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Research report

Effect of Y_1 receptor deficiency on motor activity, exploration, and anxiety

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Abstract

Neuropeptide Y (NPY) in the CNS plays an important regulatory role in anxiety-related responses as exogenous administration of NPY exerts an anxiolytic-like effect in rodents. This effect is believed to be mediated by the Y_1 receptor system as pharmacological modulation of this Y_1 receptor system results in an increase in anxiety. Here we present a comprehensive phenotyping strategy for characterizing Y_1 receptor knockout animals at different times of the circadian rhythm using several motor activity-, exploration-, and anxiety-related behavioural tasks including open field, elevated plus maze, light–dark, and hole board test. We show that Y_1 deficiency has an important effect on motor activity and explorative-like behaviours and that it results in marked alterations in anxiety-related behaviours. Importantly, the behavioural phenotype of the Y_1 receptor knockout mice is circadian rhythm-dependent and also influenced by stimuli such as restraint stress. In addition, we found evidence for increases in working memory. Taken together, these findings suggest an important role of Y_1 receptors in the regulation of motor activity, exploration, and anxiety-related behaviours. This role is also influenced by several factors such as circadian rhythm and stress exposure confirming the importance of a comprehensive strategy and of using genetic animal models in behavioural neuroscience.

Keywords: Mouse; NPY; Y_1 receptor; Motor activity; Exploration; Anxiety; Circadian rhythm; Restraint stress

1. Introduction

The neurotransmitter neuropeptide Y (NPY) is a 36 amino acid peptide, which is widely distributed in the central nervous system (CNS). At least five receptors (Y_1, Y_2, Y_4, Y_5 and y_6) are classified, which mediate the biological actions of NPY. One of the major effects of NPY in the CNS is a regulatory role in anxiolytic-like responses (for review see [21]). Exogenously administered NPY reduces experimental anxiety in a wide range of animal models including ethologically derived models, such as the elevated plus maze [16], the light-dark test [35] and the social interaction task [22]. In a pharmacological approach, Heilig could prevent these anxiolytic-like actions of NPY (Y_1 receptor expression in rats was inhibited by infusing antisense-oligonucleotides into the amygdala, the key area for the regulation of anxiety-related behaviours [13]). Further pharmacological studies confirmed that the anxiolytic-like properties of exogenous NPY administration (e.g. into the basolateral nucleus of the amygdala) could be blocked by Y_1 receptor antagonists such as BIBO3304 or BIBP3226 [39,48]. Furthermore, icv administration of BIBP3226 itself elicited anxiogenic responses in rats [23]. These pharmacological findings suggest that the pronounced anxiolytic-like properties of exogenously administered or endogenous NPY could predominantly be mediated via Y_1 receptors [13,39].

Importantly, knockout animal models for NPY and its different receptors (Y_1, Y_2, Y_4 or Y_5) offer the possibility to verify and extend pharmacological findings [43] and permit a more detailed investigation into the role of receptors than is possible using a pharmacologically based approach. For example, Bannon et al. described an anxiolytic-like phenotype in a genetic NPY deficient animal model [1]. But so far, the effect of the Y_1 receptor on anxiety and related domains has not been investigated in detail. Interestingly, there is not only evidence for high Y_1 receptor mRNA expression in the amygdala [24] but also for Y_2 receptor mRNA [32]. Evidence for an involvement of

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the Y_2 receptor in the regulation of anxiety-related behaviours in an inhibitory fashion has been demonstrated pharmacologically [30,38] and confirmed in a knockout model for the Y_2 receptor [36,46]. Therefore, in this study we used a comprehensive strategy [20,42] and tested Y_1 receptor knockout animals in several anxiety- and exploration-related paradigms to clarify the role of this Y receptor subtype in anxiety, motor activity, and exploration in a genetic animal model. As it is well known that the circadian rhythm influences locomotor activity and therefore the overall behavioural performance of rodents, we tested our animal model at different times of the circadian rhythm.

2. Materials and methods

2.1. Animals

The generation of Y_1 knockout mice was described previously [18]. A targeting vector for the Y_1 receptor gene was designed and generated (based on 129/SvJ mouse genomic BAC library), which allowed the production of both conditional and germline ($Y_1^{-/-}$) knockout mice. In this construct no original mouse genomic sequence was deleted or rearranged, thus keeping the targeted allele as similar as possible to the original gene. Chimeras carrying the Y_1 floxed gene were crossed with oozyte-specific Cre-recombinase-expressing C57BL/6 mice [40] in order to obtain heterozygotes carrying the Cre-recombinase gene and having the floxed gene already deleted (germline, $Y_1^{+/-}$). Absence of the Y_1 gene in homozygote germline $Y_1^{-/-}$ mice, which also provides verification for the functionality of the Cre-lox system, was confirmed by Southern analysis employing an Y_1 receptor coding sequence-specific DNA fragment and PCR. All further mice generated were maintained on the mixed C57BL/6-129/SvJ background. For the germline Y_1 receptor knockouts, animals no longer carrying the Cre-transgene were selected. Consistency of various phenotypes has been confirmed in Y_1 knockout and wild-type-like animals over more than 10 generations.

Wild-type-like control (WT) and Y_1 knockout mice ($Y_1^{-/-}$) were kept under standard laboratory conditions with a 12:12 h light:dark schedule (light phase: white light with illumination of 70 lx – dark phase: red light with illumination of <2 lx). Age-matched (±12 days) test animals of different sets were pair-housed. The cages were equipped with environmental enrichment: a metal ring for climbing, integrated in the cage lid, and cellulose paper as nesting material. For habituation all animals were transported to the testing room 1 h prior to behavioural testing (animals were tested repeatedly at different times of the circadian rhythm – for test order/age of animals see Table 1). All research and animal care procedures were approved by the "Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee" and were in agreement with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purpose".

Table 1
Set/age of test animals and order of the various behavioural test paradigms

Set and age (d)	Behavioural paradigm	Phase of light cycle
Set 1 (78 ± 12)	OF	Dark
Set 1 (80 ± 12)	EPM	Dark
Set 1 (81 ± 12)	HB	Dark
Set 1 (85 ± 12)	LD	Light 8 h
Set 1 (89 ± 12)	OF	Light 2 h
Set 1 (95 ± 12)	EPM	Light 8 h
Set 2 (66 ± 3)	OF	Light 8 h
Set 2 (68 ± 3)	EPM	Light 2 h
Set 2 (70 ± 3)	HB	Light 2 h
Set 2 (73 ± 3)	LD	Light 2 h
Set 2 (75 ± 3)	HB	Light 8 h
Set 2 (77 ± 3)	LD	Dark
Set 2 (80 ± 3)	EPM + stress	Light 8 h
Set 2 (82 ± 3)	OF + stress	Light 2 h
Set 2 (84 ± 3)	HB + stress	Light 2 h

2.2. Influence of circadian rhythm (context)

Animals were repeatedly tested at different times of the circadian rhythm: (a) during the dark phase – 1 h after red light onset, which is the most active phase for rodents (dark), (b) 2 h after onset of the light phase (light 2 h), at which accordingly to Kopp (2001) C57BL/6j mice still show an intermediate level of overall activity, and (c) 8 h after onset of the light phase (light 8 h), which is in the middle of the resting phase of laboratory mice [25]. To evaluate whether circadian rhythm-dependent differences in the behavioural performance of our animals could be linked to alterations in the endogenous arousal or stress level, we included one battery of tests, where animals were confronted with a common stressor to get a broader idea about the context-dependency of the evaluated phenotype. For this, test mice were exposed to restraint stress as restraint and immobilisation have become synonymous with stress [11]. Animals were restrained in a cylindrical perspex mouse restrainer (length: 8.9 cm; diameter: 2.8 cm) for 8 min just prior to open field (OF + stress; at light 2 h), elevated plus maze (EPM + stress; at light 8 h), and hole board (HB + stress; at light 2 h) performance. The stressful nature of this procedure was well documented by high defecation scores in all of the test animals (for detailed schedule of all experiments see Table 1).

2.3. Open field test (OF)

Motor activity and anxiety were evaluated by placing the mouse into an infrared photobeam controlled open field activity test chamber (MED Associates Inc., USA, Vermont). This paradigm mimics the natural conflict in mice between the tendency to explore a novel environment and to avoid a brightly lit open area [6,8]. Animals were tested for 10 min (during the dark phase for 30 min; if not mentioned otherwise, results of "dark" are based on first 10 min of testing) in an arena (43.2 cm × 43.2 cm), which was divided into a central and a peripheral zone (central zone coordinates: 3/3, 3/13, 13/3, 13/13). Different illumination levels were evident within the device during the light (illumination at floor level: 20 lx) and the dark phase (illumination: <2 lx) of the light cycle. The animal's horizontal activity (i.e. distance travelled, ambulatory frequency and episodes, resting time), time spent and vertical activity in the central and peripheral zone as well as the overall velocity were recorded automatically (software settings: box size: 4; ambulatory trigger: 2; resting delay: 1500 ms). The ratio of central to total distance travelled (distance travelled ratio) and the time spent in the central area was taken as measures of anxiety [9]. Environmental odours were removed by cleaning the arena after each trial with 30% ethanol solution.

2.4. Hole board test (HB)

The hole board test provides independent measures of motor activity and directed exploration [47]. Furthermore, it seems to be a basic task for anxiety and can be used to perform a basal screening of working memory [34]. Mice were placed into the open field activity test chamber, which was equipped with a hole board floor insert for mice (MED Associates Inc.: 16 holes; diameter: 1.6 cm). Different illumination levels were evident within the device during the light (illumination at floor level: 20 lx) and the dark phase (illumination: <2 lx) of the light cycle. The infrared photobeams provided automated measures of the distance travelled, *head dipping* frequency and working memory ratio (number of *head dippings* into novel holes/total number of *head dippings*) in a 7 min test session [28]. After each trial the apparatus was cleaned with 30% ethanol solution [41].

2.5. Elevated plus maze test (EPM)

The EPM represents the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit, elevated, open area [29]. The elevated plus maze was "+"-shaped, two alternate arms were dark and enclosed (34.9 cm × 6.1 cm; height of enclosed arm walls: 20.3 cm), while two alternate arms were open, lit and with ledges (height of ledges: 0.6 cm). The arms' surface was raised 72.4 cm above the floor. Different illumination

levels were evident within the device during the light (illumination on open arms: 220 lx) and the dark phase (illumination: <2 lx) of the light cycle. The mouse was placed into the center field of the "+" (facing an enclosed arm) and was allowed to explore the maze for 5 min. Behaviour was measured online. Frequency of *stretch-attend postures*, time spent on open arms as well as the percentage of open arm entries (open arm entry ratio) [17,34] were recorded as measurements for anxiety. An individual entry was recorded when the animal entered the arm with at least two front paws and half of its body. The number of total arm entries was recorded as a measure of general motor activity. Frequencies of *head dipping* over the edges and *rearing* in the junction area between arms were analyzed as parameters for exploration. After each trial the apparatus was cleaned with 30% ethanol solution.

2.6. Light–dark test (LD)

In the LD test the distance travelled and time spent in a brightly illuminated zone compared to a dark zone and the occurrence of associated exploratory behaviours (e.g. *rearing*) can be used to assess anxiety in rodents [4,7]. The test animals were placed into the open field activity test chamber, which was equipped with a dark box insert for mice (MED Associates Inc.). This insert (covering half the area of the chamber) was opaque to visible light while allowing the infrared photobeams to pass through. Lit and dark compartments were connected by an opening located in the center of the partition. Different illumination levels were evident within the device (lit compartment illumination: 20 lx – dark compartment illumination: <2 lx). The time spent in, entries into and distance travelled in the differentially illuminated compartments as well as distance ratio (ratio of distance travelled in lit compartment/overall distance travelled), vertical activity, and time spent in ambulatory activity were recorded for 10 min. After each trial the apparatus was cleaned with 30% ethanol solution.

2.7. Statistical analysis

Analysis of the various behavioural parameters was assessed by applying one-way analysis of variance (ANOVA) followed by the Fisher-PLSD-test for post hoc comparison, if appropriate. Differences were regarded as statistically significant if $p < .05$. The number of animals for the different sets (n) was 8–14. Significant post hoc effects of the Y_1 knockout animals versus control animals are indicated by asterisks ($^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$). All data are presented as means ± standard error of the mean (S.E.M.).

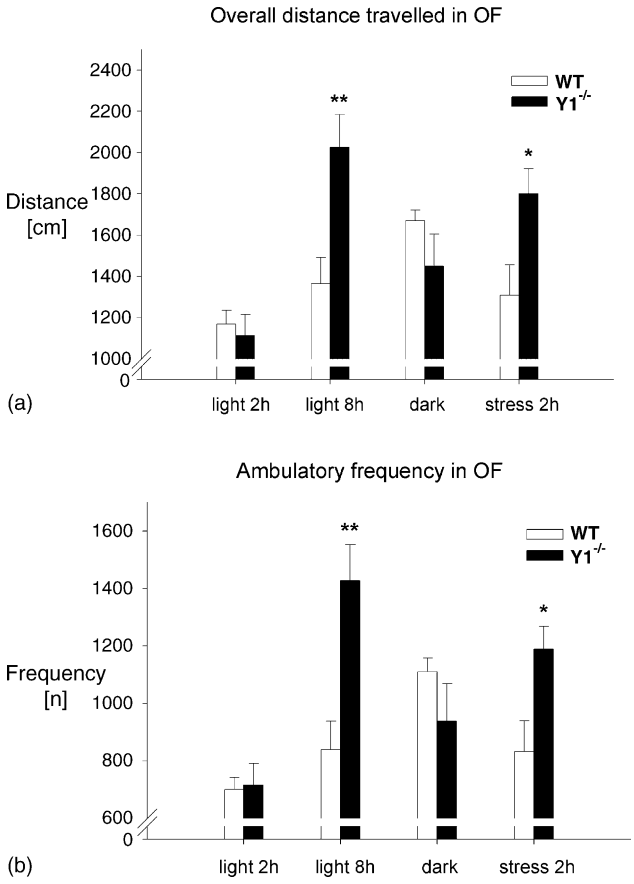

Fig. 1. Motor activity in the open field at different times of the circadian rhythm and after stress exposure: (a) overall distance travelled [cm] and (b) ambulatory frequency [n]. Significant post hoc effects of $Y_1^{-/-}$ animals ($Y_1^{-/-}$) vs. control animals (WT) are indicated by asterisks ($^{*}p < .05$ and $^{**}p < .01$). All data are presented as means ± standard error of the mean (S.E.M).

3. Results

In a recent study $Y_1^{-/-}$ mice were characterized in regard to general health, neurological reflexes, motor functions, sensory abilities, and neuromuscular strength. $Y_1^{-/-}$ mice exhibit a wild-type-like phenotype in regard to all these domains [19].

3.1. Motor activity and exploration

We have demonstrated a context-dependent elevation in motor activity and exploration caused by Y_1 deficiency. Y_1 deficient animals showed a significantly elevated overall distance travelled and increased ambulatory frequency in the OF. This hyperactivity in the OF was context-dependent (Fig. 1a and b) as it was only evident when being tested at the end of the light phase (light 8 h) or after restraint stress exposure. In the same paradigm knockout animals spent also less time in a *resting* state compared to wild-type-like animals (light 8 h: $Y_1^{-/-}$: 288.1 ± 11.9 s versus WT: 356.3 ± 13.6 s; $p = .002$; no significant differences between knockout and wild-type-like mice at other times of circadian rhythm). The importance of the circadian rhythm on this hyperactive phenotype became even more obvious using another motor activity-related parameter of the OF: ambulatory episodes. For this parameter, the knockout mice also exhibited a strongly context-dependent phenotype. Testing mutant animals at the end of the light phase (light 8 h) or after exposure to restraint stress revealed, consistent with the findings above, a hyperactive phenotype. Interestingly, a significantly hypoactive performance was recorded for the $Y_1^{-/-}$ mice when the OF was performed under light 2 h and following restraint stress. Under these test conditions, the number of ambulatory episodes decreased significantly in the Y_1 deficient animals (Fig. 2).

A hyperactive phenotype in the mutant mice was also observed in regard to exploration. This explorative phenotype was highly dependent on the circadian rhythm as the increased drive to explore the environment was most obvious 1 h after onset of the dark phase (dark). At this time of the light cycle one-way ANOVA revealed a significant increase for the explorative-like behaviours *head dipping* (in EPM and HB: Fig. 3a and b) and *rearing* (EPM; dark: $Y_1^{-/-}$: 1.2 ± 0.4 s versus WT: 0.31 ± 0.2; $p = .03$). Furthermore, restraint stress increased the frequency of the explorative-like behaviour *head dipping* in the EPM (Fig. 3a). In addition, the working memory ratio in the HB was significantly decreased in the explorative-like $Y_1^{-/-}$ mice com-

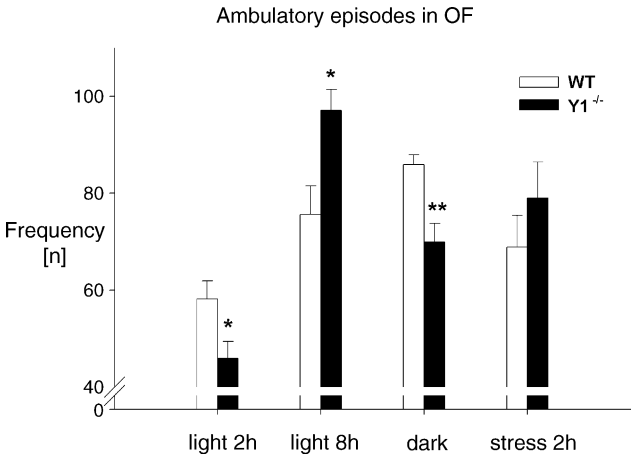

Fig. 2. The motor activity-related parameter ambulatory episodes [n] in the OF at different times of the circadian rhythm and after stress exposure. Significant post hoc effects of $Y_1^{-/-}$ animals ($Y_1^{-/-}$) vs. control animals (WT) are indicated by asterisks ($^{*}p < .05$ and $^{**}p < .01$). All data are presented as means ± standard error of the mean (S.E.M.).

pared to wild-type-like control mice (dark: $Y_1^{-/-}$: 0.35 ± 0.02 versus WT: 0.41 ± 0.03; $p < .05$). Interestingly, restraint stress exposure improved the working memory performance of the ko mice although the difference compared to the control mice failed to be statistically significant ($Y_1^{-/-}$: 0.9 ± 0.05 versus WT: 0.7 ± 0.08; $p = .052$).

3.2. Anxiety

Our analysis of anxiety-related parameters revealed marked alterations in anxiety in the $Y_1^{-/-}$ mice. For example, anxiety-related parameters such as the ratio of distance travelled in the central area (recorded in the OF) and open arm entry ratio (recorded in the EPM) were significantly increased for the Y_1 ko animals (Fig. 4a and b). This anxiolytic-like phenotype was evident during the light phase (light 8 h) for both paradigms as well as after exposure to restraint stress prior to EPM testing. Y_1 deficiency also increased the time spent on open arms

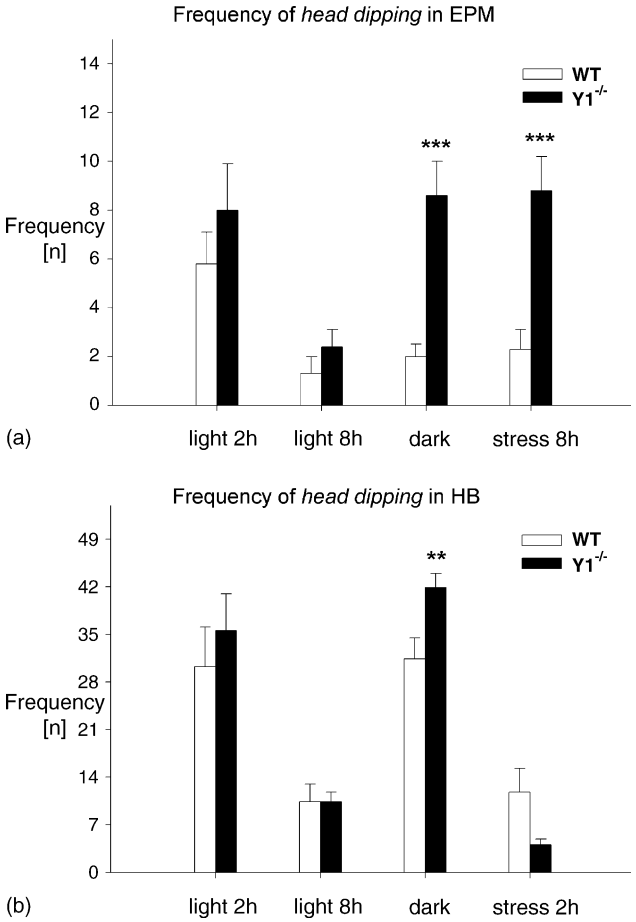

Fig. 3. The exploration-related behaviour *head dipping* [n] at different times of the circadian rhythm and after stress exposure: (a) in the EPM and (b) in the HB. Significant post hoc effects of $Y_1^{-/-}$ animals ($Y_1^{-/-}$) vs. control animals (WT) are indicated by asterisks ($^{**}p < .01$ and $^{***}p < .001$). All data are presented as means ± standard error of the mean (S.E.M.).

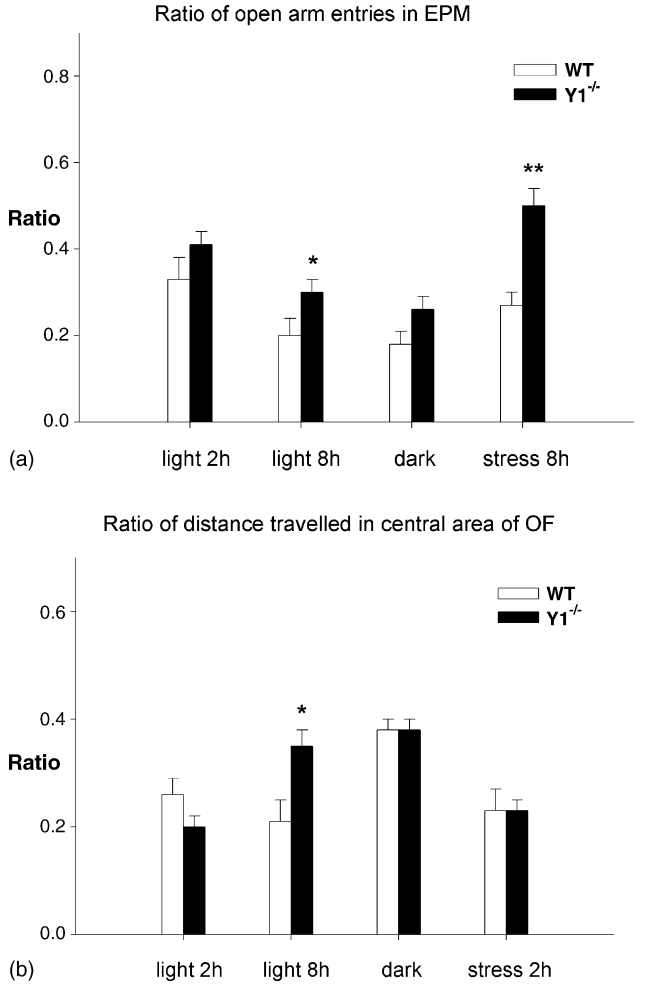

Fig. 4. Anxiety-related parameters at different times of the circadian rhythm and after stress exposure: (a) ratio of open arm entries in the EPM and (b) ratio of distance travelled in the central area of the OF. Significant post hoc effects of $Y_1^{-/-}$ animals ($Y_1^{-/-}$) vs. control animals (WT) are indicated by asterisks ($^{*}p < .05$ and $^{**}p < .01$). All data are presented as means ± standard error of the mean (S.E.M.).

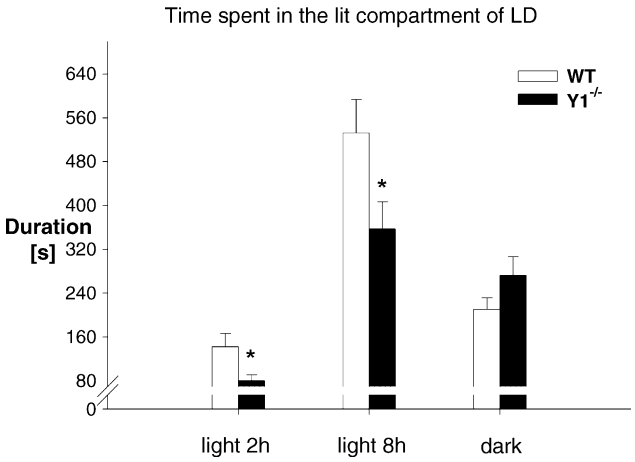

Fig. 5. The anxiety-related parameter time spent in the lit compartment [s] in the LD test at different times of the circadian rhythm and after stress exposure. Significant post hoc effects of $Y_1^{-/-}$ animals ($Y_1^{-/-}$) vs. control animals (WT) are indicated by asterisks ($^*p < .05$). All data are presented as means ± standard error of the mean (S.E.M.).

of the EPM after stress exposure ($Y_1^{-/-}$: 77.8 ± 10.4 s versus WT: 21.3 ± 5.0 s; $p < .0001$), providing additional evidence for a context-dependent anxiolytic phenotype.

Importantly, in the LD, knockout animals revealed a more anxious-like phenotype as they spent significantly less time in the lit compartment of the arena (when being tested at light 2 and 8 h; Fig. 5). Furthermore, the lit compartment to overall distance travelled ratio was significantly reduced in the Y_1 ko mice, indicating less locomotor activity in this more aversive compartment (light 8 h: $Y_1^{-/-}$: 0.29 ± 0.02 versus WT: 0.36 ± 0.03; $p = .04$). This task- and context-dependent anxious-like phenotype was confirmed in the OF. Dependent on the circadian rhythm, mutant animals spent significantly less time within the central area of the open field (light 2 h: $Y_1^{-/-}$: 36.1 ± 6.2 s versus WT: 70.4 ± 11.5 s; $p = .01$). This finding was supported by the reduced central distance travelled ratio in the ko mice (Fig. 4b; light 2 h: $p = .066$) in the same paradigm.

4. Discussion

In this study, we used a comprehensive strategy to characterize Y_1 receptor knockout animals in different tasks for motor activity, exploration, and anxiety with an emphasis on the influence of the circadian rhythm and stress on these behaviours. We demonstrate an important role of the Y_1 receptor in the regulation of motor activity and exploration, since ablation of this receptor causes significant changes in these parameters. Furthermore, a lack of Y_1 receptors results in marked alterations in anxiety-related behaviours. Importantly, the behavioural phenotype of the Y_1 receptor deficient mice is strongly circadian rhythm-dependent and can be modified by restraint stress.

Motor activity as measured in the open field test varies between hyper-(during resting period and after restraint stress) and hypoactive (during intermediate to high activity periods of the mouse circadian rhythm) in the Y_1 knockout animals revealing a more complex effect of Y_1 deficiency on motor activity than described previously [5,15]. In general, the diurnal rhythm has a significant impact on locomotor activity in mice [25] and NPY itself is known to modify circadian rhythm (e.g. by inhibiting phase-shifting effects of light during the dark phase; [50]). But so far nothing is known about a diurnal rhythm for endogenous NPY release, which could explain the observed circadian rhythm-dependent effect of Y_1 deficiency on motor activity. Stress is known to significantly increase NPY expression in the brain in order to combat this state of overexcitement [3]. The lack of a sedative-like effect [31] of NPY after restraint stress exposure in the open field test suggests a critical role for this Y receptor in coordinating stress- and locomotion-related responses. This coordinative link is confirmed by an increase in the explorative-like phenotype of these animals after stress exposure in the EPM. This finding potentially explains the previously reported strong increase in territorial aggression [19] as exploration correlates positively with aggression [33].

Pharmacological evidence has implicated the Y_1 receptor as the major mediator of anxiolytic-like effects of NPY [21]. Our results confirm and extend this evidence. Dependent on the diurnal rhythm, Y_1 receptors mediate anxiolytic- or anxious-like effects. The anxiolytic-like behaviours (recorded in EPM and OF test) were positively correlated with the hyperactive phenotype in Y_1 knockout mice. However, testing Y_1 deficient mice at a different time of the circadian rhythm in the OF test or in a different anxiety-related paradigm (the LD test) revealed an anxious-like phenotype in the same mutant animals. This multi-faceted, “circadian” phenotype [2] shows the limitation of pharmacological studies [13,39,49] and the potent diurnal rhythm- and task-dependent impact of Y_1 deficiency on anxiety-related behavioural domains. Furthermore, it highlights the importance of a comprehensive strategy in behavioural phenotyping using different behavioural paradigms and times of testing. Interestingly, restraint stress causes a decrease in anxiety in mutant mice in the EPM test. Restraint stress is known to decrease NPY mRNA expression in the amygdala [45], a key brain structure for processing anti-stress actions of NPY [14]. Reduced levels of endogenous NPY of generally more anxious and therefore more stressed Y_1 knockout mice could cause a diminished activation of pre-synaptically expressed anxiogenic-like Y_2 receptors [36] in these animals and therefore result in a more anxiolytic-like phenotype of Y_1 knockout mice. Furthermore, compensatory mechanisms during ontogeny are reported for this germline knockout model [5,12] leading to the hypothesis that the overall Y_2 receptor expression could be altered in the germline Y_1 knockout mice [26,27]. The NPY system and its Y_2 receptor have also been implicated in learning and memory [37]. The weak but significant differences in working memory obtained in the hole board task support such an involvement and suggest that the Y_1 receptor participates in the effect of NPY on memory [10,44]. However, additional tests are necessary to define the specific role of the Y_1 receptor subtype in this process.

In summary, we show for the first time an important role for the Y_1 receptor in the regulation of motor activity, exploration, and anxiety in a genetic animal model. We also demonstrate that the lack of Y_1 receptors leads to a more complex behavioural phenotype than pharmacological studies previously revealed.

Factors such as the circadian rhythm and stress exposure have a significant impact on the role of the Y_1 receptor. In addition, the study confirms the importance of a comprehensive strategy in behavioural phenotyping and the significance of genetic animal models in behavioural neuroscience.

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