L1 vs. L3, $p < 0.001$) and licks (L1 vs. L3, $p = 0.014$) for *Gpr88*^{-/-} mice. Thus, in their resting period, *Gpr88*^{-/-} mice do not differ from *Gpr88*^{+/+} mice, except for the number of licks that was higher in *Gpr88*^{+/+} mice on L1 ($p = 0.034$) and L2 ($p = 0.025$).

Spontaneous alternations—To test hippocampal-dependent navigation, as was done previously using a Y-maze (Meirsman et al., 2016a), we used the four identical corners of

the IntelliCage to quantify spontaneous alternations over the first hundred visits of each mouse to stay in the acclimation phase. As for the Y-maze, we divided the alternation in 3 groups 1) spontaneous corner alternation (SCA), 2) alternate corner return (ACR) and 3) same corner return (SCR). Two-way ANOVA on the number of alternations revealed no effect of Genotype but an effect of the type of alternation ($p = 0.043$) and an interaction between both factors ($p = 0.004$). Pairwise comparison tests revealed that *Gpr88*^{+/+} mice made a significantly higher number of spontaneous corner alternations compared to alternate corner returns ($p = 0.028$), same corner returns ($p = 0.011$), and compared to *Gpr88*^{-/-} mice ($p = 0.007$) (Figure 2D). Thus, although there was no significant difference in the total number of alternations between the groups ($p = 0.066$) (Figure 2E), *Gpr88*^{+/+} mice displayed an exploration behavior going successively in each corner, whereas *Gpr88*^{-/-} mice displayed an increase sequence of repeated actions by visiting the same corner several times in a row. We also characterized the alternation pattern of visits over the entire free adaptation phase. As shown in Figure 2F, two-way ANOVA on the number of alternations revealed an effect of Genotype ($p = 0.008$), of the type of alternation ($p = 0.003$) and an interaction between both factors ($p = 0.011$). Pairwise comparison tests revealed that *Gpr88*^{+/+} mice displayed the three types of alternations to similar levels, and made significantly more spontaneous corner alternations ($p = 0.002$) and alternate corner returns ($p = 0.015$) than *Gpr88*^{-/-} mice. In contrast, *Gpr88*^{-/-} mice made significantly more same corner returns than spontaneous corner alternations ($p = 0.001$) and alternate corner return ($p < 0.001$). Total alternations over the 4 days of free adaptation phase were also lower for *Gpr88*^{-/-} mice ($p = 0.04$) (Figure 2G). Again, therefore, *Gpr88*^{+/+} mice displayed a randomized visit behavior with no difference in the alternation pattern, whereas *Gpr88*^{-/-} mice showed an altered exploratory behavior with more same corner returns.

Taken together, data from the free adaptation phase show a clear habituation pattern for *Gpr88*^{+/+} mice, including decreasing number of visits, nose-pokes and licks along dark periods. *Gpr88*^{-/-} mice did not display this habituation pattern, as shown by stable number of visits and increased number of nose-pokes throughout this phase. Further, *Gpr88*^{-/-} mice displayed a preference to return to the previously visited corner (for statistical detail see Supplementary Table 1). Observations from this phase confirm non-habituation behaviors, which we previously described for *GPR88*^{-/-} mice (Meirsman et al., 2016a) and an exploratory behavior distinct from control mice.

Nose-poke adaptation - *Gpr88*^{-/-} mice remain more active 7 days after entering the IntelliCage

Following the Free adaptation phase and prior to any behavioral tests in the IntelliCage, female mice were exposed to three days of behavioral training, in which they needed to perform a 5s nose-poke per visit to access the water bottles, the nose-poke adaptation phase.

Dark period—In Figure 3A, Two-way RM-ANOVA revealed an effect of Time on the number of visits ($p = 0.001$) and nose-pokes ($p = 0.004$), but not on the number of licks. Genotype had an effect on the number of nose-pokes ($p = 0.041$) and licks ($p = 0.006$) but not on the number of visits. Interaction between Time and Genotype had an effect on all three parameters ($p_{\text{visits}} < 0.001$, $p_{\text{nose-pokes}} = 0.023$ and $p_{\text{licks}} = 0.018$). Pairwise comparison

tests revealed a significant decrease in the number of visits (D5 vs. D7, $p = 0.014$), and no significant changes in the number of nose-pokes and licks for *Gpr88*^{+/+} mice. Whereas, *Gpr88*^{-/-} mice showed a trend to decrease their number of visits (D5 vs. D7, $p = 0.053$), a steep decrease followed by an increase in the number of nose-pokes (D5 vs. D6, $p < 0.001$ and D6 vs. D7, $p = 0.009$) and licks (D5 vs. D6, $p = 0.004$ and D6 vs. D7, $p = 0.003$). Thus, there was a higher number of visits ($p = 0.008$), nose-pokes ($p = 0.047$) and licks ($p = 0.011$) on D7 for *Gpr88*^{-/-} mice compared to their controls.

Light period—In Figure 3B, Two-way RM-ANOVA revealed an effect of Time on the number of visits ($p < 0.001$), nose-pokes ($p < 0.001$) but not licks, no effect of the Genotype and no Interaction between Time and Genotype on all three parameters.

Overall—In Figure 3C, *Gpr88*^{-/-} mice showed a higher total number of licks ($p = 0.009$) and nose-pokes ($p = 0.05$).

Taken together, these data show a stabilization of control mice behavior after habituation phase, whereas *Gpr88*^{-/-} mice still showed more activity with significantly higher numbers of visits, nose-pokes and licks on D7 during the dark. Notably however, and as for control mice, mutant animals showed no difficulty in acquiring 5s nose-poke conditioning to obtain water (for statistical detail see Supplementary Table 2)

Place learning and reversal - *Gpr88*^{-/-} mice show delayed preference learning but reversal learning is intact

GPR88 deficiency affects learning and memory in different classical paradigms (Meirsmann et al., 2016a; Quintana et al., 2012). Here, *Gpr88*^{-/-} mice were tested for place learning and reversal procedures. These tasks consisted in two phases of three days each in the IntelliCage protocol (Figure 1A). First, in the place learning phase, the water of one bottle was replaced by an 8% sucrose solution in each corner. Pairs of cage-mates were assigned to one corner (correct corner) in which they could access water or sucrose in response to a consecutive 5s nose-poke. The other corners were accessible but access to the bottles was blocked. Second, in the reversal phase, the corner with accessible bottles was switched to the opposite side for each pairs of cage-mates. For each phase, we analyzed the total number of visits, nose-pokes and lick per dark and light periods. We also analyzed the correct number of visits and nose-pokes, defined as visits or nose-pokes in the corner with access to bottles, as well as the percentage of correct visits (% visits) and nose-pokes (% nose-pokes). The licks were separated in total lick and sucrose licks. For the percentage of licks on the sucrose side (% sucrose licks), the data are shown per day (not per dark and light periods) because several mice (8 per group) did not perform licks during the light phase (Figure 4F and 4M).

Place learning—The total number of visits (Figure 4A), nose-pokes (Figure 4C) and licks (Figure 4E) in all corners during the place learning phase were not different between the two genotypes. Similarly, the number of visits and nose-pokes in the correct corners and licks in the sucrose side were not significantly different between the two genotypes. Two-ways RM-ANOVA revealed an effect of Time on the percentage of nose-pokes in the correct corner ($p = 0.007$) with no effect of the percentage of correct visits. No effect of Genotype or

interactions was detected for the two parameters. Further, pairwise comparison revealed lower % visits ($p = 0.014$, Figure 4B) and % nose-pokes ($p = 0.009$, Figure 4D) in the correct corner for *Gpr88*^{-/-} mice on D9 compared to their wild-type littermates. Indeed, *Gpr88*^{+/+} mice displayed a preference to visit the correct corner with more than 34% on D9 (chance level of visit is 25%), whereas *Gpr88*^{-/-} mice were only at 25.8% correct visits. Finally, both groups displayed a similar preference to lick (Figure 4E) on the sucrose side (> 60% for each day of the experiment) over the three days of the experiment (Figure 4F). Taken together, these data show that the development of the preference for the correct corner was slightly delayed in *Gpr88*^{-/-} mice, yet the preference for sucrose was similar in the two groups.

Reversal—Our previous study showed enhanced behavioral flexibility of *Gpr88*^{-/-} mice in a cross-maze (Meirsman et al., 2016a). To challenge this phenotype, we tested reversal learning after the place learning phase was completed. In the reversal phase, the two groups displayed a similar number of total visits (Figure 4G), as well as correct number of visits (Figure 4H) and sucrose licks (Figure 4M). Also, *Gpr88*^{-/-} mice displayed significantly more total number of nose-pokes ($p = 0.028$) (Figure 4J) and total number of licks ($p = 0.047$) (Figure 4L). Further, the two groups showed a percentage of correct visits and nose-pokes above 30% and 40% respectively, and a preference for the sucrose solution higher than 67%, during the reversal phase (Figure 4H, 4K and 4M). Next, we tested whether place reversal learning and strategy switching occurred immediately after the corner switch, we specifically analyzed the percentage of correct visits in the reversal corner over the first 25, 50, 100 and 200 corner visits (Figure 4I). A Two-ways RM-ANOVA revealed an effect of the number of visits on the Percentage of correct visits ($p < 0.001$) but no Genotype or interaction effect. This result shows that both groups increased their percentage of new correct corner visits in D11.

Altogether, data from place learning and reversal indicate that *Gpr88*^{-/-} mice are capable to learn the task, although with some delay, and show no obvious behavioral flexibility abnormality (for statistical details see Supplementary Table 3) and for the full dataset -% visits to correct and incorrect corners during both learning and reversal phases- see Supplementary Figure 2).

Fixed schedule drinking - *Gpr88*^{-/-} mice show altered anticipatory and persistent behavior

Gpr88^{-/-} mice show striatal alteration of basal dopamine and an enhanced amphetamine-induced hyper-locomotion (Logue et al., 2009) suggesting a modification in reward-related responses. To test temporal learning abilities as well as motivation for a natural reward, we used the Fixed Schedule drinking task. During 9 days mice had access to water bottles for one hour twice a day, starting at 11am and 4pm (light phase). The rest of the time, doors in front of the spouts remained closed.

12 hours bins—Prior to the statistical analysis, we controlled whether all the mice were activating the lickometer during the two access hours. As shown in Figure S3A, three-way RM-ANOVA revealed an effect of Days ($p < 0.001$), and Hour ($p < 0.001$), but not Genotype on the number of licks. Interactions effect were also found between Days and Hour ($p <$

0.001) and Days x Hour x Genotype ($p = 0.004$) but not Days and Genotype. Pairwise comparison tests revealed no difference in the number of licks for hour 11am and hour 4pm between the two genotypes. However, both genotypes displayed a significantly higher number of licks in hour 11am compared to 4pm ($p < 0.001$ for both). In addition, an average of 86% ($\pm 10\%$) of all mice went to drink on hour 11am, against only 43% ($\pm 5\%$) on hour 4pm over the nine days of experiment. The difference between 11 am vs 4 pm strongly suggests that all mice were drinking sufficiently, despite restricted access to water.

In this task however, *Gpr88*^{-/-} mice showed a different response compared to their counterparts. Two-ways RM-ANOVA revealed a significant effect of Time, Genotype and interaction on the number of visits (Figure 5A; $p_{\text{time}} < 0.001$, $p_{\text{genotype}} = 0.016$ and $p_{\text{interaction}} < 0.001$) and nose-pokes (Figure S3B; $p_{\text{time}} < 0.001$, $p_{\text{genotype}} < 0.01$ and $p_{\text{interaction}} < 0.001$) in the corner. Pairwise comparison tests revealed that control mice modified their corner visit activity after three days, as the difference between dark and light periods disappeared ($p > 0.05$ from days 16 to 22) (Figure 5A), with no significant changes in the number of nose-pokes (Figure S3B). *Gpr88*^{-/-} mice also decreased their number of corner visits during the dark period. However, a marked difference remained between dark and light periods ($p < 0.01$ from days 14 to 22) (Figure 5A). Also, the difference in nose-poke number between dark and light periods decreased until D16 and then stabilized ($p > 0.1$) (Figure S3B). While observing only the active periods (dark periods) in Figure S3C, a two-way RM-ANOVA, on the number of visits, showed an effect of the Days ($p < 0.001$), of Genotype ($p < 0.001$) and an interaction between both ($p = 0.006$). Pairwise comparison revealed a significant decrease in the number of visits on D15 for both *Gpr88*^{+/+} ($p = 0.036$) and *Gpr88*^{-/-} mice ($p = 0.022$) followed by slow decrease before stabilization of the number of visits until D18 for *Gpr88*^{+/+} mice and D19 for *Gpr88*^{-/-} mice. Overall, *Gpr88*^{-/-} mice displayed a significantly higher number of visits during the dark periods ($p < 0.001$) (Figure 5B), and lower number of visits in the light periods ($p < 0.01$) (Figures 5C & S3D). At 11 am, more than 80% mice accessed the water bottles on average over the 9 days of the experiment, whereas less than 50% mice made licks at 4 pm (Figures S3A, E & F), making statistics less robust for the latter time period. We therefore focused on the 11 am period and compared the number of visits and nose-pokes one hour before (hour 10), during (hour 11) and after (hour 12) mice had access to the water, during the 9 days of the experiment.

Hours before water access—During hours preceding water access, *Gpr88*^{-/-} mice showed a different response compared to their counterparts. Two-way RM-ANOVA on the number of visits showed a significant interaction between Days and Genotype for the three hours before water access (p 's < 0.05) (Figure 5D). During the fourth (-4h) and fifth hours (-5h) before water access period both genotypes visited the corners equally over days of the experiment. However, overall the number of visits increased in *Gpr88*^{+/+} mice compared to *Gpr88*^{-/-} mice during the 3 hours preceding water access (p 's < 0.05). Two-way RM-ANOVA on the number of nose-pokes only revealed an interaction between Days and Genotype two hours before water access ($p < 0.05$). (Figure 5E). Pairwise comparison revealed differences between the two groups only at the beginning of the phase, on day 14 ($p < 0.05$) and 15 ($p < 0.001$).

Hour during water access—As showed in Figure 5D, during the hour when water was accessible, two-way RM-ANOVA on the number of visits showed no effect of the Genotype, a significant effect of Days ($p < 0.001$) and an significant interaction between Days x Genotype factors ($p < 0.001$). Two-way RM-ANOVA on the number of nose-pokes showed an effect of Days only ($p = 0.004$), with no Genotype or interaction effect for the two factors. Pairwise comparison tests on the number of visits revealed a difference between the groups only on day 14 ($p = 0.001$), showing that when water was accessible, both groups had a similar visit behavior during almost the entire experiment.

Hour after water access—During the 2 hours following water access, two-way RM-ANOVA on the number of visits showed a significant interaction between Days and Genotype factors (p 's < 0.05) (Figure 5D). Pairwise comparison tests revealed that for the first hour following water access time a higher number of visits for *Gpr88*^{-/-} from day 15 to 19 compared to *Gpr88*^{+/+} mice ($p < 0.05$, except at day 17 $p = 0.07$). However, for the second hour after water access period, only higher visits for *Gpr88*^{-/-} were observed on day 15 and 16 compared to *Gpr88*^{+/+} mice. Two-way RM-ANOVA on the number of nose-pokes also showed a significant interaction between Days and Genotype factors ($p = 0.002$) between the two factors an hour after water access (+1h) but not on the second hour (+2h) (Figure 5E). Pairwise comparison tests revealed that for the first hour following water access time a higher number of nose-pokes for *Gpr88*^{-/-} from day 15 to 19 compared to *Gpr88*^{+/+} mice ($p < 0.05$, except at day 17 $p = 0.07$).

Taken together, these data first indicate that *Gpr88*^{-/-} mice show a delay in adjusting their behavior to the new rule (late stabilization of the number of corner visits during the dark phase). Further, and contrary to control mice also, mutant animals did not seem to develop an anticipatory behavior (higher number of visits) in the time period preceding the water-accessible hour. Finally, mutant mice were more persistent (higher number of visits) to visit corners after water was not accessible anymore (for statistical detail see Supplementary Table 4).

Discussion

Previous studies of *Gpr88* null mutant mice were done using standard behavioral tests, which provide snapshots of individual mouse behavior in novel testing environments, (Logue et al., 2009 ; Meirsman et al., 2016a; Quintana et al., 2012). In this study, we performed longitudinal analysis of group-housed mutant females in an automated home cage system. We designed a 22-days protocol in the IntelliCage apparatus where mouse behavior was monitored in 4 different phases: 1) Free adaptation: *Gpr88*^{-/-} mice showed delayed habituation to the home cage, and altered exploratory behavior in their alternation pattern; 2) Nose-poke adaptation: non-habituation in *Gpr88*^{-/-} mice continued through this phase, however mutant mice acquired the 5s nose-poke conditioning similarly to their control counterparts; 3) Place learning and reversal: *Gpr88*^{-/-} mice showed a slight delay in developing preference for the water/sucrose accessible corner, but showed no difference from controls in the reversal phase; 4) Fixed schedule drinking: *Gpr88*^{-/-} mice showed delayed adaptation to the fixed drinking hour with an elevated light/dark activity along the entire phase. Importantly in this phase, control animals showed higher activity during the

hour preceding accessibility to water, and highly reduced activity after access to water was terminated, while on the contrary, mutant mice showed a lack of anticipatory behavior followed by persistent higher activity after water was not available anymore.

Results from the IntelliCage study are summarized in Table 1 and confronted to published data using conventional testing methods. In order to be able to compare data from the IntelliCage set-up to those from conventional testing, we have classified behavioral responses collected throughout the five IntelliCage phases in five phenotypic categories: hyperactivity (section 1), non-habituation (section 2), altered exploratory behavior (section 3), learning (section 4) and anticipatory behavior (section 5). While the three former are fully concordant with already reported phenotypes, the learning phenotype appears complex and is refined in this study, and the lack of anticipatory behavior is novel.

The IntelliCage protocol confirms hyperactivity, non-habituation and altered exploratory behavior in *Gpr88* deficient mice

In previous reports, deletion of *Gpr88* in mice was reported to caused hyperactivity, non-habituation and repetitive exploratory behavior (detailed in Table 1, first three sections). This phenotype was reported through different tests and in both males and females. Our data using group-housed females in a home cage environment are in line with these results. In the five different phases of the protocol, *Gpr88*^{-/-} mice showed a higher number of visits, nose-pokes and/or licks (Table 1). Under IntelliCage conditions also, the *Gpr88* deletion effect could not be detected during the light (resting) phase, hyperactivity was only observed during the active phase (dark phase) and did not affect diurnal rhythm (Figure 2A). Further, *Gpr88*^{-/-} mice showed a non-habituation behavior, with no decrease in the number of visits after the free adaptation, more same corner returns and maintained a marked light-dark rhythm in the fixed schedule (Figures 2B, E, G and 5A). Finally, the higher percentage of same corner returns (Figures 2E and 2G) can be interpreted as an altered exploratory behavior, as detected in *Gpr88*^{-/-} mice in our previous study (Meirsman et al., 2016a). Together, data throughout the IntelliCage protocol strengthen the characterization of *Gpr88*^{-/-} mice displaying a non-habituating hyperactivity and confirm the tendency to repeat certain behaviors. Compared to standard behavioral test, automated home cage recording brings advantages in behavioral screening in that, with only one experiment using relatively few animals (n = 16/group) and time, we could reproduce previous findings that necessitated ten different assays (Meirsman et al., 2016a). Of note, our data are focused on female animals, whereas previous standard testing used both males and females and showed few statistical gender differences, such as nest building, startle response and rotarod (Logue et al., 2009; Meirsman et al., 2016a; Quintana et al., 2012). Hence, it would be interesting to repeat this IntelliCage experiment with groups of male siblings raised together.

The Intellicage long-lasting protocol reveals only subtle learning deficit

The total deletion of *Gpr88* in mice affects several forms of learning, which involve both striatal and hippocampal function (see Meirsman et al., 2016a; Quintana et al., 2012 and Table 1). In a first study (Quintana et al., 2012), *Gpr88*^{Cre/Cre} mice showed normal turned-based (egocentric) learning involving the striatum (Rubio et al., 2012) but cue-based (allocentric) learning involving the hippocampus was delayed (Kleinknecht et al., 2012). In a

further study using different testing paradigms (Meirsman et al., 2016a), we reported facilitated hippocampal-dependent behaviors in *Gpr88*^{-/-} mice, based on less repetitive arm re-entries in the Y-maze, higher preference for the displaced object in the novel object recognition test, and a faster acquisition and behavioral shift in the dual solution task using a cross-maze, all requiring hippocampal integrity (Kleinknecht et al., 2012; Oliveira et al., 2010). In the IntelliCage, learning occurs in yet another different experimental setting, and likely recruits striatal and hippocampal functions differently from the previous studies. In this case, *Gpr88*^{-/-} mice mutant mice showed slightly delayed place learning, and reversal learning was intact indicating that spatial cues remain correctly interpreted (Figure 4). In fact, the learning phenotype of mutant mice in the IntelliCage set-up remained extremely subtle. It seems therefore that, despite previous evidence of altered striatal/hippocampal balance in *Gpr88*^{-/-} mice (Meirsman et al., 2016a), the non-stressful home cage conditions allowed optimal learning performance in mutant mice. This is clear evidence that the IntelliCage system offers a very distinct environmental and emotional context for animal testing, compared to conditions of traditional behavioral analyses.

The IntelliCage system reveals delayed anticipatory behavior in *Gpr88* deficient mice

The longitudinal aspect of the IntelliCage protocol allowed analyzing the behavior before and after the one-hour water access in the fixed schedule-drinking phase. The need to drink water was comparable in the two groups because, when water was accessible, no major difference was observed between the groups in number of visits, nose-pokes and licks at both 11 am or 4 pm (Figure 5D & E and Fig. S3A), suggesting that thirst-activated circuits in the hypothalamus (Oka et al., 2015) are unaffected in mice lacking *Gpr88*. Interestingly however, after four days, control mice increased their number of visits during the hour preceding water access, which may be interpreted as an anticipation of the up-coming event (water access), whereas *Gpr88*^{-/-} mice did not show this behavior (Figure 5D). This interpretation is based on the fact that, although the 24h water deprivation step does not produce robust physiological changes (Bekkevold et al., 2013), mice are subjected to 19-hour water deprivation for nine consecutive days and that, under these conditions, drinking water is considered an innately rewarding behavior (Rolls and Rolls, 1982).

Reward anticipation neural networks involve both the striatum and cortical regions including visual association cortex and the somatosensory cortex (Jia et al., 2016) and the fact that *Gpr88*^{-/-} mice did not anticipate the water access suggests a possible alteration of this neural network. This is consistent with the prominent expression of GPR88 in both striatum and cortex (Massart et al., 2009, Massart et al., 2016). This conclusion also fully accords our recent discovery of specific GPR88 expression in layer 4 of the somatosensory cortex, paralleling delayed sensory processing in *Gpr88*^{-/-} mice (Ehrlich et al., 2017), as well as our recent fMRI data from *Gpr88*^{-/-} mice indicating disrupted functional connectivity predominantly at the level motor and sensory cortices, as well as the striatum in live mutant mice (Arefin et al., 2017). In further support of our interpretation, the ventral striatum was shown activated in response to ingestive behavior (Pitchers et al., 2010; Yoshida et al., 1992), and dopamine levels in lateral hypothalamic area and the nucleus accumbens are associated with anticipatory and consummatory phases of feeding (Legrand et al., 2015). Altogether therefore, we propose that GPR88 plays a role in reward anticipation, involving

striatum-cortex communication. This function has not been described as yet, and was revealed by refined temporal data analysis from the IntelliCage approach. Further experiments using highly palatable food schedule in the IntelliCage (similar to Hsu et al., 2010) or selected standard testing paradigms (5-choice serial reaction time task) and conditional knockout approaches (see for example Meirsmann et al., 2016b) will further investigate this aspect of Gpr88 knockout mice behavior, and determine the exact brain site for this particular GPR88 function.

Conclusion

Standard behavioral testing is designed to study hypothesis-driven behavioral modifications, and provide reasonable interpretations in the least amount of time. A large number of classical testing paradigms are based on the behavioral reaction to a novel environment (e. g. open-field, elevated plus maze) for a short time period (minutes to maximum one hour). Thus, standard testing provides no baseline condition, and reactions are always recorded in response to challenging conditions, hence in a state of arousal (“stress”) of the animal. However, psychiatric diseases are chronic illnesses and their diagnosis, treatment and recovery are long lasting processes and depend on the environmental factors including pharmacotherapies. Their complexity compels the development of long-term, unbiased behavior assays taking place in familiar environment for the mice. In Long-term-based behavioral analysis, such as IntelliCage, the animal is constantly monitored, allowing a multidimensional behavioral profiling. Through the development of an IntelliCage protocol, we were able to observed hyperactivity (Figure 2, 3, 4 and 4), non-habituation (Figure 2 and 5), altered exploratory behavior (Figure 2 and 5) and learning alteration (Figure 4), that were previously described using ten different classical behavioral tests (Table 1). Home cage monitoring therefore extends the characterization of these mutant mice, revealing another facet of GPR88 function, and providing yet another useful endophenotypic profile in the context of genetic mouse models for neuropsychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The IntelliCage system

A. Schematic representation of the IntelliCage five-phase protocol. 1) Free adaptation: all four corners and door were opened, giving access to 2 water bottles (blue circles) per corner (D1 to L4). 2) Nose-poke adaptation: all doors were closed and could be opened once per visit in response to a minimum of successive 5s nose-poke (D5 to L7). 3) Place learning: one bottle of each corner was replaced with an 8% sucrose solution (green circle). Each pair of mice had a designated corner (correct corner) in which they could access water or sucrose in response to a 5s nose-poke. The other corners were accessible but not the bottles (red circles) (D8 to L10). 4) Reversal Learning: the correct corner was set as the opposite corner (D11 to L13). 5) Fixed schedule drinking (FSD): water access was restricted to two one-hour time periods during the light phase (11 am and 4 pm) (D14 to L22). Timeline: grey and white rectangles correspond to dark (D) and light (L) periods, respectively and yellow rectangles represent the change of protocol period; blue arrows show hours of accessible water in the FSD. **B.** Conditioned chamber: accessible via a ring containing a transponder reader antenna and presence is confirmed by temperature-differential sensor. **C.** 5s Nose-poke water access.

Figure 2. Free adaptation

A. Total corner visits per hour over 4 days: both groups displayed a contrasted activity between dark and light periods. **B.** Number of visits (left), nose-pokes (middle) and licks (right) during the dark period. **C.** Number of visits (left), nose-pokes (middle) and licks (right) during the light period. **D.** Distribution of alternation types over the first hundred visits. Spontaneous corner alternation (SCA), alternate corner returns (ACR) and same corner returns (SCR). **E.** Total number of alternations over the first hundred visits. **F.** Distribution of alternation types over four days. **G.** Total number of alternations over four

days. Black bars, *Gpr88*^{+/+} mice; white bars, *Gpr88*^{-/-} mice; grey bars, dark periods. Numbers in bar graphs represent the n for each group. All graphs show means $\pm$ S.E.M, except for cumulative plots that show individual data. Statistical significance shown here are pairwise comparison, *p<0.05, **p<0.01. (for statistical details see Supplementary Table 1).

Figure 3. Nose-poke adaptation

A. Number of visits (left), nose-pokes (middle) and licks (right) during the dark period. **B.** Number of visits (left), nose-pokes (middle) and licks (right) during the light period. **C.** Total number of visits (left), nose-pokes (middle) and licks (right). Black, *Gpr88*^{+/+} mice; white, *Gpr88*^{-/-} mice. Grey bars represent dark periods. Numbers in bar graphs represent the N for each group. All graphs presents means $\pm$ S.E.M, except for the cumulative plot which represents individual data. Statistical significance shown here are pairwise comparison, * $p < 0.05$, ** $p < 0.01$. (for statistical detail see Supplementary Table 2).

Figure 5. Fixed schedule drinking

A. Number of visits in 12h bins in periods Dark 14 to Light 22. **B.** Total Number of visits during the dark period. **C.** Total Number of visits during the light period. **D.** Number of visits per day during the 5 hours before (-5h to -1h), during (water access) and 2 hours (+1h and +2h) after water accessibility. **E.** Number of nose-pokes per day during the 5 hours before (-5h to -1h), during (water access) and 2 hours (+1h and +2h) after water accessibility. Black, *Gpr88*^{+/+} mice; White *Gpr88*^{-/-} mice. Grey bars represent dark periods. All graphs presents means $\pm$ S.E.M. Statistical significance shown here are pairwise

comparison * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (for statistical detail see Supplementary Table 4).

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Summary table (left) and comparison to phenotypes previously describe using traditional behavioral testing (right).

Table 1

	IntelliCage findings in this study		Previous studies using conventional testing		References
	Phase	Parameter (–/– compared to +/+)	Behavioral test	Parameter (–/– compared to +/+)	
Hyperactivity	Free adaptation	higher number nose-pokes during the dark phase	Open-field	longer distance moved under 3mg/kg Amphetamine	Logue et al., 2009
	Nose-poke adaptation	higher number of visits, nose-pokes and Licks	Y-maze	higher number of arm entries	Meirsmann et al., 2016
	Place learning & reversal fixed schedule drinking	higher number of nose-pokes higher number of visits during the dark phase	object recognition grid floor home cage 48h	higher number of visits to object longer distance moved during dark period	Meirsmann et al., 2016 Quintana et al. 2012
Non-habituation	Free adaptation	stable number of visits	Open-field	no decrease of activity after repeated exposure	Meirsmann et al., 2016
	fixed schedule drinking	keep a dark-light pattern			
Altered exploratory behavior	Free adaptation	higher number of same corner returns	Stereotypies	More burying duration and more circling	Meirsmann et al., 2016
	fixed schedule drinking	higher perseverative visits and nose-pokes after access to water	Stereotypies	more climbing and sniffing licking and gnawing under 0.3mg/kg apomorphine	Logues et al., 2009
Learning	Place learning & reversal	preference development delayed	dual solution cross-maze task	faster acquisition and shift	Meirsmann et al., 2016
			Morris water maze	longer latency to escape	Quintana et al. 2012
Anticipatory behavior	fixed schedule drinking	no visit increase before water access	water U-maze	delayed development of the correct choice	Quintana et al. 2012
Unprecedented					