Metaplastic contribution of Neuropeptide Y receptors to spatial memory acquisition

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Highlights:

Hippocampal neuropeptide Y, is significantly involved in hippocampus-dependent spatial memory.

Intrahippocampal NPY Y2 antagonism improve spatial reference memory, without affecting anxiety levels, or spontaneous motor activity.

Rapid changes in expression levels of Y_2R in the hippocampus and prefrontal cortex after Y_2R receptor antagonism support metaplastic regulation of NPY receptors

Abbreviated Title: Metaplastic role of NPY receptors on spatial memory

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ABSTRACT

Neuropeptide Y (NPY) is highly abundant in the brain and is released as a co-transmitter with plasticity-related neurotransmitters such as glutamate, GABA and noradrenaline. Functionally, its release is associated with appetite, anxiety and stress, regulation. NPY acting on Y2 receptors (Y_2R), facilitates fear extinction suggesting a role in associative memory. Here, we explored to what extent NPY action at Y_2R contributes to hippocampus-dependent spatial memory and found that dorsal intrahippocampal receptor antagonism improved spatial reference memory acquired in a water maze in

rats, without affecting anxiety levels, or spontaneous motor activity. Water maze training resulted in an increase of Y2R, but not Y1R expression in the hippocampus. By contrast, in the prefrontal cortex there was a decrease in Y2R, and an increase of Y1R expression. Our results indicate that neuropeptide Y_2R are significantly involved in hippocampus-dependent spatial memory and that receptor expression is dynamically regulated by this learning experience. Effects are consistent with a metaplastic contribution of NPY receptors to cumulative spatial learning.

Keywords: NPY Y_2R antagonist, hippocampus, prefrontal cortex, Morris water maze, Extinction learning.

Abbreviations:

CS: Conditioned Stimulus; LC: coeruleus; LTD: Long-term depression; LTP: Long Term Potentiation; mGlu: Metabotropic glutamate; NMDA: N-methyl-D-aspartate; NMDAR: N-methyl-D-aspartate receptors ; NPY: Neuropeptide Y; MTHFR: methylenetetrahydrofolate reductase gene; PVDF: polyvinylidene difluoride membrane; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; US: Unconditioned Stimulus; Y_1R: Neuropeptide Y Y1receptor ; Y_2R: Neuropeptide Y Y2 receptors.

1. INTRODUCTION

A neuropeptide is a proteinaceous substance produced and released by neurons that acts on neural substrates. Humans possess a diverse assortment of neuropeptides that can influence a variety of activities. Over 100 different neuropeptides have been currently identified in the brain. These neuropeptides modulate the activity of co-released neurotransmitters to increase or decrease the strength of synaptic signalling. Within the periphery, neuropeptides can act like peptide hormones, modulating different bodily functions [See review by Russo [1]]. Neuropeptide Y (NPY) is a 36-amino acid peptide that is intrinsically involved in a multitude of functions including homeostatic control and the regulation of fundamental physiological processes [2-6]. In mammals, its effects are mediated by six G-protein coupled receptors (Y_1R- Y_6R) [7], all of which, with the exception of Y_2R, mediate their actions postsynaptically [8, 9]. Of these receptors, whereas Y_1R and Y_5R promote feeding behavior [10], Y_2R and Y_4R mediate appetite inhibition and satiety [11-13]. A functional role for Y_6R has not yet been described: although this receptor is present in primate and mouse, it is not expressed in rat [7]. A specific role for NPY in mediating anxiety and anxiolysis has been reported [2, 14].

NPY and its receptors are highly expressed in brain regions that contribute to learning and memory [7, 15]. Y_1R shows highest expression levels in the amygdala cortex, hippocampus, hypothalamus and thalamus. Within the hippocampus levels are highest in the cornus ammonis although expression also occurs in the dentate gyrus [7]. Y_2R exhibits high expression levels the amygdala, hippocampus and hypothalamus. In the hippocampus this receptor is strongly expressed in the pyramidal cell layer of the cornus ammonis, moderately expressed in the dentate gyrus and hilus, and more weakly expressed in Stratum oriens, Stratum radiatum and the molecular layer of the dentate gyrus. The CA3 region exhibits the highest levels of Y_2R expression in the hippocampus [7]. In contrast to the widespread expression of Y_1R and Y_2R in the brain, Y_4R expression is largely restricted to the brain stem, hippocampus and hypothalamus and Y_5R

78 expression is mostly confined to hippocampus and hypothalamus and associated
79 structures [7].

80 A wide body of evidence supports a central role of the hippocampus and diencephalic
81 nuclei in spatial memory processes, both in humans, with its most famous case study of
82 the patient H.M. by Scoville and Milner [16] and in rodents [see for review [17, 18]]. The
83 most common behavioural test of hippocampus-dependent, spatial learning and memory
84 is the water-maze task [19]. However, after decades of intensive investigations, it still
85 remains controversial how rodents solve this task, and how the spatial specificity of
86 hippocampal neurons contributes to it [20, 21] .
87 There are two anatomical axes in the hippocampal formation that have functional
88 consequences for spatial orientation, the “long axis” (posterior-to-anterior in humans)
89 and the “proximodistal” axis. [17]. Recent studies support that hippocampal subfields
90 along its proximodistal axis are differentially involved in the processing of positional and
91 directional information [22-25]. For the longitudinal axis, (reviewed in [26]) the dorsal
92 pole (‘posterior pole’ in humans) has been traditionally associated with spatial cognition
93 and memory [27-33], whereas the ventral pole is associated with anxiety and emotional
94 processing [34, 35]. With the recent discoveries on hippocampal physiology, this dualism
95 seems untenable. For example, hippocampal theta rhythm occurs during spatial,
96 mnemonic or emotional and/or anxiety-related information processing, resembling a
97 single traveling wave along the longitudinal hippocampal axis [for a review see [36]].
98 While the traditional view is that only the ventral hippocampus supports unconditioned
99 anxiety, at least some studies [37], including those on neuropeptides implicate the
100 dorsal/whole hippocampus in anxiety [38-40]. The dorsal hippocampus role in
101 unconditioned (unlearned) anxiety, evaluated here with the Elevated Zero Maze (EZM),
102 is on the contrary still poorly understood [39, 41]. The influence of hippocampal NPY in
103 unconditioned anxiety has been studied in the elevated plus-maze and EZM, with mixed
104 results [42, 43]

105 In line with the extensive expression of NPY and NPY receptors in the hippocampus, a
106 specific role for NPY receptors in associative learning and memory has recently become
107 evident, particularly in the context of aversive experience: Activation of Y_2R promotes
108 fear extinction and reduces reinstatement of fear memory [44], and both Y_1R and Y_2R
109 contribute to valence-encoding of fear memory [45-47]. Furthermore, intracerebral NPY
110 administration in rodents enhances aversive memory, as shown in passive avoidance
111 tests in mice [48]. Much less is known about the involvement of NPY receptors in other
112 forms of memory such as appetitive associative memory, or spatial memory.

113 Indirect evidence that NPY may modulate *spatial* memory derives from the extensive
114 expression of NPY receptors in the hippocampus [7]. In the dentate gyrus, for example,
115 it is co-released from GABA interneurons, and to a lesser extent from glutamatergic
116 neurons [49]. Consistent with the fact that NPY is also co-released with noradrenaline
117 from the locus coeruleus [50, 51] and that locus coeruleus afferents terminate in high
118 density in the hippocampus [52, 53], interneurons often co-express beta-adrenergic and
119 NPY receptors [54]. This latter observation brings an intriguing link to memory function
120 and memory encoding in the hippocampus, given that both activation of the locus
121 coeruleus and of beta-adrenergic receptors are critically involved in determining the
122 direction of change in synaptic strength and in spatial content encoding by the
123 hippocampus and GABA uptake [55-57].

124 Despite the abovementioned correlative implication of NPY receptors in hippocampus-
125 dependent spatial memory, to our knowledge a role for NPY receptors in this form of
126 associative memory has not yet been demonstrated. In the present study, we therefore
127 explored to what extent NPY may contribute to spatial memory acquired cumulatively in
128 a water maze. We specifically targeted Y_2R, because this receptor is present at high
129 levels in brain areas considered essential for memory processes [7], are present on
130 Schaffer collateral inputs to the CA1 region [9], on dentate gyrus granule cells [58] and
131 because these receptors are highly expressed on mossy fiber terminals in the

hippocampal CA3 region [49] a hippocampal subfield that serves as a hub for memory acquisition and retrieval [59]. We observed that antagonism of Y_2R within the hippocampus, results in an improvement of escape latency in the acquisition phase of water maze learning and improves platform localization in trained animals. Furthermore, antagonism of Y_2R resulted in changes of both of Y_1R and of Y_2R expression in the hippocampus and prefrontal cortex suggesting that NPY receptors are subjected to metaplastic regulation.

2. MATERIAL AND METHODS

2.1. Animals

All experimental procedures carried out with animals were approved in advance by the local Animal Ethics Committee of the University of Oviedo and closely complied with both the European Communities Council Directive 2010/63/UE and the Spanish legislation on care and use of animals for experimentation (Royal Decree 53/2013). All efforts were made to minimize the suffering and number of animals used.

Male adult Wistar rats (*Rattus norvegicus, source:* University of Seville central animal facility*)* weighing between 250-330g (n=51) were housed in a temperature controlled-room (23 ± 2ºC). Lighting was kept on a reversed 12-h light/dark cycle with lights on from 08:00–20:00 h. Animals were group-housed in standard laboratory cages (20 × 35 × 55 cm) with four rats in each group. During the entire experimental period, rats had *ad libitum* access to food and water.

2.2. Surgery

Rats were deeply anaesthetized with xylazine (5 mg/kg, intramuscularly (i.m.)) and ketamine (80-100 mg/kg, intraperitoneal (i.p.)) and placed in a stereotaxic frame. Stainless steel cannulae (inner diameter 22G) (Becton Dickinson S.A., Spain) were stereotactically implanted bilaterally in the CA1 region of the dorsal hippocampus

(coordinates from bregma: anterior-posterior (AP) -3.6, L ±2.6, dorsoventral (DV) -2.1 mm). Cannulae were fixed to the skull using dental acrylic cement (Glaslonomer Cement, Shofu Inc., UK) and anchor screws. Animals were allowed to recover for 5 days after surgery, after the surgery a battery of neurological tests were carried out in order to discard motor impairments attributable to the surgical intervention. For a timeline of the experiments, see Figure 1.

2.3. Pharmacological treatment

Rats received a bilateral injection of a Y_2R antagonist, or vehicle, through the implanted bilateral cannulae 30 min before the spontaneous locomotor or elevated zero maze test. To explore the effect of acute Y_2R antagonism of reference memory acquisition and reference memory retrieval, animals received antagonist or vehicle treatment 30 minutes before starting the training sessions on day 3. (Figure 1 A)

Animals were divided in two groups as follows:

1. an experimental group that received 1 nmol/µl BIIE0246 in 0.9% physiological saline (1 µl/hemisphere). Experimental group.
2. a vehicle group that received 0.9% saline (1 µl/hemisphere). Saline group.

Solutions were infused at 0.5 µl/min and the cannulae were left in place for one minute after conclusion of treatment to ensure that fluid did not remain in the cannula.

BIIE0246 has been reported to act as a potent and selective Y_2 receptor antagonist devoid of high affinity for the Y_1, Y_4 and Y_5 subtypes [60].

Given the length of the behavioral procedures, some of the permanently implanted bilateral cannulae were lost or became blocked with time, (Two before the EZM and nine during the spatial memory training, or before the spatial memory test), thus, preventing pharmacological treatment. The animals affected were therefore not included in the following tests.

2.4. Behavioral tests

2.4.1. Spontaneous locomotor activity

Thirty min after the infusion of Y2R antagonist or vehicle the spontaneous locomotor activity was monitored. Each activity monitoring system consisted of a closed acrylic transparent cage that incorporated a recording camera on the top (Noldus PhenoTyper, Wageningen, The Netherlands). During each session, automatic recording of distance travelled was obtained using video-tracking analysis software (EthoVision XT, Noldus, Wageningen, The Netherlands). Rats were transported to the experimental room in their home cages. Once there, they were placed into the activity monitoring cages, and allowed to freely explore the new environment during a habituation phase (5 min). Spontaneous locomotor activity was measured during 30 min and analyzed every 5 min. The cages were cleaned between rats with 70% ethanol and then washed with water between each usage by an individual rat to remove any possible odor cue.

2.4.2. Elevated zero maze

Two days after the spontaneous locomotor activity test (Figure 1), animals carried out the elevated zero-maze test. The apparatus was made of black acrylic in a circular track 10 cm wide, 81 cm in diameter, and was elevated to a height of 82 cm from the floor (Noldus, Wageningen, The Netherlands). The maze divided into four sections of equal lengths, two open sections and two closed sections with black acrylic walls 35 cm in height. The test commenced 30 min after infusion of the treatment/ vehicle control. Rats were placed in the center of the open arm and their movements were recorded for 5 min. After finishing each session, the maze was cleaned with 70% ethanol and then washed with water to remove any odor cues. Variables measured included total distance moved and time in open sectors. Rat movements were recorded with a camera connected to a computer with a video-tracking system (EthoVision XT; Noldus, Wageningen, The Netherlands).

2.4.3. Spatial learning in a water maze

Two days after the EZM, the spatial learning on the Morris water maze began (Figure 1). The maze comprised a circular water tank made of black fiberglass, measuring 1.5 m in diameter by 75 cm in height. The pool was filled with tap water and an escape platform was placed hidden beneath the water surface. The water temperature was kept at 20 ± 1°C during the entire training period. The pool was surrounded by numerous distal visual cues such as colored boxes, patterns and an air balloon fixed in three black panels surrounding the pool. Additionally, the room was illuminated by two halogen spotlights (500 W) facing the lab walls. Each trial was video-recorded and later analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). Variables measured included the mean time spent to reach the platform (latencies) and time spent in each of the four virtual quadrants in which the pool was divided (A, B, C and D).

Rats were released facing the pool walls from the central part of each quadrant following a pseudo-random sequence. Rats were allowed to swim for up to 60 s to locate the platform in each trial, or gently manually guided to the platform location by the experimenter if they have not found it by themselves within 60 s. The animals were left on the platform for 15 s, followed by a rest period of 5 s in a black plastic bucket until the next trial.

Pre-training

In an initial pre-training trial, and to expedite comprehension of the task principle, the target platform was first rendered visible to the animals by allowing its surface to extend 2 cm above the water in the center of the maze. (For a timeline and procedure, please see Figure 1)

Training

This pre-training trial was followed on day 1 by four training sessions of four trials each, where the platform surface was submerged to 1.5 cm beneath the water surface. The

intersession period was approximately 30 min. On day 2, animals participated in three training sessions of four trials each, by which them the animals had reached the learning criterion of finding the platform within 20s.

The platform was kept in the same quadrant (escape quadrant, C) on each day of the experiment. (Figure 1 C)

Each day after conclusion of the acquisition learning on days one and two, the animals were evaluated in a probe test to examine memory retention of the platform location. The retention test was conducted as a single probe trial. During this test, the platform was removed from the pool, and rats were released from the quadrant that was diagonally remote from the target quadrant. They were allowed to swim for 60 s. After this period, animals were removed from the pool. In order to prevent early extinction of the previously learned task, all animals then underwent an additional training (Post-training) trial in which the platform was available again in its original place. In this last trial, all animals were released into the pool from the quadrant B.

Test

After the acquisition learning criteria was reached on day two, animals were submitted to a spatial memory test. For this purpose, animals underwent a single session of 4 trials in the same conditions as described before, 30 min after the infusion of saline control or Y2R antagonist. A last retention probe in which the platform was not present in the maze was carried out after the test session.

2.5. Western Blotting

Rats (n = 4 per group) were sacrificed by decapitation immediately after the final water maze procedure, brains were removed, rapidly frozen in isopentane at −70 °C and stored

263 at −80 °C. The interval between antagonist or vehicle-treatment and brain removal was
264 ca. 40 min.

265 Brains were subsequently defrosted and the hippocampi, striata and cortices were
266 collected, whereby the prefrontal cortex was separated from the parietal, occipital and
267 temporal cortices that were denominated as “other cortices”. Afterwards, brain regions
268 were homogenized in a lysis buffer supplemented with a protease inhibitor cocktail and
269 protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL,
270 USA). Samples (25 to 60 μg) were separated onto sodium dodecyl sulfate–
271 polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene
272 difluoride membrane (PVDF; Millipore, Madrid, Spain) and blocking medium.
273 Membranes were then incubated overnight at 4°C, with the following primary antibodies:
274 NPY Y_1 receptor (1:1000, AbD Bio-Rad Cat# 6732-0150, RRID:AB_620417) and NPY Y_2
275 receptor (1:200, Alomone Labs, Jerusalem, RRID:AB_2818974). Afterwards,
276 membranes were washed and incubated with the respective secondary antibodies for 1h
277 at room temperature. Immunoreactive bands were detected by enhanced
278 chemofluorescent detection (ECF kit, Amersham) and visualized on a Typhoon 9000
279 system (GE Healthcare Europe GmbH). The membranes were then re-treated with an
280 antibody against β-Actin, which was used as loading control (1:2000; Sigma-Aldrich Cat#
281 A5316, RRID:AB_476743). The immunoblots were analyzed with ImageJ Software
282 (NIH, Bethesda, MD, USA) to measure the optical density of the bands.

283 **2.6. Statistical analysis**

284 Student’s t tests were used to evaluate group differences in the variables measured with
285 regard to spontaneous activity (total distance moved) and the zero maze (total distance
286 moved and time in open arms). Hedge´s g unbiased test was used as an effect size
287 measurement.

288 A two-way repeated-measures analysis of variance (ANOVA) was applied to evaluate
289 possible group differences in the escape latencies across training sessions in the water

maze task. Post-hoc tests (Holm Sidak tests) were used to further analyze group differences in case of significant interactions between group and training sessions. Holm-Sidak's post-hoc tests were used to evaluate differences across training sessions in each experimental group. Group differences in swim time spent in the previously reinforced quadrant across transfer tests were evaluated by two-way repeated-measures ANOVA. Holm Sidak's post-hoc tests were used in case of significant ANOVA results. A Kruskall Wallis as a non-parametric test was used to evaluate differences in between groups and trials after the drug infusion, using a Dunn´s method as a post-hoc test.

For western blot analysis, an unpaired Student's *t* test was used to evaluate Y_1R and Y_2R expression in brain regions of vehicle and antagonist-treated rats. A non-parametric Mann–Whitney U test was used to analyze these values when normality or equal variances failed. A p value ≤ 0.05 was considered as statistically significant. Hedge´s g unbiased test was used as an effect size measurement. Data were analyzed using *SigmaStat* 3.5 (Systat Software, Chicago, USA).

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3. RESULTS

Antagonism of Y_2R has no effect on spontaneous locomotor activity or anxiety-related behavior.

Activity monitoring of animals that were treated with a Y_2R antagonist (n = 28), or vehicle (n = 25), revealed no differences in spontaneous locomotor activity when measured as the total distance moved ($t_{(51)} = 0.03$; $p = 0.97$). Hedge´s g unbiased = 0.01, 95% CI (Figure 2 A).

Assessment of the total distance moved in an elevated zero maze was equivalent in animals that received the Y_2R antagonist (n = 26), or vehicle (n = 25) ($t_{(49)} = 0.32$; p = 0.75) Hedge´s g unbiased = 0.09, 95% CI. The time spent in open sectors of the elevated

zero maze was also similar in both animal's cohorts ($t_{(49)} = -0.52$; $p = 0.605$) Hedge´s g unbiased = 0.14, 95% CI (Figure 2 B, C).

Antagonism of Y_2R prior to commencing task acquisition in a water maze transiently improves escape latency

When animals were treated with an Y_2R antagonist four and two days prior to commencing training sessions in a water maze on day 1 (n = 23), escape latency was improved compared to vehicle treated animals (n = 19). ($F_{(1,119)} = 4.67$; $p = 0.04$) (Figure 3 A).

These effects were short-lived: no difference in escape latency were evident on day 2, and both animal cohorts successfully reached the learning criterion of 20s escape latency by the last training session on day 2. ($F_{(1,80)} = 0.207$; $p = 0.65$. (Figure 3 A). Thus, although antagonism of Y_2R prior to commencing training on day 1 brought transient benefits, no advantage in reaching learning criterion was obtained through this treatment.

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Acute antagonism of Y2R prior to commencing task retrieval in a water maze transiently improves escape latency

After achieving learning criterion on the previous day, animals were treated with an Y_2R antagonist (n = 23) or vehicle (n = 19) 30 minutes prior to commencing water maze sessions on day 3. Vehicle-treated rats showed in initial marked disimprovement in escape latency in trial 1, that recovered to near criterion levels by trial 2 (Figure 3 A). Escape latency was significantly better in antagonist-treated animals in trial 1 compared to vehicle treated animals (Kurskall Wallis $H_{(1)} = 4.48$; $p = 0.03$) and overall performance during all trial session was significantly better in antagonist-treated animals ($F_{(1,120)} = 6.66$; $p = 0.014$) (Figure 3 A).

Antagonism of Y2R has acute and prolonged effects on reference memory

After the conclusion of the escape latency monitoring sessions on days 1 through 3, a probe test was conducted to determine the efficacy of platform location memory in Y_2R antagonist-treated or vehicle-treated rats. Significant interaction was found between the factors group, and the specific transfer test that was presented ($F_{(1,133)} = 22.39$; $p \leq 0.01$). Specifically, on day 1 no difference in time spent in the target quadrant was detected when performance in both cohorts was compared (Holm Sidak method t = 1.48; $p = 0.14$) (Figure 3 B, C). On day 2, target quadrant performance was significantly better in Y antagonist-treated animals however (Holm Sidak method t = 2.95; $p = 0.04$) (Figure 3 B, D). This was also the case on day 3, where we detected that antagonist-treated animals spent more time in the target quadrant (Holm Sidak method t = 6.26; $p = 0.001$) (Figure 3 B, E). Thus, antagonism of Y_2R improves reference memory retention.

Neuropeptide Y receptor expression is altered by intrahippocampal antagonism of Y_2R

After the conclusion of the behavioral tests on day 3, brains were removed for western blot analysis. Here, differences were found between groups with regard to Y_2R expression in the hippocampus, whereby levels were higher in antagonist-treated animals (n = 4) compared to vehicle-treated controls (n = 4) ($t_{(6)} = -2.72$; $p = 0.03$) Hedge´s g unbiased = -1.68, 95% CI. By contrast decreased Y_2R expression was found in the prefrontal cortex compared to controls) ($t_{(6)} = -2.72$; $p = 0.033$) Hedge´s g unbiased = 1.72, 95% CI (Figure 4 A, C). No differences in Y_2R expression were found between groups in the striatum or other cortices (Figure 4 E, G)

Although no changes in expression of Y_1R were found in the hippocampus following Y_2R antagonist-treatment (Figure 4 B, 4 C), a significant increase in expression was detected in the prefrontal cortex ($t_{(6)} = 3.80$; $p = 0.009$) Hedge´s g unbiased = -2.34, 95% CI. Expression was unchanged in the striatum or other cortices (Figure 4 F, H).

4. DISCUSSION

In this study, we provide novel evidence that NPY acting on Y_2R contributes to spatial reference learning and that the expression of NPY receptors in the hippocampus and prefrontal cortex is dynamically regulated by the modulation of NPY binding to Y_2R. We observed that antagonism of Y_2R improves the acquisition of platform-location learning in a water maze task, improves the accuracy of platform localization in trained animals and enhances performance in the probe test. In addition, Y_2R antagonism caused an increase in Y_2R expression in the hippocampus and a decrease in Y_2R expression in the prefrontal cortex. Although no changes in Y_1R expression were caused in the hippocampus by antagonist treatment, Y_1R expression was increased in the prefrontal cortex.

Despite the fact that NPY has been implicated in the modulation of anxiety and stress both in rodents and in human studies [61-64], antagonism of Y_2R had no effect on anxiety behavior in the present study. No change in anxiety state was also reported in transgenic mice that lack Y_2R [65] suggesting that NPY effects on anxiety/anxiolysis are not mediated by this receptor. We also detected no effects of Y_2R antagonism on motor behavior, as determined by assessment of spontaneous locomotor activity, or the total distance moved during the elevated zero-maze test. Accordingly, previous studies have shown that transgenic mice that lack Y_2R do not show alterations in their locomotor activity in the elevated plus maze [66], or in an open field paradigm [67].

Spatial navigation is a complex skill. Tasks that assess navigational ability have multiple perceptual, mnemonic, and executive components and rely on a broad network of cortical and subcortical circuits and structures [68-70]. Since the first early efforts to evaluate the spatial navigation in rodents [19, 71], adaptations of the original hidden platform test have been broadly used in the study of spatial memory, and thus, associative memory in rodents [20]. Virtual reality adaptations of the spatial task have also been used to evaluate human place learning [72, 73]. However, perhaps given its complexity, only a handful of human genetic studies using the water maze can be found

393 in the literature. To cite some, previous genetic studies have analyzed the influence of
394 MTHFR C677T genetic polymorphism [68] the androgen receptor GAC -repeat number
395 [74], the MAO-A and androgen receptor [75, 76], or six genes previously associated with
396 memory or executive functioning: APOE, SORL1, BDNF, TOMM40, KIBRA, and COMT
397 [77]. Despite the abovementioned well-studied contribution of NPY to anxiety disorders
398 in humans, to our knowledge about the involvement of NPY in spatial human navigation
399 is, nevertheless, still to be addressed.

400 To evaluate the influence of acute and prolonged effect of NPY on spatial memory, we
401 treated paradigm-naïve rats with a Y_2R antagonist into the hippocampus two days prior
402 to commencing water maze training sessions and retreated the animals with the
403 antagonist after they had reached learning criteria and thus had fully acquired the
404 platform search strategy. We observed a small improvement in escape latency on day 1
405 that was not sustained on day 2. Treatment with the antagonist prior to starting water
406 maze sessions on day 3 improved escape latencies once more. The lack of ostensible
407 differences in escape latencies on day 2 suggests that the improvements on day 3 are
408 related to transient and acute effects of Y_2R on platform search behavior. As mentioned
409 above, this is unlikely to have been mediated by changes in anxiety status or motor
410 behavior. Thus, acute effects on neurotransmitter release in the hippocampus, may have
411 mediated these effects (see below). This modulation was not without functional
412 consequences, however: both on day 2 and day 3 a significant improvement was
413 detected in antagonist-treated animals in the probe test, indicating that antagonism of
414 Y_2R also elicited long-term effects on reference memory.

415 The dynamic changes in Y_1R and Y_2R expression in the hippocampus and prefrontal
416 cortex after Y_2R antagonist treatment were striking and may serve to explain the effects
417 of receptor antagonism on spatial memory that we observed. We detected an increase
418 of Y_2R expression in the hippocampus and a decrease of Y_1R in the prefrontal cortex in
419 animals that had been treated with a Y_2R antagonist prior to beginning anxiety test and

spontaneous activity test and prior to the probe test in the water maze on day 3. No significant changes in expression of either receptor were found in the striatum or other cortices suggesting that these effects were restricted to brain structures involved in memory processing. Metaplastic changes in neurotransmitter receptor expression following learning or synaptic plasticity have been reported for plasticity-related neurotransmitter receptors. For example, induction of hippocampal long-term potentiation (LTP) alters the hippocampal expression of metabotropic glutamate (mGlu) receptors [78] and allosteric modulation of mGlu5 receptors in conjunction with cumulative spatial learning alters expression of mGlu1 receptors [79]. Metaplasticity alters the subunit composition of N-methyl-D-aspartate (NMDA) receptors [80] and changes the NMDAR contribution to memory acquisition in task-adept rodents [81].

Y_2R are located presynaptically and thus, are likely to function as autoreceptors for neurotransmitter release [82, 83]. Activation of these receptors results in opening of potassium channels and closure of calcium channels [84]. Y_2R receptors are present on mossy fiber terminals in the hippocampal CA3 region [49], on Schaffer collateral inputs to the CA1 region [9], on dentate gyrus granule cells [58] and on the fimbria [85]. Evidence for metaplastic changes in Y_2R expression in the hippocampus have been reported in the context of epilepsy: here, kainic acid induced seizures caused increases in Y_2R expression in granule cells and enhanced NPY affinity on Schaffer collaterals [58]. A dynamic state-dependent shift from high affinity to low affinity states of this receptor, as a result of inhibition of agonist binding has also been reported [86, 87]. Taken together with our own findings, strong evidence exists that Y_2R undergo experience-dependent metaplastic regulation.

The question now arises as to how this could support hippocampus-dependent spatial learning, given that an inevitable consequence of the inhibition of Y_2R receptors at glutamate terminals in the hippocampus will be an *increase* of glutamate release. This can be expected to enhance the propensity of hippocampal synapses to express

447 synaptic plasticity under these specific conditions. Conversely, our findings suggest that
448 under physiological circumstances, the *suppression* of glutamate release by NPY acting
449 on Y_2R receptors would rather act against information encoding by means of LTP.
450 However, memory encoding in the hippocampus is not enabled by LTP alone. Long-term
451 depression (LTD) is an intrinsic component of the long-term acquisition of complex
452 representations [88, 89]. Whereas LTP may serve to select a hippocampal neuronal
453 network that serves as the scaffold for a spatial representation, LTD may serve to prune
454 and modify this representation thus ensuring its uniqueness and preventing
455 generalization of similar spatial representations [90]. The relative degree of activation of
456 NMDA receptors determines whether LTP or LTD occurs as a result of glutamate binding
457 to this receptor [91] and LTD is an intrinsic component of cumulative acquisition of
458 reference memory in a spatial learning task that is accumulated over days [92]. LTD is
459 tightly related to the acquisition of information about spatial content [88, 93]. Thus, NPY
460 acting on Y_2R receptors may contribute to memory acquisition by acting permissively
461 towards the encoding of the spatial experience by means of LTD.

462 Hippocampal Y_2R are not only present on glutamatergic terminals in the hippocampus,
463 they are also present on locus coeruleus (LC) terminals [94] and NPY is a co-transmitter
464 of the LC [50, 51]. Thus, NPY release from the LC is likely to modulate the release of
465 both NPY and noradrenaline. The LC sends afferents to all areas of the hippocampus
466 [52, 53]. Activity in the LC promotes the induction of LTD [56, 95], regulates the
467 magnitude and expression of LTP[24] and modulates hippocampus-dependent memory
468 acquisition and stabilization [55]. It has been proposed that the LC supports memory
469 encoding by means of 'hot-spots' of activity in LC terminals, whereby local noradrenaline
470 release is accentuated by glutamate acting on NMDA receptors on LC terminals [96].
471 This process, in turn, may support the dynamic changes in expression of Y_2R in the
472 hippocampus that we detected in the present study. Y_2R-mediated regulation of
473 noradrenaline release may also serve to explain why LC activity can modulate both

hippocampal LTP and LTD [55] .Furthermore, the autoreceptor regulation by Y_2R of neurotransmitter release from LC terminals may serve to prevent excessive neurotransmitter release that could support pathophysiological events in the hippocampus related to excessive excitatory responses.

The hippocampus sends afferents to the medial prefrontal cortex [97], and intrinsic, prolonged, elevations in hippocampal excitability changes excitation-inhibition balance in the medial prefrontal cortex by altering GABA receptor expression [98]. Disinhibition of glutamatergic (and noradrenergic) terminals in the hippocampus by means of Y2R antagonism, can be expected to change the excitatory output of the hippocampus to the medial prefrontal cortex. This, in turn, may have triggered the changes in NPY receptor expression that we detected in this structure. Whereas Y1R receptor expression was increased, Y2R was decreased as a result of Y2R antagonism. Y1R are postsynaptically localized and by means of G-protein coupling inhibit cAMP accumulation in the rodent brain [99]. These receptors are present on glutamatergic neurons and their activation attenuates neuronal excitation [46]. Thus, the changes in NPY expression that occurred as a result of Y2R antagonism may not have elicited a net effect on excitation-inhibition balance, but may have altered intrinsic information processing in the prefrontal cortex. In line with this, it has been reported that NPY reduces the robustness of CS-US associations during eyeblink conditioning [100] and it has been proposed that NPY acting on Y1R modulates context saliency [46].

Surprisingly, because no manipulation was performed during the second transfer test, increased time spent in the target quadrant over training days was found in the treated animals, though the time in target quadrant remained the same in saline controls on the second day. Although the extinction of the previously conditioned place preference is a tempting interpretation, previous of our studies using the water maze [57, 101] demonstrated that 16 non-reinforced consecutively performed trials are necessary to acquire an extinction learning of the previously reinforced location, thus, a single

unreinforced trial cannot solely explain these effects. Western blot also revealed an increased Y1R expression in the medial prefrontal cortex (mPFC) in those animals treated with intrahippocampal Y2R antagonist, two days before. These results are in agreement with previous experiments reporting an important role of the mPFC on the reactivation of the former memories. In particular, the mPFC is known to plays an important role in short-term memory, especially in a delayed matching- or non-matching-to-sample tasks, in which a correct choice response for a stimulus, as an object or a spatial location, is required after a delay period [102, 103]. Furthermore, rats with lesions in the PFC show acquisition and retrieval deficits in the spatial memory task [104]. Alternatively, the role of the mPFC in behavioral persistence, conditioned extinction and drug-seeking paradigms has been extensively described [105-107]. The mPFC also mediates attentional and motivational components of spatial memory test performed in the water maze [108] [109]. Additionally, it is known that extinction learning of conditioned fear memories requires plasticity in the infralimbic mPFC, and that brain-derived neurotrophic factor (BDNF), key mediator of synaptic plasticity in multiple brain areas, prevents persistence and extinction failures when directly infused in the mPFC. [110]. Among others, we have previously suggested a role of this brain region in the modulation of early extinction learning of an spatial memory task [57, 101], in which persistence of the previously reinforced quadrant needed to be decreased. This aspect of mPFC function, together with the above mentioned NPY metaplastic changes in the hippocampal-mPFC circuit may account for the improved recall observed in treated animals in the spatial navigation task.

In conclusion, the results of this study indicate that neuropeptide Y acting on Y_2R contributes to the acquisition of spatial reference memory. Given that the Y_2R antagonist used in our study was applied directly into the hippocampus, we can assume that effects on memory were mediated by alterations in neurotransmission in the hippocampus. Our results also support that there is a tight interrelationship between the expression of Y_2R

and Y_1R, given that antagonism of Y_2R altered Y_1R expression in the prefrontal cortex. Furthermore, rapid changes in expression levels of Y_2R in the hippocampus and prefrontal cortex after Y_2R receptor antagonism support that NPY receptors undergo metaplastic regulation. This in turn may support dynamic and experience-dependent contributions of NPY to spatial memory processing by the hippocampus and related structures.

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FIGURE LEGENDS

Figure 1: Spatial memory procedure and timeline.

A) Experimental timeline. The Neuropeptide Y2-Receptor antagonist, or vehicle were infused intrahippocampally 30 min before the spontaneous activity test, before the elevated zero maze test and before the spatial memory test day in the water maze**.**

B) Temporal structure of the experiment. The period in between test was two days.
C) Water maze procedure. The schema shows the positioning of quadrants in the water maze and placement of the platform in every phase. All visual cues were situated in panels around the maze to support allocentric orientation (not depicted). For the pre-training phase, the platform remained visible in the center of the maze, whereas for the training and test day, it remained hidden below the water surface. For the retention probe, the platform was absent, and the total amount of time spent in each quadrant was measured. An additional trial was added at the end of each day, with the platform in place, to avoid early extinction learning of the previously acquired place preference.

Figure 2: Anxiety-related behavior:

A) Spontaneous activity. No differences were found in the open field in between the vehicle and the Y2R antagonist-treated. (p = 0.972).

B-C). Elevated zero maze: There was no difference in the distance animals ran in the zero maze (p = 0.753), or the time they spent in the open arms (p = 0.605). This indicates that no anxiogenic or anxiolytic activity occurred due to the treatment. Averages per group are indicated by a horizontal dash.

Figure 3: Spatial memory in the water maze

A) Latencies. In animals that were treated with an Y_2R antagonist four and two days prior to commencing the training sessions in the water maze on day 1, escape latency was improved compared to vehicle treated animals. These effects were short-lived: no

difference in escape latency were evident on day 2, and both animal cohorts successfully reached the learning criterion of 20s escape latency by the last training session on day 2. Thus, although antagonism of Y_2R prior to commencing training on day 1 brought transient benefits, no advantage in reaching learning criterion was obtained through this treatment. After achieving learning criterion on the previous day, animals were treated with an Y_2R antagonist again 30 minutes prior to commencing water maze sessions on day 3. Escape latency was significantly better in antagonist-treated animals in trial 1 compared to vehicle-treated animals and overall performance during all session was significantly better in antagonist-treated animals. *($p < 0.05$) ** ($p < 0.01$).

B) Quadrant preference in the transfer test. Immediately after each training session a transfer test was carried out in absence of the platform in the water maze, in which animals were released from the contralateral quadrant. Mean time spent in the virtual quadrant that used to contain the previously reinforced platform was analyzed. No differences were found between vehicle and Y2R antagonist treated animals in the first session, however, both in the second session, and after animals received the drug before the test day, animals showed an improvement in the time they spent in the previously reinforced quadrant *($p \leq 0.05$). Averages per group are indicated by a horizontal dash.

C-D) **Examples of trajectories during the transfer test of a vehicle (left) and Antagonist treated animal (right).** Images represent an example trajectory in the transfer probe test performed after the first **C)** after the second acquisition day **D)** and after the test day (under a new drug treatment) **E).**

Figure 4: NPY receptor expression profiles. The expression of Y1 (44 kDa) and Y2 (50 kDa) receptor proteins in different brain areas was evaluated by western blot.

A-C) Increased Y2R expression was found in the in the hippocampus of the experimental group compared to the vehicle-treated group. By contrast, decreased Y2R expression was found in the prefrontal cortex.

605 **D**) Scrutiny of Y1R revealed increased expression in the prefrontal cortex.

606 **E-H**) No significant differences in Y1R or Y2R receptor expression were found in the
607 Striatum or in the rest of the cortices (expression in the prefrontal cortex was not included
608 in this analysis). Group averages indicated by a horizontal dash. * ($p < 0.05$).

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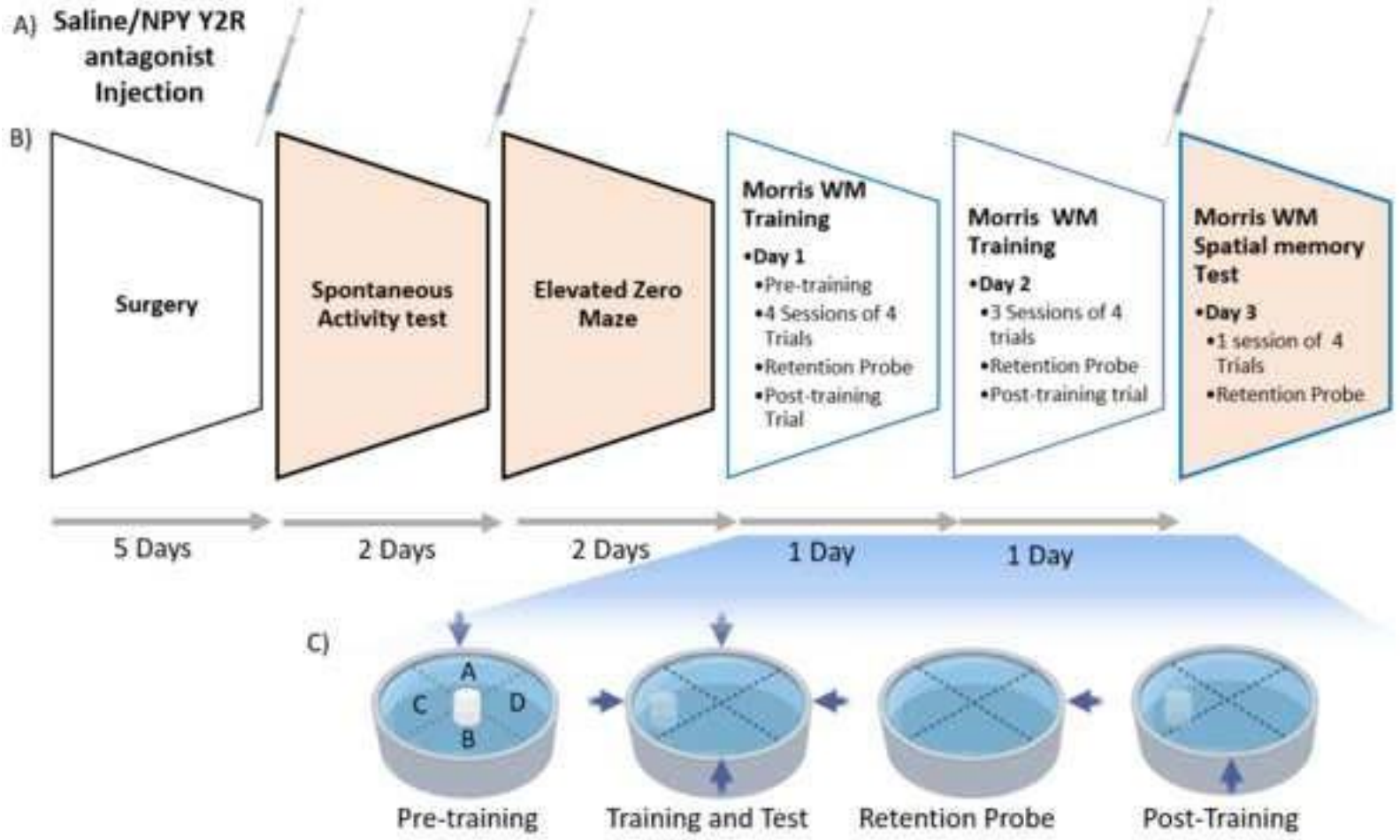
Figure



Figure

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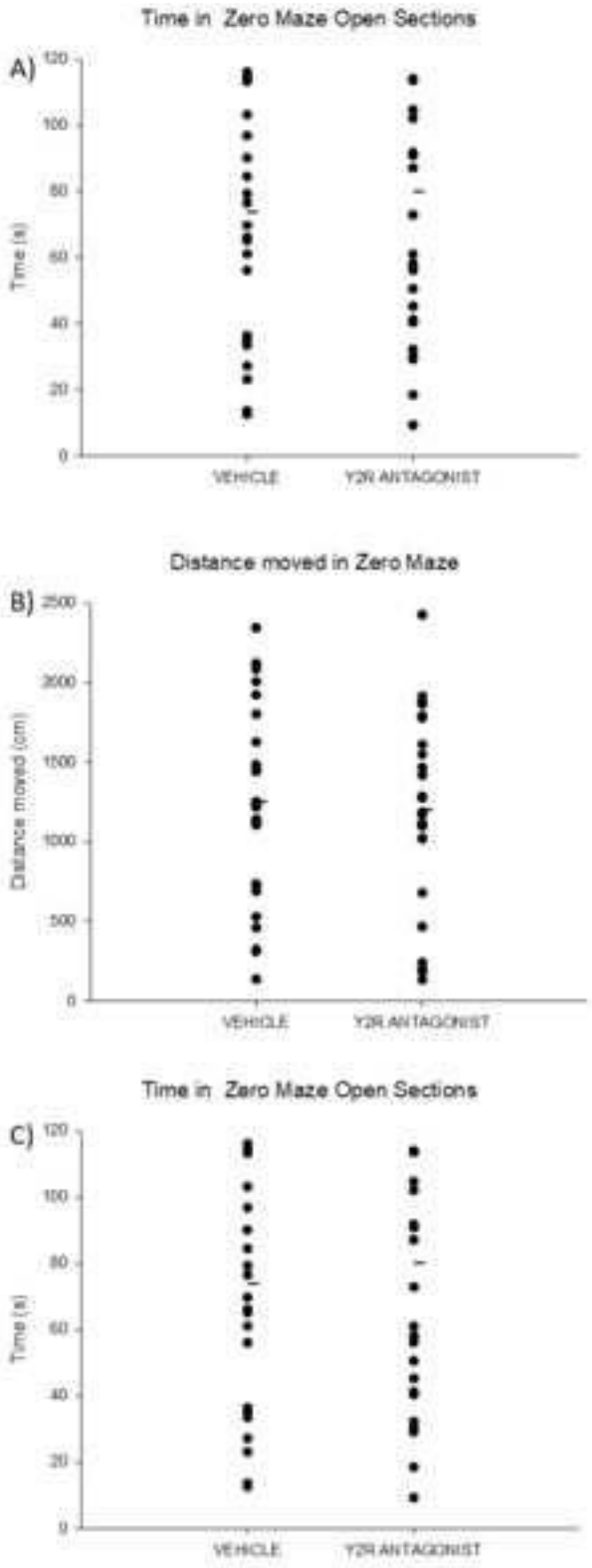

Figure

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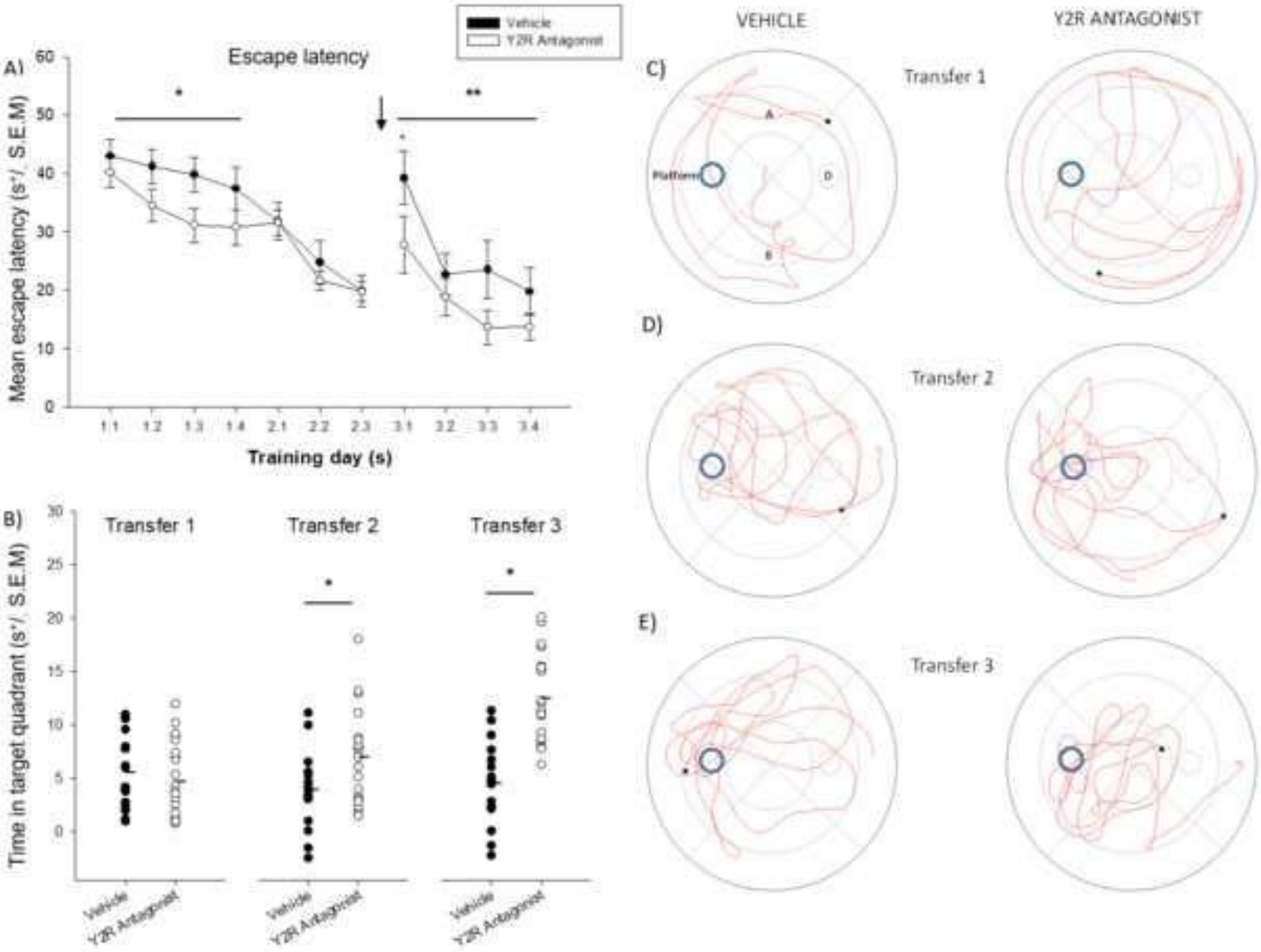

Figure

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