Behavioral phenotyping of mice lacking the deubiquitinase USP2

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Abstract

Ubiquitin specific peptidase 2 (USP2) is a deubiquitinating enzyme expressed almost ubiquitously in the body, including in multiple brain regions. We previously showed that mice lacking USP2 present altered locomotor activity rhythms and response of the clock to light. However, the possible implication of USP2 in regulating other behaviors has yet to be tested. To address this, we ran a battery of behavioral tests on *Usp2* KO mice. Firstly, we confirmed our prior findings of increased daily activity and reduced activity fragmentation in *Usp2* KO mice. Further, mice lacking USP2 showed impaired motor coordination and equilibrium, a decrease in anxiety-like behavior, a deficit in short-term recognition memory and in sensorimotor gating. On the other hand, no effects of *Usp2* gene deletion were found on spatial memory. Hence, our data uncover the implication of USP2 in different behaviors and expands the range of the known functions of this deubiquitinase.

Introduction

Ubiquitination is the process of covalent attachment of a 76 kDa long protein called Ubiquitin to other proteins. While ubiquitination has multiple roles in the functioning of the cell, including protein localization and trafficking [1] , its most salient function is the targeting of tagged proteins for degradation [2]. Ubiquitination is carried out by a group of enzymes known as E3 ubiquitin ligases [2, 3]. The process of ubiquitination is counteracted by the process of deubiquitination, which is mediated by enzymes known as deubiquitinases or DUBs. These opposing processes can, in essence, determine the timing of various processes such as degradation, activity and localization of proteins.

While various DUBs are found in different tissues and cell types, Ubiquitin Specific Peptidase 2 or USP2 is one of the DUBs that has been found to be expressed almost ubiquitously in the organism [4–7]. More interestingly, it is the only DUB that has been found to have a circadian pattern of expression in virtually all the tissues where it is expressed [7, 8].

Circadian rhythms are self-sustaining oscillations with a period of approximately 24 hours, which are sustained at the molecular level by negative feedback loops involving circadian clock genes [9]. The counterbalance between ubiquitination and deubiquitination is essential to accurately control the timing of degradation of several clock proteins and hence, to the maintenance of periodicity in the clock [10]. Previous studies have shown that the deubiquitinase USP2 modulates the stability and/or localization of clock gene products BMAL1, PER1 and CRY1 [11–14]. Surprisingly though, despite its interaction with many of the core clock components, knocking out *Usp2* in mice did not lead to proportionally severe circadian deficits [11, 12]. Our group showed that USP2 modulates the response of the clock to light [11]. We also showed that *Usp2* KO mice have a slightly longer period than WT littermates [11]. While no other significant

52 phenotypes were noticed under a normal light:dark cycle, a trend towards increased total activity
53 over 24 hours was observed in the *Usp2* KO mice, compared to WT mice [11].

54 Ubiquitination, and ubiquitinating/deubiquitinating enzymes, have been associated with
55 various behaviors as well as neurological and psychiatric disorders, such as bipolar disorder [15,
56 16], neurodevelopmental disorders [15, 17, 18], and Parkinson's disease [19]. Searching the mouse
57 brain expression data in the Allen Mouse Brain Atlas [20], we noticed that *Usp2* is expressed in
58 many brain regions. Hence, we aimed to test the hypothesis that USP2 plays a role in modulating
59 various behaviors beyond circadian rhythms. To test this, we subjected *Usp2* KO and WT mice to
60 a battery of tests, to assay various behaviors such as daily wheel running behavior, motor
61 coordination, anxiety-like behavior, sensorimotor gating and memory. We confirmed that the *Usp2*
62 KO mice showed increased and more consolidated running wheel activity. We also found that they
63 have reduced balance and motor coordination, as well as a reduction in anxiety-like phenotypes
64 and short-term recognition memory. There were limited or no effects of *Usp2* gene deletion on
65 sensorimotor gating and spatial memory.

66

Materials and Methods

Animals

Usp2 KO mice [21] were obtained from Dr. Simon S. Wing and bred in house. *Usp2* KO and WT littermates (on a C57BL/6J background) were generated by breeding heterozygotes. In some cases, PER2::LUC knockin mice (on a C57BL/6J background) were used as controls. This PER2 gene modification was shown not to affect PER2 function, mouse health, general activity or wheel-running activity [22]. Four such mice were used in the wheel running, rotarod, novel object recognition and pre-pulse inhibition tests. In all cases, similar effects of the KO were seen irrespective of whether or not these mice were included in the analyses.

Mice were weaned at 3 weeks of age, into cages containing no more than 5 mice per cage. They were maintained on a 12h:12h light:dark cycle (with light at ~200 lux). At 2 to 3 months of age, male mice were designated to running wheel or (non-circadian) behavioral cohorts. All procedures involving animals were carried out in accordance with guidelines of the Canadian Council on Animal Care and approved by the Animal Care Committees of McGill University and Douglas Mental Health University Institute (protocol no. 2001-4586). For euthanasia at the end of the experiments, the animals were first anesthetized by isoflurane inhalation, followed by CO_2 euthanasia; all efforts were made to minimize suffering.

Wheel-running behavior

For running wheel experiments, 2 to 3-month-old WT and KO mice (n = 6) were transferred to running wheel-equipped cages (Actimetrics, Wilmette, IL, USA), where they were singly housed, with ad-libitum access to food and water. After a baseline week for acclimatization, wheel

running activity was recorded for three weeks, in the same LD cycle. The last 10 days of recorded data were analysed on the Clocklab software Version 6 (Actimetrics, Wilmette, IL, USA). Daily wheel rotations, number of bouts of activity per day, length of each activity bout and number of wheel rotations in each bout were quantified. One mouse (KO) was excluded from the analysis because its wheel running activity was not recorded on the software.

Non-circadian behavioral tests

WT mice (6-12/group) and *Usp2* KO mice (10-12/group) went through Rotarod, Elevated plus maze (EPM), Novelty suppressed feeding (NSF), Forced swim test (FST), Novel object recognition (NOR), Morris water maze (MWM) and Pre-pulse inhibition (PPI) of acoustic startle. To account for time-of-day effects on behavior, all behavioral tests were carried out between one and five hours after lights on. Tests were performed under dim light conditions (~15 lux) unless noted otherwise. The groups (WT and KO) were counterbalanced between tests and between trials. Mice were habituated to the testing room for 30 minutes, prior to the start of each session of behavioral testing. EPM, NSF and NOR tests were video recorded to minimize interaction with the mice and to reduce the resulting stress levels. All the mice in the video recordings of the behaviors were tracked using the TopScan 2.0 software (Clever Sys, Restin, VA, USA). Since some tests measured anxiety-like behaviors or could be affected by stress, all mice were given 2 "recovery days" between each test, during which they were not handled or disturbed.

Rotarod

The Rotarod test assays motor coordination, equilibrium and balance [23]. This test was carried out over two days. On the first day, each mouse was habituated to the rotarod (Bioseb,

Valbonne, France). For the habituation phase, the ridged cylinder was made to rotate at a constant speed of 5 rotations-per-minute (rpm) for two 3-minute long trials and 10 rpm for two 3-minute long trials. During each of these trials, the mice were put back on the rod if they fell, until the timer ran out. Each mouse got a rest period of at least 15 minutes in its home cage between each subsequent trial.

For the testing day, each mouse was tested for three trials. It was placed on the rotarod, which accelerated at a constant rate, from 4 to 40 rpm, over a 5-minute span (acceleration = 7.2 rpmpm). When the mouse fell off the cylinder, onto the press plate placed 20 cm below the rod, the time taken to fall off the rod got recorded. After the fall, they were returned to their home cage. The greater their latency to fall, the greater is their motor coordination, equilibrium and/or balance. Two mice were excluded from this analysis (1 WT and 1 KO) as they refused to run on the rotarod.

Elevated plus maze

Anxiety-like behavior was measured using the elevated plus maze (EPM) [24]. The maze is elevated 75 cm from the floor and consists of 4 arms shaped like a cross and painted black. Two opposing arms are enclosed by 10 cm high walls on three sides, while the other two are open. For the assay, each mouse was placed in the center of the maze, facing an open arm. The mouse was then allowed to freely explore the maze for 5 minutes, after which it was returned to its home cage. The total time spent in the open and closed arms, as well as in the center were measured. The longer a mouse spends in the open arms, the less anxiety-like behavior it is considered to show. Proportion of time spent in open arms is calculated as:

$$\%\ time\ in\ open\ arms = \frac{(time\ spent\ in\ open\ arms\ (s)) + 0.5 * (time\ spent\ in\ the\ middle\ (s))}{300\ s}$$

One mouse (WT) was excluded from analysis as it jumped out of the EPM during the trial.

Novelty-suppressed feeding

The novelty suppressed feeding (NSF) test is an assay of anxiety-like behavior in a conflict-based environment [25, 26]. Mice were fasted for 24 hours before the start of the test. Then, each mouse was placed in a grey 1.5ft X 1.5ft X 1.5ft box, in a well-lit area. At the center of the box, a fixed food pellet (standard chow) was available for the mouse to feed on. Thus, the mouse needs to choose between the anxiogenic setting of feeding in an open, well lit area as opposed to the safety of walls, but without any food availability. The mouse was allowed to explore this setting for 10 minutes and the latency to feed was measured, being careful to not include food manipulation or sniffing behaviors in this measure. The longer a mouse takes to feed, the higher its anxiety-like phenotype.

After this, the mouse was transferred to a quiet area, into a cage containing pre-weighed quantities of food and the amount of food they ate over a further 10-minute span was recorded. This measure assesses the hunger levels of the mice and whether differences due to the food deprivation itself influences the observations within the arena.

Additionally, to factor in the possibility of a general difference in appetite between KOs and WTs, at the end of the NSF protocol, the mice were weighed daily for 9 days and the amount of food that they consumed was noted over these days.

Novel object recognition

The novel object recognition (NOR) test measures learning and memory in mice [27]. NOR involves three phases spread over two days. On the first day, each mouse was acclimatized to an

157 empty grey 1.5ft X 1.5ft X 1.5ft box by allowing it to freely explore the box for 10 minutes. On
158 the second day, the first phase of the day is the habituation phase where the mouse was allowed to
159 freely explore the grey box for 10 minutes. Each box contained two identical objects (25 mm tissue
160 culture flasks filled with corn cob bedding or boxes of coverslips) placed at opposite corners of
161 the box, 4.5 inches away from each of the two nearest walls. The mouse was then returned to its
162 home cage. The second phase of the day is the novel object recognition phase, which tests short-
163 term retention of recognition memory. In this phase, which starts 4 hours after the habituation
164 phase, one of the objects in each box was exchanged for a differently shaped object (tissue culture
165 flask as a replacement for coverslip box and vice-versa). The mouse was then allowed to freely
166 explore the arena for 10 more minutes. The time spent exploring the two objects was separately
167 recoded, in both phases. A mouse was considered to be involved in object exploration if its head
168 was directed towards the object, within a radius of approximately 5 cm from it. This analysis was
169 carried out using the TopScan 2.0 software. Mice tend to prefer exploring novel objects, over
170 familiar objects. Hence, the exploration of the novel object is a measure of the extent to which the
171 animal remembers the previous encounter with the familiar object. The ability to discriminate
172 between the novel and familiar object was measured by the Discrimination Ratio (DR), calculated
173 as:

174 $$DR = \frac{Time\ spent\ exploring\ novel\ object}{Time\ spent\ exploring\ familiar\ object}$$

175 A DR of 1 shows a lack of discrimination between the two objects. DR > 1 shows that the
176 mouse is exploring the novel object more than the familiar object.
177

Morris water maze

The Morris water maze (MWM) assesses spatial memory [28, 29]. The maze consists of a large circular swimming pool (diameter of 150 cm) filled with opaque water with a platform (225 cm^2) submerged 1 cm below the surface of the water. The mice must reach the platform in order to be able to stop swimming and rest. Spatial cues were placed on the pool walls to allow the mice to learn the location of the platform. The learning stage was 4 days long, with each day consisting of 4 trials, each with the mouse beginning at a different location in the pool. The inter-trial interval was at least 30 min. The time to reach the platform was recorded at every trial, to compare the patterns of learning. On day 5 of the experiment, the platform was removed, and the time spent in each quadrant of the pool in the span of 1 minute was recorded, to test the robustness of spatial learning (probe trial). Then, a cue trial, where the platform is placed in a new location along with a visual cue, was administered to verify that the visual system was intact in the animals. The latency to reach the platform and time spent in the appropriate quadrant in the probe trial were analyzed and calculated using HVS Image Analysis (HVS Image, Hampton, UK).

Pre-pulse inhibition of acoustic startle

Pre-pulse inhibition (PPI) of acoustic startle reflex is a measure of sensory-motor gating [30, 31]. Testing is carried out using the SR-Lab software connected to 6 sound attenuating chambers equipped with plexiglass animal enclosure tubes (San Diego Systems, San Diego, CA, USA). These chambers are ventilated by an electrical fan that produces a constant 70 dB background. Speakers positioned directly above the enclosure present tone pulses of differing loudness and the startle of the animal is recorded by an accelerometer attached to the base of the enclosure. Following a 5-minute acclimatization period in the tube, there were 3 phases in the paradigm. In

the first and third phases, 6 startle pulses of 120 dB loudness and lasting for 30 msec each, were administered. In the second phase, 38 trials were administered. The first 8 trials were pulse only (startle only) trials. In the next 30 trials, the mouse received a 30 msec prepulse of 0 (pulse alone), 6, 9, 12 or 15 dB intensity above the background, 100 msec prior to the actual 120 dB pulse. These pre-pulses were randomly varied across the 30 trials spaced 15 msec apart, 5 of each of the pre-pulse trials presented to the animals. The average amplitude of startle in the last 15 startle-only trials is the baseline startle. % Pre-pulse inhibition (% PPI) is calculated as:

$$\% \, PPI = 100 - \frac{Startle\ response\ to\ trials\ with\ pre-pulse}{Startle\ response\ to\ pulse\ alone\ trials} * 100$$

The higher the % PPI, greater is the inhibition of acoustic startle when subjected to a low intensity sound pulse prior to the startle. In essence, a higher PPI shows higher sensorimotor gating. Two mice were excluded from this analysis (1 WT and 1 KO) for having impossibly high baseline startle levels, pointing to a recording error in the data of these mice.

Statistical Analysis of Data

All the data were analyzed first for homogeneity of variances as well as normality of data. The data were plotted and analyzed on GraphPad Prism, using the appropriate statistical tests. For all the tests with data distributed normally and having equal variance across the groups (WT, KO), unpaired two-tailed t-tests were used (Wheel running data, Rotarod, EPM, NSF, FST, probe and cue trials of MWM). In the cases where variances were not normal (Discrimination during NOR phase), Welch's correction was applied to the two-tailed, unpaired t-test. If the normality assumption was violated (Average startle, Average % PPI), the two-tailed Mann-Whitney test was used. For comparing between Habituation and NOR phases in the NOR task, paired, two-tailed t-tests were used. Mixed model 2-way ANOVAs were used to compare the daily consumption of

224 food and weights of the mice in the days after NSF, learning across days (MWM) and % PPI across

225 different pre-pulse intensities (PPI). Differences were considered to be significant if $p < 0.05$.

Results

Mice show altered activity patterns in the absence of USP2

WT and *Usp2* KO mice were placed in running wheel cages and locomotor activity was recorded under a 12h:12h light:dark cycle (representative actograms in Fig 1A). *Usp2* KO mice showed a trend ($p = 0.06$) for more total daily activity than WT mice (Fig 1B). Further, the duration of activity bouts was significantly longer in KO mice ($p = 0.049$, Fig 1C) and the average number of bouts per day was lower ($p = 0.14$, S1A Fig). The total counts of wheel rotation per bout of activity was also significantly increased in *Usp2* KOs, compared to WTs ($p = 0.0481$, S1B Fig.). Overall, these data indicated increased and less fragmented activity in mice lacking USP2. These data are consistent with those previously obtained in our laboratory [11].

Fig 1. Increased daily activity and less fragmentation of activity in *Usp2* KO mice. (A) Representative actograms for the wheel running activity of WT and *Usp2* KO mice over 10 days under a 12h:12h light:dark cycle. (B, C) Quantification, averaged over the 10 days, of the total daily activity (B) and the average bout length (C). Individual data points represent independent mice and data are represented as mean ± SEM. Unpaired two-tailed t-tests, *: $p < 0.05$.

Motor coordination is reduced in mice lacking USP2

The increased activity and reduced fragmentation in running wheels led us to wonder if this phenotype could be the result of a change in motor coordination in the *Usp2* KO mice. To assay this, mice were subjected to the rotarod protocol, which assesses motor coordination and balance

by evaluating the ability of the mouse to stay on top of a rotating cylinder. *Usp2* KO mice spent significantly less time ($p = 0.02$) on the accelerating cylinder ($p = 0.02$, Fig 2) and fell from the cylinder at a lower speed (WT: 18.11 ± 1.35 rpm, KO: 13.94 ± 1.31 rpm, $p = 0.038$) compared to WT mice, pointing towards a reduction of motor coordination in mice lacking USP2. Hence, a change in motor coordination does not seem to explain the increased activity phenotype seen in the running wheel experiments.

Fig 2. Reduced motor coordination of *Usp2* KO mice. Measurement of time spent on the accelerating rotarod by WT and *Usp2* KO mice. Individual data points represent independent mice and data are represented as mean ± SEM. Unpaired two-tailed t-tests, *: $p < 0.05$.

Anxiety-like behavior is decreased in mice lacking USP2

We surveyed *Usp2* gene expression in the mouse brain expression database in the Allen Mouse Brain Atlas (https://mouse.brain-map.org/experiment/show?id=76098316) [20]. As shown in Table 1, *Usp2* is expressed throughout the brain. Therefore, we subjected the WT and *Usp2* KO mice to a battery of tests for affective and cognitive behaviors.

Table 1. Relative expression of *Usp2* transcript in the mouse brain

Brain region	*Usp2* expression level *
Isocortex	++++++
Olfactory bulb	+++
Hippocampus	++++

Striatum	++++
Thalamus	+++
Hypothalamus	+
Midbrain	+
Pons	+
Cortical sub-plate	+++++
Pallidum	++
Medulla	+
Cerebellum	++

* Relative expression from in situ hybridization data for *Usp2*, in the Allen mouse brain atlas (each '+' sign represents ~1 unit of raw expression value).

Expression of the gene in regions such as the hippocampus and cortex, which are associated with anxiety-like behavior [32], prompted us to assess such behavior, using the Elevated Plus Maze (EPM) and the Novelty-suppressed Feeding (NSF) test.

The EPM assay builds on the natural aversion of mice to heights and open spaces, counterbalanced by their drive to explore novel surroundings. When subjected to the EPM, *Usp2* KO mice exhibited a trend towards reduced anxiety-like phenotypes compared to WT mice: they spent a greater proportion of time in the open arms as compared to the WTs ($p = 0.09$, Fig 3A). The latency to enter open arms, on the other hand, did not differ significantly between genotypes (WT: 34.67 ± 29.7 s, KO: 18.23 ± 5.98 s, Mann-Whitney test $p = 0.2275$).

Fig 3. Reduced anxiety-like behavior in *Usp2* KO mice. (A) Proportion of time spent in the open arms in the elevated plus maze (EPM). (B) Latency to the start feeding in the

testing arena of the novelty-suppressed feeding (NSF) test. (C) Food consumed over 9 days following the NSF test. Individual data points represent independent mice and data are represented as mean ± SEM. Two-way ANOVA (C) or unpaired two-tailed t-tests (A, B, D), **: $p < 0.01$.

The NSF test opposes the desire for safety with the desire to feed. The *Usp2* KO mice started feeding faster than the WT mice ($p = 0.0046$, Fig 3B). When feeding was assayed in home cages right after the test, for 10 minutes, all mice ate equally ($p = 0.882$, S2A Fig). To verify if the increased latency to feed was a result of metabolic changes, or changes in hunger, mice were weighed daily, and their daily food consumption was measured. There were no differences between WT and KO mice in their general appetite, as both genotypes consumed equal amounts of food over the span of a week ($F(8, 120) = 1.63$, $p = 0.1232$, Fig 3C). Similarly, there were no differences in the weights of the mice recorded over a 9-day span ($F(4, 60) = 0.1$, $p = 0.9812$, Fig S2B). Thus, the observed phenotype of an increased latency to feed reflects mainly on a decrease in the anxiety-like behavior in mice lacking the *Usp2* gene, consistent with the trend observed in the EPM test results.

Recognition memory, but not spatial memory, is attenuated in mice lacking USP2

Since *Usp2* is highly expressed in the cortex and hippocampus, we questioned whether knocking it out would result in effects on short-term recognition memory [33]. In the NOR test, WT mice had a higher DR in the NOR phase of the test ($p = 0.08$, Fig 4A). On the other hand, for the *Usp2* KO mice, the DR is unchanged between the habituation and NOR phases ($p = 0.916$, Fig

4B). Comparison between the discrimination ratios of WT and KO mice showed that the KOs had a significantly lower ability to distinguish novel objects ($p = 0.032$, Fig 4C). Therefore, recognition memory is impaired in mice lacking *Usp2*.

Fig 4. Impaired object recognition learning and memory in *Usp2* KO mice. (A, B) Proportion of time spent by WT mice (A) or *Usp2* KO mice (B) to explore the novel object compared to the familiar object (discrimination ratio). (C) Comparison of the discrimination ratios of the WT and KO mice during the novel object recognition phase. Paired, two-tailed t-tests (A, B) or unpaired two-tailed t-tests with Welch's correction (C), *: $p < 0.05$.

We also tested for spatial memory using the Morris water maze (MWM). Learning was observed in all the mice, over the first 4 days ($F(3, 45) = 12.71$, $p < 0.0001$) (Fig 5A). In the probe trial, both genotypes of mice spent more than 25% of the test time in the appropriate quadrant (WT: $p = 0.051$, KO: $p = 0.055$, Fig 5B), suggesting that the mice had learnt the position of the platform. In the cue trial, all the mice were equally adept at finding the platform at a new location ($p = 0.9235$, Fig 5C), confirming that performance factors unrelated to place learning were not involved in the probe trial results. However, in all these procedures, no differences were found between the WT and *Usp2* KO mice.

Fig 5. Unaltered spatial learning and memory of *Usp2* KO mice. (A) Time taken by the mice to find the platform on subsequent days, as a proxy for learning. (B) Probe trial: Proportion of time spent in the target quadrant in the absence of the platform on day 5.

(C) Cue trial: Time taken by the mice to find the platform at a new location. Individual data points represent independent mice and data are represented as mean ± SEM. Two-way ANOVA (A) or unpaired two-tailed t-tests and one sample t-test (B, C), ****: $p < 0.0001$ for effect of time in (A), interaction and effect of genotype n.s.

Effects on sensorimotor gating in mice lacking USP2

Usp2 expression is high in the olfactory bulb, striatum and prefrontal cortex, which are associated with sensorimotor gating [34–36]. Hence, we tested for pre-pulse inhibition (PPI) of acoustic startle in *Usp2* KO mice. There was no difference between the genotypes in their baseline startle response to a 120 dB startle pulse (Fig 6A). The % PPI averaged across the different pre-pulse intensities were not different between the genotypes ($p = 0.285$, Fig 6B). However, when the % PPI data were analyzed separately for each pre-pulse intensity, a Genotype x Pre-pulse intensity interaction was seen ($F(3, 63) = 2.79$, $p = 0.043$, Fig 6C). A simple main effect analysis revealed a trend for a difference between genotypes for a 12 dB pre-pulse intensity ($p = 0.06$). This suggested that *Usp2* may affect sensorimotor gating.

Fig 6. Reduced sensorimotor gating in *Usp2* KO mice. (A) Amplitude of baseline startle when a 120 dB pulse is given in the absence of any pre-pulse. (B) Percent pre-pulse inhibition (% PPI) when any non-zero intensity of pre-pulse was administered. (C) % PPI in response to the different intensities of pre-pulse. Individual data points represent independent mice and data are represented as mean ± SEM. Two-way ANOVA (C) or two-tailed Mann-Whitney test (A, B), *: $p < 0.05$ for the interaction in (C).

Discussion

In this report, we present evidence for a role of the deubiquitinating enzyme USP2 in the central nervous system. More specifically, we have uncovered alterations of anxiety-like behavior, learning and memory, and motor coordination in mice with a deletion in the *Usp2* gene. This work represents, to our knowledge, the first characterization of behaviors in the absence of USP2 function, beyond its established role in the regulation of circadian rhythms.

USP2 is a well-studied DUB with multiple established functions. The first known substrate of USP2 was the fatty acid synthase protein (FAS). In 2004, Graner and colleagues showed that USP2 binds to and stabilizes FAS [37], a protein known to be upregulated in many cancers including prostate cancer [38]. USP2 was shown to be involved in regulating the degradation of oncogene p53 by targeting the ubiquitin ligase Mdm2 [39]. MdmX, another target of Mdm2, is also deubiquitinated by USP2. Further, USP2 controls the cell cycle by deubiquitinating Aurora-A, a centrosomal kinase required for mitosis [40], as well as cyclins such as cyclin A1 and cyclin D [41–43], which are regulators of meiosis. Hence, unsurprisingly, the dysregulation of USP2 levels was associated with the development of various kinds of cancers, such as colorectal cancer [42], prostate cancer [41] and oral squamous cell carcinoma [44]. In the circadian system, USP2 regulates the ubiquitination of several clock proteins and contributes to the response of the circadian clock to light [11–14]. Apart from its functions within the clock, USP2 also mediates clock output. For instance, USP2 regulates the membrane scaffolding protein NHERF, in a circadian manner [45]. NHERF, in turn, regulates cellular homeostasis of calcium absorption in a clock-dependent manner [45], hence making USP2 a clock output mediator.

While several functions of USP2 have been uncovered, little is known about its possible roles in the central nervous system. Based on data from the Allen Mouse Brain Atlas, *Usp2* gene

is expressed in various brain regions (Table 1), which suggests that this DUB could be regulating behavioral processes. We addressed this using a battery of tests aimed at assaying various behaviors. This was initially prompted by our analyses of wheel-running behavior, in which we noted increased daily activity and a more consolidated activity pattern in *Usp2* KO mice. The reduction in activity fragmentation could be the result of an effect of the *Usp2* KO on the circadian system but our prior work using constant conditions, where limited effects on the endogenous period of the rhythms were found, argues against this. Thus, we used the rotarod to check a possible impact of *Usp2* gene deletion on motor coordination: the decrease in motor coordination of the KO mice suggests that this is also not the source of the altered activity patterns in mice lacking *Usp2*. Another possible source for this phenotype could be in the light-response pathways; however, the increased activity and consolidation is seen not only in the light phase of the 12:12LD cycle but also in the night, which suggests that an alteration of the light-response pathways (e.g. reduced masking of activity by light) is not involved. Finally, running wheel activity being a motivated behavior [46, 47], the phenotype in these assays could be due to changes in the reward pathways in the KO mice [48, 49]. Indeed, *Usp2* is highly expressed in the striatum (Table 1). Therefore, although we have not tested the *Usp2* KO mice for reward behaviors, this could represent an interesting avenue for future research.

The hippocampus and the cortex show the highest levels of *Usp2* expression. These are regions associated with the control of anxiety-like behavior. Accordingly, we found a decrease in anxiety-like behavior in mice lacking *Usp2*, both in the EPM and the NSF tests. In the former, the mice lacking *Usp2* spent more time in the open arms than their WT littermates, showing a reduction in anxiety-like behavior. In line with these findings, in the NSF test (which is a more sensitive measure of anxiety-like behavior, due to the pressure to feed), the mice lacking *Usp2* fed

396 significantly quicker than the WT mice, reiterating the reduction in anxiety-like behavior in mice
397 lacking USP2.

398 Further exploring the effects of cortical and hippocampal expression of *Usp2*, we looked at
399 the effects its deletion might have on cognition. In the NOR test, the KOs showed no distinction
400 in interacting with the novel and the familiar object (DR of 1 in both, the habituation and NOR
401 phases), pointing to a deficit in either the learning of the two objects or to the lack of short-term
402 memory retention. On the other hand, the MWM test showed that spatial learning and memory
403 were intact in the *Usp2* KO mice. Interestingly, a prior study has shown a correlation between a
404 reduction of spatial memory in rats following stress with a downregulation of USP2 protein levels
405 in the hippocampus, both reductions being concomitantly rescued by treatment with retigabine, an
406 opener of Kv7 potassium channels [50]. This suggested that USP2 might play a role in spatial
407 memory, at least under stress conditions, in rats. On the contrary, our data indicate no role for
408 USP2 in spatial memory in mice under basal, unchallenged conditions.

409 Finally, using the PPI test, we looked at sensorimotor gating, or the ability of sensory inputs
410 to guide motor responses in the organism. This behavior is guided by the striatum and olfactory
411 bulbs, where the expression of *Usp2* is high as well. There seemed to be an effect of genotype on
412 the startle response, but not at all pre-pulse intensities. Thus, further work will be required to
413 delineate the effects of USP2 on sensorimotor gating.

414 In conclusion, our data indicate that *Usp2* plays a role not only in the circadian system, but
415 also in controlling various other behaviors such as anxiety-like phenotypes, motor coordination
416 and short-term memory retention. A limitation of our study is that it does not provide insights on
417 what might be the substrates of USP2 in the brain regions where it is highly expressed. It will be
418 important in the future to find what these substrates are, and how USP2 action on these proteins

419 can lead to behavioral alterations like the ones we have observed in this study. Nevertheless, this
420 report sets the stage for the study of the roles of USP2 in the central nervous system, and shows
421 that this deubiquitinase has many roles beyond those already described in peripheral organs.

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Supporting information

S1 Fig. Locomotor activity patterns of *Usp2* KO mice. Quantification averaged over 10 days of number of bouts of activity per day (A) and number of wheel rotations per activity bout (B). Individual data points represent independent mice and data are represented as mean ± SEM. Unpaired two-tailed t-tests, * $p < 0.05$.

S2 Fig. Control measures of novelty-suppressed feeding (NSF) test. (A) Quantity of food consumed within 10 minutes post-NSF test, in the home cage. (B) Weight of the mice over 9 days following the NSF test. Individual data points represent independent mice and data are represented as mean ± SEM. Unpaired two-tailed t-tests (A) or two-way ANOVA (B), all n.s.

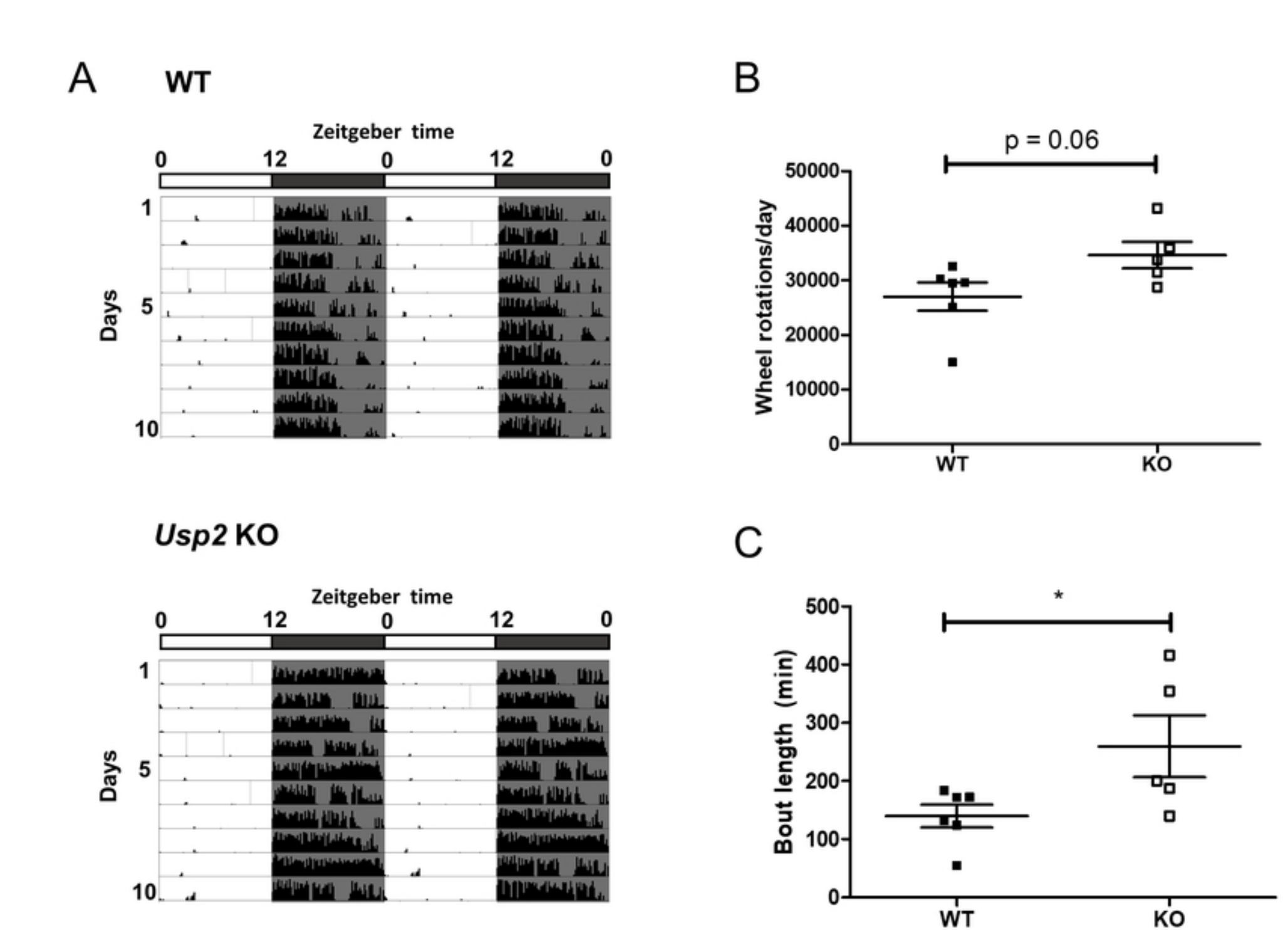
A
WT
Zeitgeber time
0
12
0
12
0
1
5
10
Days
Usp2 KO
Zeitgeber time
0
12
0
12
0
1
5
10
Days
B
p = 0.06
Wheel rotations/day
50000
40000
30000
20000
10000
0
WT
KO
C
*
Bout length (min)
500
400
300
200
100
0
WT
KO

Figure 1

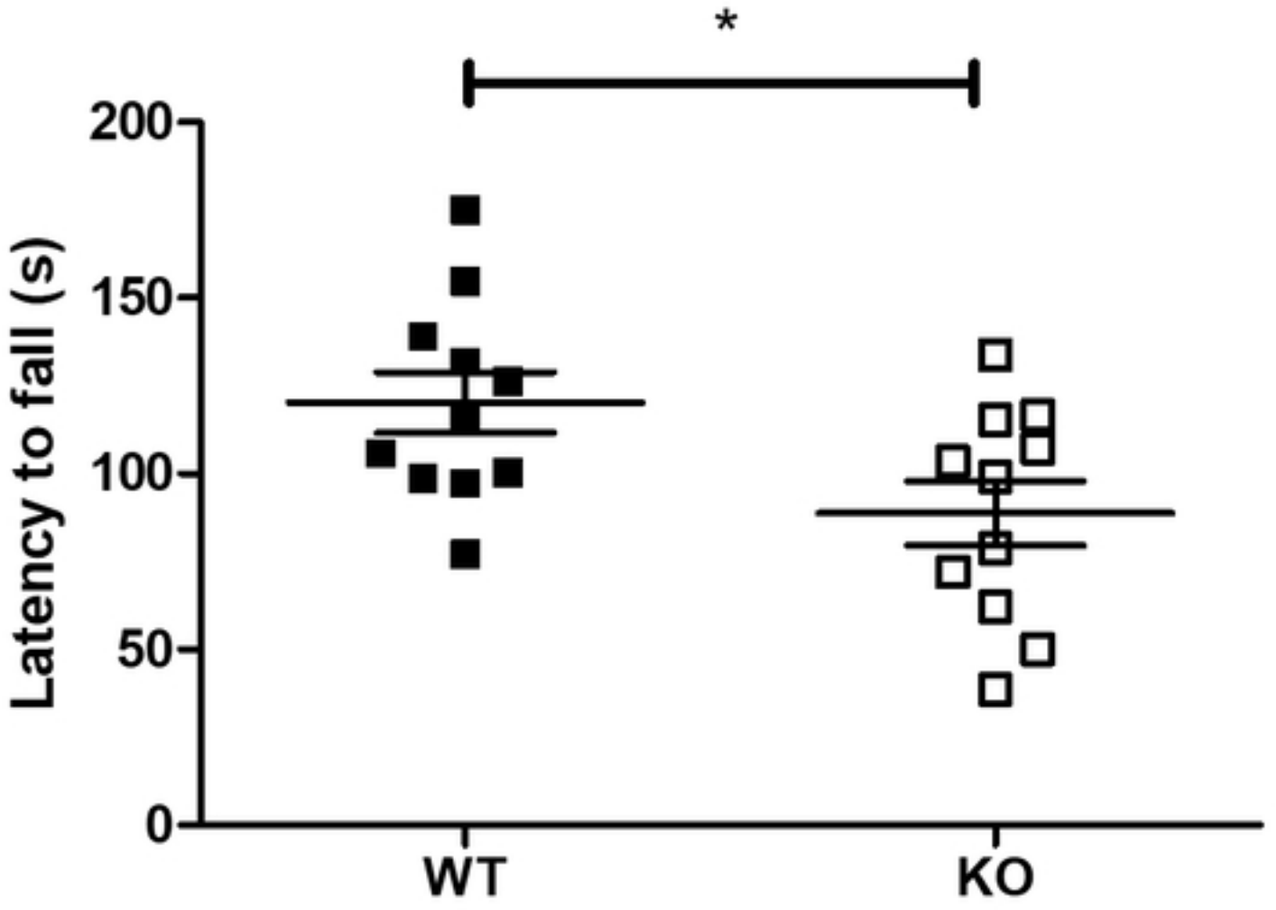

Figure 2

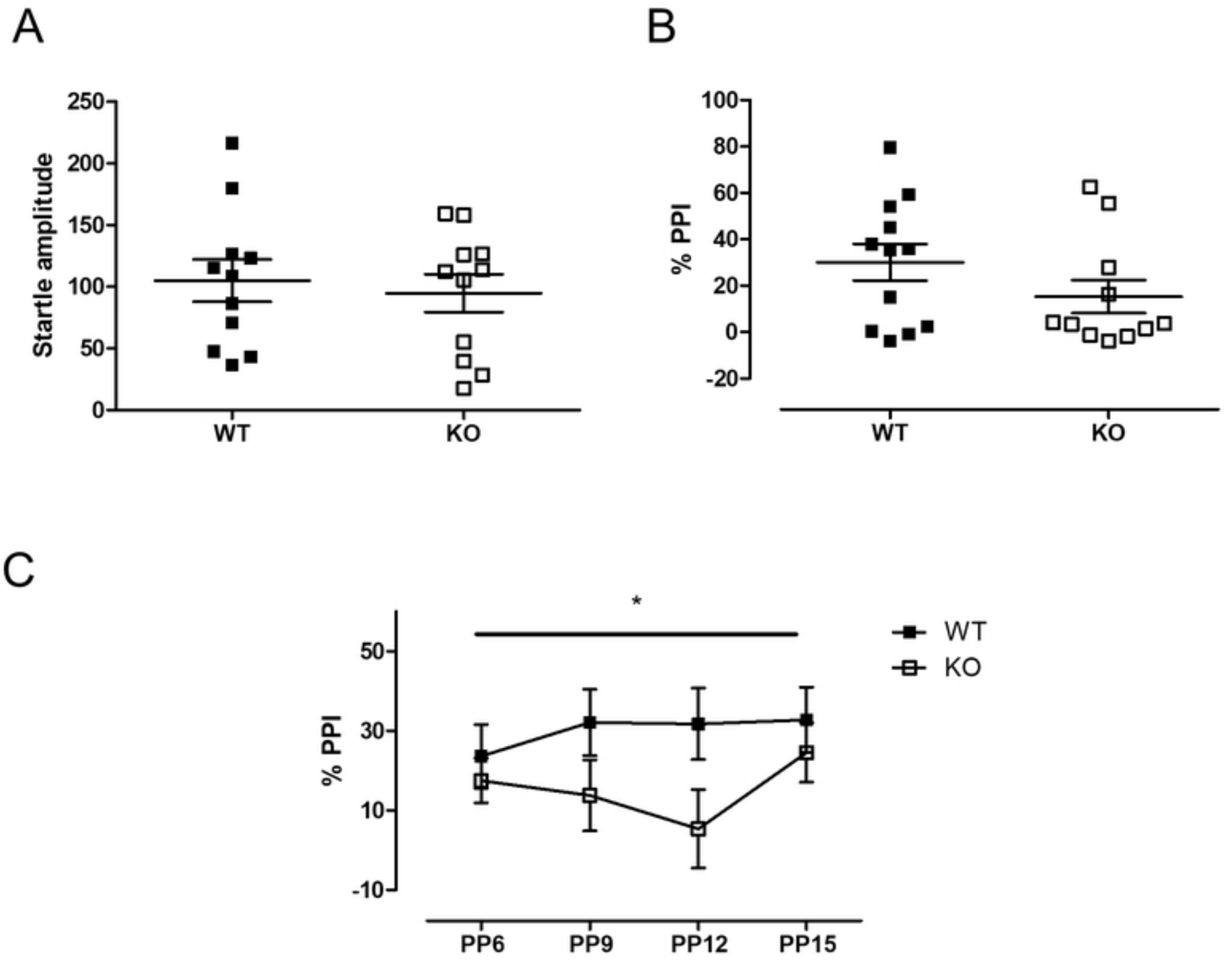
A
Startle amplitude
250
200
150
100
50
0
WT
KO
B
% PPI
100
80
60
40
20
0
-20
WT
KO
C
*
% PPI
50
30
10
-10
PP6
PP9
PP12
PP15
WT
KO

Figure 6

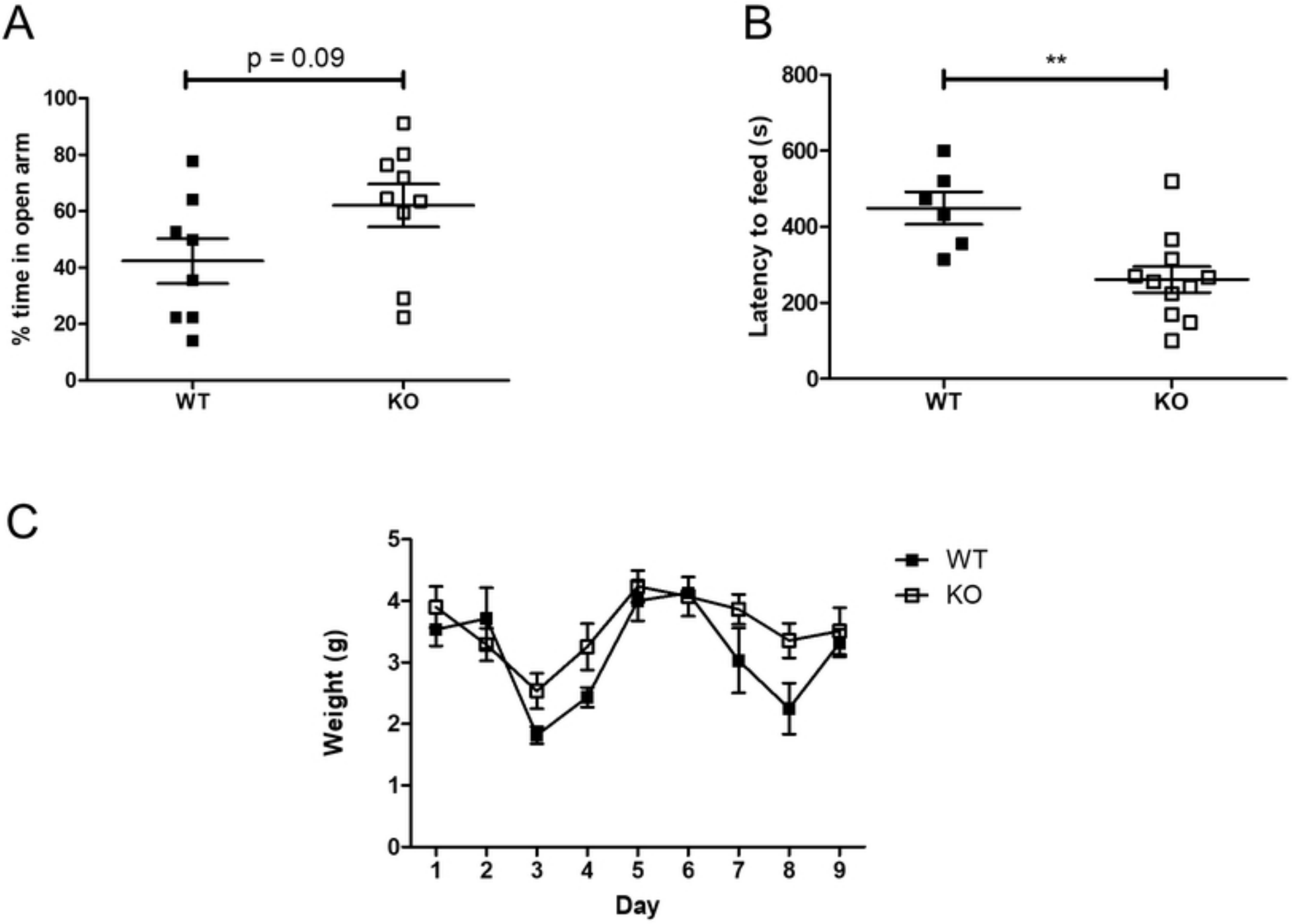
A
p = 0.09
% time in open arm
100
80
60
40
20
0
WT
KO
B
**
Latency to feed (s)
800
600
400
200
0
WT
KO
C
Weight (g)
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Day
WT
KO

Figure 3

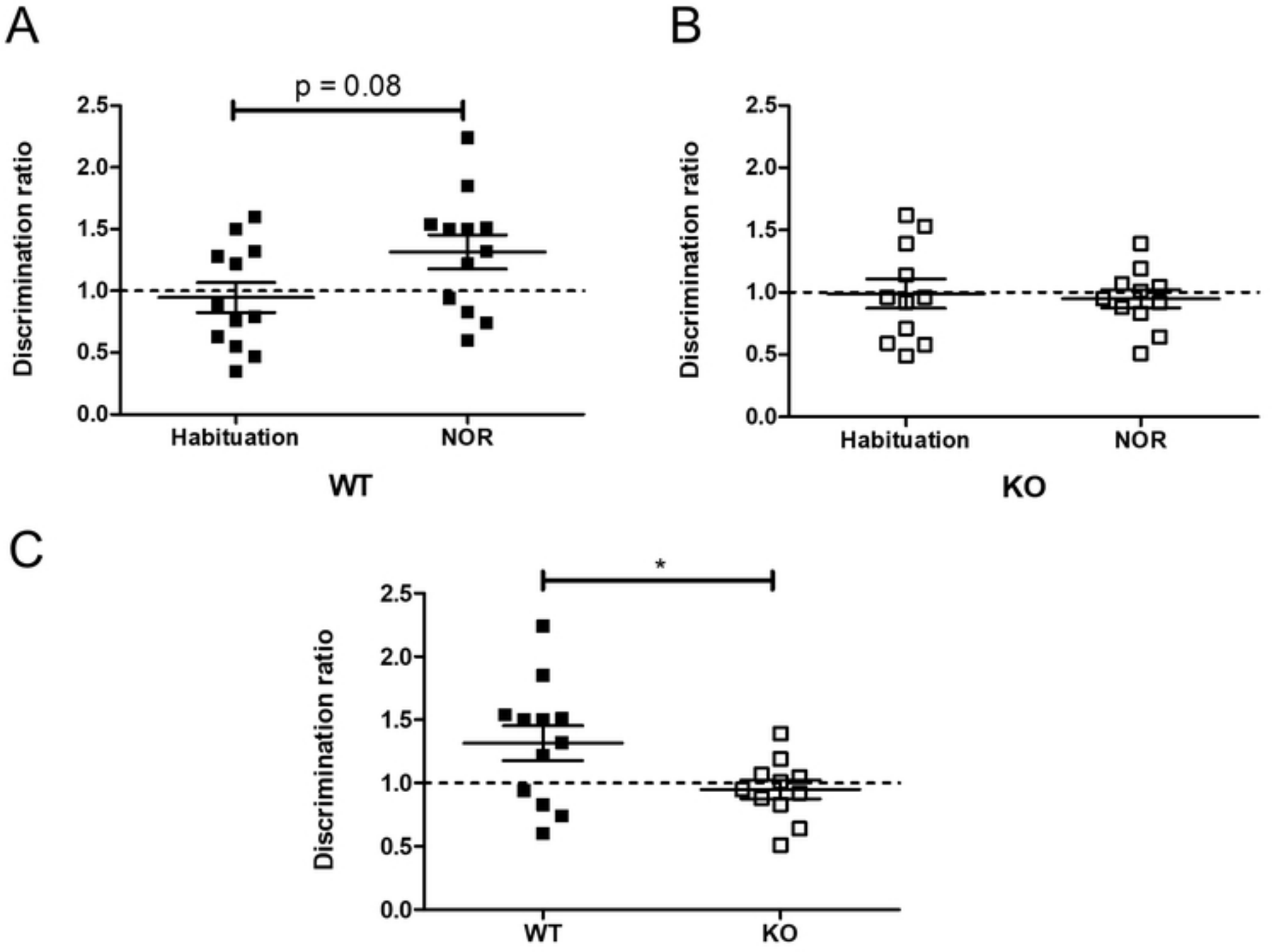
A
p = 0.08
Discrimination ratio
2.5
2.0
1.5
1.0
0.5
0.0
Habituation
NOR
WT
B
Discrimination ratio
2.5
2.0
1.5
1.0
0.5
0.0
Habituation
NOR
KO
C
*
Discrimination ratio
2.5
2.0
1.5
1.0
0.5
0.0
WT
KO

Figure 4

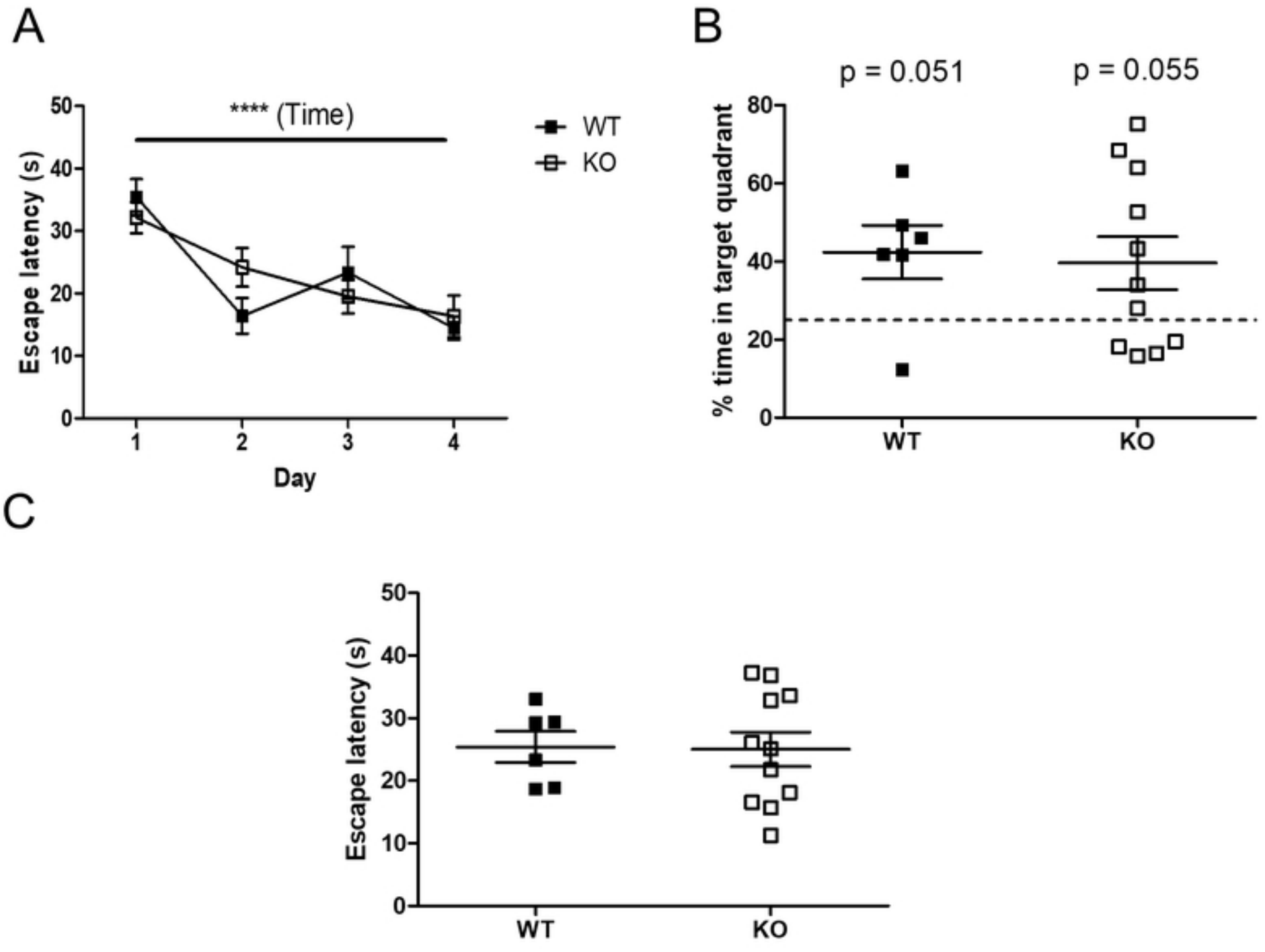
A
**** (Time)
WT
KO
Escape latency (s)
Day
B
p = 0.051
p = 0.055
% time in target quadrant
WT
KO
C
Escape latency (s)
WT
KO

Figure 5