Conditional inactivation of Npy1r gene in mice induces behavioural inflexibility and orbitofrontal cortex hyperactivity that are reversed by escitalopram

Elsevier Editorial System(tm) for Neuropharmacology

Manuscript Draft

Manuscript Number: NEUROPHARM-D-17-00623R2

Title: Conditional inactivation of Npy1r gene in mice induces behavioural inflexibility and orbitofrontal cortex hyperactivity that are reversed by escitalopram.

Article Type: Research Paper

Keywords: NPY-Y1R; behavioural flexibility; orbitofrontal cortex; c-Fos; escitalopram; obsessive-compulsive disorder; 5-HT.

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Abstract: Cognitive flexibility is the ability to rapidly adapt established patterns of behaviour in the face of changing circumstance and depends critically on the orbitofrontal cortex (OFC). Impaired flexibility also results from altered serotonin transmission in the OFC. The Y1 (Y1R) and Y5 (Y5R) receptors for neuropeptide Y (NPY) colocalize in several brain regions and have overlapping functions in regulating cognition and emotional behaviour. The targeted disruption of gene encoding Y1R (Npy1r gene) in Y5R containing neurons (Npy1rY5R-/- mice) increases anxiety-like behaviour and spatial reference memory. Here we used the same conditional system to analyse whether the coordinated expression of the Y1R and Y5R might be required for behavioural flexibility in reversal learning tasks, OFC serotoninergic tone and OFC neural activity, as detected by immunohistochemical quantification of the immediate-early gene, c-Fos. In addition, we investigated whether the acute treatment of Npy1rY5R-/- mice with the selective serotonin reuptake inhibitor escitalopram affected behavioural flexibility and OFC c-Fos expression. Npy1rY5R-/- male mice exhibit an impairment in performing the reversal task of the Morris water maze and the water T-maze but normal spatial learning, working memory and sociability, compared to their control siblings. Furthermore, Npy1rY5R-/- male mice display decreased 5-hydroxytriptamine (5-HT) positive fibres and increased baseline neural activity in OFC. Importantly, escitalopram normalizes OFC neural activity and restores behavioural flexibility of Npy1rY5R-/- male mice. These findings suggest that the inactivation of Y1R in Y5R containing neurons increases pyramidal neuron activity and dysregulates serotoninergic tone in OFC, whereby contributing to reversal learning impairment.

Cover letter

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Turin, January 9, 2018

Dear Dr. Hurren,
we thank you and the Referee for the careful review of our manuscript NEUROPHARM-D-17-00623R1 entitled “Conditional inactivation of Npy1r gene in mice induces behavioural inflexibility and orbitofrontal cortex hyperactivity that are reversed by escitalopram” by Angela Longo et al.
The revised version of the manuscript deals with all the criticisms and text has been changed according to the requests of the Referee. All changes made to the manuscript have been highlighted in yellow.

We thank you and the Referees for the helpful suggestions and we hope that this version of manuscript NEUROPHARM-D-17-00623R1 meets your approval and that you will recommend it for publication.
Sincerely

Carola Eva, PhD

Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest.

***Response to Reviewers**

Answer to Reviewer n. 1

Qa: Were separate or same cohorts used for three chamber and marble burying tests (page 3)? In the same paragraph it is mentioned that these test were performed on independent cohorts while also stating that two weeks after the three chamber test, mice were tested for marble burying test. Please clarify
Answer to Qa: The same cohorts of mice were used for three chamber and marble burying tests. This has been clarified in the Methods Section, (page 3, line 20 and line 22)

Qb: When was cfos tissue collected post behavior? 2hours after the end of 1st day of reversal task (page 7) or two hours after the end of final session when the choice behavior activation of OFC has been reported to return to baseline levels (page 13)?
Answer to Qb. C-Fos tissue was collected 2 hours after the end of 1st day of reversal task. To clarify this point, we have changed the text in the Result section, page 13, line 5 from the bottom.

HIGHLIGHTS

- Reversal learning, a form of behavioural flexibility, depends on OFC functional integrity
- Deletion of *Npy1r* gene in Y5R neurons increases inflexibility and OFC neuronal activity
- Deletion of Npy1r gene in Y5R neurons decreases 5-HT fibers in OFC
- SSRI treatment rescues reversal learning impairment and OFC hyperactivity of Npy1r$^{Y5R-/-}$ mice
- NPY-Y1R transmission in OFC plays a role in behavioural flexibility that is impaired in OCD

*Title page

Conditional inactivation of Npy1r gene in mice induces behavioural inflexibility and orbitofrontal cortex hyperactivity that are reversed by escitalopram.

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Abbreviations

α-CamKII, α-calcium/calmodulin-dependent protein kinase II; BLA, basolateral amygdala; CNS, central nervous system; Cre, Cre recombinase; dig, digoxigenin; DRC/DRI, dorsal raphe caudal part and inferior part; DR(DRD-DRV), dorsal raphe, dorsal and ventral part; 5-HT, 5-hydroxytriptamine; IR, immunoreactivity; MWM, Morris water maze; NGS, normal goat serum; NPY, Neuropeptide Y; *Npy1r*, gene encoding the Y1R for NPY; OCD, obsessive-compulsive disorder; OD, optical density; OFC, orbitofrontal cortex; PaV, Parvalbumin; PBS, phosphate buffered saline; PD, postnatal day; SSRI, selective serotonin reuptake inhibitors; SW, south west; Y1R, Y1 receptor; Y5R, Y5 receptor; WTM, water T-maze.

Abstract

Cognitive flexibility is the ability to rapidly adapt established patterns of behaviour in the face of changing circumstance and depends critically on the orbitofrontal cortex (OFC). Impaired flexibility also results from altered serotonin transmission in the OFC. The Y1 (Y1R) and Y5 (Y5R) receptors for neuropeptide Y (NPY) colocalize in several brain regions and have overlapping functions in regulating cognition and emotional behaviour. The targeted disruption of gene encoding Y1R (*Npy1r* gene) in Y5R containing neurons (Npy1r$^{Y5R-/-}$ mice) increases anxiety-like behaviour and spatial reference memory. Here we used the same conditional system to analyse whether the coordinated expression of the Y1R and Y5R might be required for behavioural flexibility in reversal learning tasks, OFC serotoninergic tone and OFC neural activity, as detected by immunohistochemical quantification of the immediate-early gene, c-Fos. In addition, we investigated whether the acute treatment of Npy1r$^{Y5R-/-}$ mice with the selective serotonin reuptake inhibitor escitalopram affected behavioural flexibility and OFC c-Fos expression. Npy1r$^{Y5R-/-}$ male mice exhibit an impairment in performing the reversal task of the Morris water maze and the water T-maze but normal spatial learning, working memory and sociability, compared to their control siblings. Furthermore, Npy1r$^{Y5R-/-}$ male mice display decreased 5-hydroxytriptamine (5-HT) positive fibres and increased baseline neural activity in OFC. Importantly, escitalopram normalizes OFC neural activity and restores behavioural flexibility of Npy1r$^{Y5R-/-}$ male mice. These findings suggest that the inactivation of Y1R in Y5R containing neurons increases pyramidal neuron activity and dysregulates serotoninergic tone in OFC, whereby contributing to reversal learning impairment.

Introduction

Cognitive inflexibility is observed in various psychiatric disorders such as obsessive-compulsive disorder (OCD) (Chamberlain et al., 2006; Remijnse et al., 2006), schizophrenia (Leeson et al., 2009) and autism (Yerys et al., 2009). Reversal learning is a domain that involves cognitive flexibility and is defined as the ability to rapidly and flexibly change established patterns of behaviour when faced with changing circumstances (Boulougouris et al., 2007; Fellows and Farah, 2003; McAlonan and Brown, 2003). It measures the ability to actively suppress reward-related responding and to uncouple from ongoing behaviour, both of which are phenomena that are biologically related to impulsivity and compulsivity (Izquierdo et al., 2016).

Convergent evidence indicates that OFC is crucial for reversal learning (Hamilton and Brigman, 2015; Ragozzino, 2007; Rudebeck et al., 2013; Schoenbaum et al., 2009). OFC damage results in a range of symptoms that include impulsivity, perseveration, compulsive behaviours and deficit in cognitive flexibility in both humans and rodents (Boulougouris et al., 2007; Fineberg et al., 2011; Gu et al., 2008). OFC hyperactivity, resulting from a decrease in inhibitory signals converging on neurons projecting to dorsomedial striatum and amygdala, is associated with reversal learning impairment (Lagemann et al., 2012; Simon et al., 2010). Impaired flexibility also results from altered serotonin transmission in the OFC. Acute treatment with selective serotonin reuptake inhibitors (SSRIs) in rats has been shown to improve reversal learning performance and behavioural flexibility (Bari et al., 2010; Barlow et al., 2015; Brown et al., 2012).

NPY is one of the most abundant and widely distributed peptides in the central nervous system (CNS) where it is involved in the control of several emotional and cognitive behaviours including anxiety, stress reactions, depression, learning and memory (Dumont and Quirion, 2014; Gøtzsche and Woldbye, 2016; Reichmann and Holzer, 2016). Genetic studies as well as plasma and cerebrospinal fluid examinations in humans suggest a protective action of NPY in stress, anxiety and post-traumatic stress disorder (Enman et al., 2015; Kautz et al., 2016; Schmeltzer et al., 2016).

Moreover, Y1R null mice display a significant reduction in mRNA expression of the 5-HT synthesis-limiting enzyme tryptophan hydroxylase in the dorsal raphe nucleus, suggesting that the NPY-Y1R mediated transmission regulates the 5-HT system (Karl et al., 2004) .

Genes encoding the Y1R and Y5R receptors for NPY (*Npy1r* and *Npy5r*, respectively) are located on the same chromosome in humans and rodents, displaying an opposite transcriptional orientation and a partly overlapping gene structure (Herzog et al., 1997). In rodents, Y1Rs and Y5Rs have an overlapping role in regulating anxiety (Bertocchi et al., 2011; Eva et al., 2006; Sørensen et al., 2004) and colocalize in several brain regions belonging to circuits of cognitive and emotional functions, including layers II-III of cerebral cortex, basolateral amygdala (BLA), hippocampal neurons and dorsal raphe (Oberto et al., 2007; Wolak et al., 2003).

To investigate whether the coordinated expression of the Y1R and Y5R might be required for the regulation of anxiety, spatial learning and memory, and to exclude effects induced by the *Npy1r* gene inactivation in early development, we have previously generated a conditional knockout mice in which the inactivation of *Npy1r* was induced in Y5R-expressing neurons of adolescent mice (Npy1r$^{Y5R-/-}$ mice). Npy1r$^{Y5R-/-}$ mice show an anxious phenotype that might be related to inactivation of the Y1R in the BLA (Longo et al., 2014; Longo et al., 2015). Additionally, Npy1r$^{Y5R-/-}$ mice display increased spatial reference memory, suggesting an inflexible-perseverative phenotype and habit learning (Longo et al., 2014).

In the present study, we demonstrate that a targeted disruption of *Npy1r* gene in Y5R containing neurons reduces cognitive flexibility in two reversal learning tasks, increases the expression of a marker for neuronal activity (the immediate early gene, c-Fos) (Morgan and Curran, 1991) and decreases serotonin immunoreactivity (IR) in the OFC. Moreover, treatment with the SSRI escitalopram normalizes OFC neuronal activity and restores behavioural flexibility in the Npy1r$^{Y5R-/-}$ mutant mice.

2. Materials and Methods

2.1 Animals

Mice were housed in a temperature (22 ± 1 °C) and humidity (50 ± 10%) controlled room, in groups of 2-6, with a 12-hour light/dark cycle (08:00 AM - 08:00 PM) with ad libitum access to food and water. All experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/EEC) and approved by the University of Turin Ethical Committee for animal research and by the Italian Ministry of Health (license no. 180/2006-B).

The generation of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice (C57BL/6J/129/SvJ-derived strain) was achieved as previously described (Longo et al., 2014). Briefly, to generate the conditional deletion of *Npy1r* gene, a targeting vector for homologous recombination in ES cells was designed to introduce *loxP* sites around exons 2−3, which code for the entire region of *Npy1r* gene. The obtained *Npy1r* gene floxed mice were crossed with transgenic mice carrying a Dox-sensitive tTA-regulated Cre recombinase under the control of the *Npy5r* gene promoter. Using this combination of the tTA and Cre regulated gene expression systems, we achieved the deletion of *Npy1r* gene specifically in Y5R expressing neurons of limbic system between postnatal day (PD) 45 and PD 50.

2.2 Behavioural tests

Behavioural tests were carried out between the ages PD 60 and PD 90.

The Morris water maze (MWM), the water T-maze (WTM) and the T-maze tests were performed on independent cohorts of male mice (PD 90) from 02:00 PM to 05:00 PM.

Three chamber test and marble burying test were performed on the same cohorts of male mice (PD 60) from 10:00 AM to 1:00 PM.

For the three chamber test, on test day, mice were transported to a dimly illuminated (2 x 40 W, indirect) testing room, adjacent to the animal housing area, and left undisturbed for at least 1 h before testing. At the end of each trial, the apparatus was accurately cleaned up with ethanol 2% and water.

Two weeks after the three chamber test, mice were tested for the marble burying test and the following day they were single-housed, habituated to a reverse light/dark cycle (01:00 PM - 01:00 AM) for 1 week and then live scored from 10:00 AM to 12:00 AM for biting and twirling stereotypic behaviours by a single investigator blind to genotype.

Data from three chamber test and MWM were recorded automatically and analysed by a single observer blind to genotype using a video tracking software (Ethovision XT9 video track system; Noldus Information Technology, Wageningen, The Netherlands).

2.2.1 Morris Water Maze. The acquisition phase and the probe trial (day 1-5) were performed as previously described (Longo et al., 2014). Briefly, an escape platform was placed in the centre of one quadrant (target zone) of a circular pool and it was hidden 1 cm beneath the water surface. The water (24 ± 1°C) was made opaque with powdered milk. The acquisition phase consisted of four trials per day with a 20 minute inter-trial interval for four consecutive days (day 1–4). Four points equally spaced along the circumference of the pool (North, South, East, West) served as the starting position, which was randomized across the four trials each day. Mice were given 90 seconds to find the hidden platform. If an animal did not reach the platform within 90 seconds, it was gently guided to the platform, where it had to remain for 30 seconds. The escape latencies were recorded.

On day 5 (probe trial) the platform was removed and animals were allowed to swim freely for 90 seconds in the maze. Percentage of distance travelled in the target zone was calculated.

On day 6 and 7 a reversal training task was carried out. This involved changing the location of the escape platform in the opposite quadrant of the pool compared to its position on the acquisition phase. Mice received four trials per day with a 20 minute inter-trial interval and were given 60 seconds to find the hidden platform. The escape latencies were recorded.

2.2.2 Water T-Maze. The WTM consisted of a white Plexiglas T-maze with a start arm (34×13 cm) and two goal arms (31×13 cm) with walls (height 25 cm). The apparatus was kept in a room without visual cues and data were collected manually by a single observer blind to genotype. The maze was filled with water to a depth of about 16 cm, made opaque with powdered milk and

maintained at 24 ± 1°C. On day 1, mice were placed in the WTM for 90 seconds without the platform to acclimatise them to the task environment. In the training phase (day 2) an escape platform (12×15 cm) was placed at the end of one goal arm and submerged 1 cm below the surface of the water. Each mouse was placed in the starting arm and allowed up to 15 seconds to find the hidden platform. On each trial, a correct choice was recorded when the mouse entered the arm and climbed onto the platform directly. When a mouse failed to find the platform, it was picked up and placed onto it for 10 seconds. Each mouse underwent 20 trials divided into 4 blocks. In the test phase (day 3), mice were tested to see if they achieve an escape success criterion of 4/5 correct choices. Only mice that achieved this criterion underwent the reversal task. In the reversal learning session, the platform was switched to the opposite arm of the T maze. This time, each mouse received 30 trials, divided into 6 blocks and they were allowed 15 seconds (maximum) of time to make an arm choice. Entry into the arm where the platform was originally located was recorded as an error. Performance in reversal phase was indexed by percentage of correct trials, total number of errors made during the reversal learning session and total number of trials to complete the task. The total number of trials was calculated as the number of trials required to achieve criterion performance or, for mice that did not achieve criterion, total number of trials over the 6 blocks of 5 trials (30 trials).

2.2.3 T-Maze. The T-maze was made of transparent Plexiglas, comprising of three arms (6×35 cm), namely, a start arm and two choice arms, surrounded by transparent Plexiglas walls (11.5 cm high). The food reward (0.1 ml of powdered milk) was positioned 3 cm from the choice arm's distal end. The mice were housed individually and were maintained on a food deprivation diet, with one pellet per day until they lost 20% of their initial weight. Animal body weight was monitored daily and the diet was maintained for the entire duration of the test. First, the animals were habituated to the T-maze over two consecutive days, by being individually placed in it, and allowed to explore freely for 5 minutes. On day 1 of the testing phase, a reward was placed in each of the three arms, while, on day 2, the rewards were placed only in the choice arms. In the forced choice phase on day 3, the

mice were forced either left or right by the presence of a block, according to a pseudorandom sequence (with no more than two consecutive turns in the same direction). The reward was available in the food well at the end of the arm. After a delay of 5-10 seconds, rewards were placed in each of the choice arm and the block was removed. The mouse was placed, facing the observer, at the end of the start arm and was allowed one minute to make a free choice. The animal was rewarded only for choosing the previously unvisited arm. The completion of the test was when the mouse ate the food. Each mouse received 25 trials in total over 5 days of testing (5 trials/day).

2.2.4 Three Chamber Test. A Plexiglas box (60×42×22 cm) was divided into three equal regions (20×42×22 cm). Each partition had a square opening in the bottom centre. In the first 10-minute session all the doorways were closed and the test mouse was allowed to habituate in the central chamber. In the second 10-minute session the doorways were removed to let the mouse acclimatise to the whole box. To test for sociability, the test mouse was placed in the centre of the three-chamber unit, where two inverted empty black wire cups (www.spectrumdiversified.com/products/galaxy-pencil-utility-cup) were located in the left and right chambers. A FVB/J male mouse (Mouse and Familiar mouse) that had never been exposed to the test mouse, was placed in one of the two wire cups, while the wire cup on the other side remained empty (Object). For the testing of the social memory, a second FVB/J stranger mouse (Novel mouse) that had never been exposed to the test mouse, was placed in the wire cup, which had previously served as an empty cup. For both sociability and social memory tests, the time spent and distance travelled in each chamber were automatically recorded for 10 minutes.

2.2.5 Marble burying test. Mice were housed individually in clean cages (36×20×14 cm) with 4.5 cm sawdust bedding and 20 glass marbles (15 mm diameter) arranged in five evenly spaced rows of four marbles. During the test, mice had access to food and water. The mouse was removed from the cage after 30 minutes and the number of buried marbles was counted (a marble was scored as buried if more than two-thirds of it was covered with sawdust).

2.2.6 Stereotypic behaviours. Observations were taken for 2 hours, 1 minute every 20 minutes, across 5 days for a total of 30 minutes of home cage observation and were conducted during the dark cycle, two hours before light on, using red light illumination. Male mice were single-housed in the test room for all the days of the observations. Behaviour was scored as "stereotypic" if it lasted at least 3 consecutive seconds during the 1 minute window of observation.

2.3 Histological Examination

2.3.1 Immunohistochemistry. Mice were given an anesthetic overdose of Zoletil (25 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). The Y1R immunohistochemistry reaction was performed as previously described (Bertocchi et al., 2011). Primary Y1R antibody was kindly provided by Janice Urban (Wolak et al., 2003) and was used at 1:2000 dilution (Bertocchi et al., 2011; Longo et al., 2014; Longo et al., 2015). For the immunostaining of c-Fos, mice were perfused two weeks (for baseline expression) or 2 hours after the end of the 1st day of the reversal task of the MWM (Brigman et al., 2013).

Immunostaining was carried out on free floating coronal brain sections (25 µm thick). After endogenous peroxidase blocking by 0.5% H_2O_2 in 0.1 M PBS for 15 minutes, sections were blocked with 10% normal goat serum (NGS) in PT buffer (PBS containing 0.02% Triton X-100 and 1% bovine serum albumin) for 1 h and incubated overnight at 4°C with primary antibody rabbit anti-c-Fos (Santa Cruz Biotechnology) at 1:10000 dilution with 1% NGS in PT buffer. After washing with PBS 0.1 M, sections were incubated for 1 h with a secondary biotinylated goat anti rabbit antibody (1:500; Vector Laboratories) followed by the ABC Vectastain kit (Vector Laboratories). Peroxidase was reacted with 0.04% DAB and 0.003% hydrogen peroxide, mounted on slides and air-dried overnight. The slides with DAB-developed sections were then coverslipped with DPX mounting medium and analysed with a Leica microscope.

For 5-HT immunostaining, sections were incubated over-night at room temperature with a primary antibody rabbit anti-5-HT (1:6000; Peninsula Laboratories) and subsequently with a secondary biotinylated goat anti rabbit antibody (1:300; Vector Laboratories).

For each animal, six to eight sections of OFC (from 2.68 to 2.10 mm relative to Bregma) and of dorsomedial striatum (from 0.98 to 0.38 mm relative to Bregma) were analysed. In the area of interest (OFC, from 0.09 to 0.96 mm^2; dorsomedial striatum, 0.29 mm^2) circular particles larger than 20 μm in diameter were manually counted using ImageJ software (NIH); the counts were expressed as the number of c-Fos-positive cells per mm^2. Quantification of 5-HT immunopositive fibres was performed on five to eight sections of OFC as previously described (Longo et al., 2014).

2.3.2 Immunofluorescence. The immunofluorescence analysis was performed using free floating coronal brain sections (25 μm thick) from Npy1r$^{Y5R-/-}$ mice transcardially perfused with 4% paraformaldehyde. For the double immunofluorescence detection of α-calcium/calmodulin-dependent protein kinase II (α-CamKII) and Cre recombinase (Cre) or Parvalbumin (PaV) and Cre, sections were incubated with 10% NGS for 1 h followed by an overnight incubation at 4°C with primary mouse anti α-CamKII (1:200; Chemicon) or anti-PaV (1:1500; Swant Inc.) antibodies in 0.1M PBS, 0.2% Triton. After washing, sections were incubated for 1 h with secondary biotinylated anti-mouse antibody (1:300; Vector Laboratories), followed by 1 h incubation with Streptavidin Texas Red (1:500; Vector Laboratories). Sections were then incubated overnight at 4°C with rabbit anti Cre antibody (1:3000; Novagene) in 0.1M PBS, 0.2% Triton, followed by 1 h incubation with secondary Alexa Fluor 647 goat anti-rabbit (1:400; Life Technologies, Monza MB, Italy). The sections were washed in PBS and then mounted with Mowiol. Three sections per animal were analysed using a confocal Leica SPX-microscope.

For the double immunofluorescence detection of c-Fos and α-CamKII, sections were blocked with 10% NGS in PT buffer for 1 h and incubated overnight at 4°C with primary mouse anti α-CamKII (1:200; Chemicon) and rabbit anti-c-Fos (1:5000; Santa Cruz Biotechnology) antibodies in PT buffer 1% NGS. After washing, the sections were incubated for 1 hour with Alexa Fluor 647 goat anti-mouse (1:400; Life Technologies, Monza MB, Italy) and Cy3 anti rabbit (1:800; Jackson ImmunoResearch). For each animal, eight to twelve OFC sections (from 2.68 to 2.10 mm relative to Bregma) were analysed using a confocal Leica SPX-microscope.

2.4 Non-radioactive *in situ* hybridization for *Npy1r* mRNA

2.4.1 Synthesis of probes for non-radioactive *in situ* hybridization

RNA from mouse brain was isolated by a standard acid guanidinium thiocyanate phenol/chloroform extraction. To synthesize digoxigenin (dig)-labelled RNA probes for *Npy1r*, first-strand cDNA was synthesized from 2 micrograms of total RNA by using a cDNA synthesis kit from Invitrogen. Then, the target cDNA was amplified by PCR, with primers designed on *Npy1r* mouse cDNA sequence: Y1R-FW: TTCTCCCTCCAGTGACACTC; Y1R-RV: GGAGACACATGACCGCAAC. The PCR product (489 bp) was amplified using a 5′ primer containing a T7 phage promoter sequence and a 3′ primer containing a SP6 phage promoter sequence, generating a template for transcription of a sense and an antisense probe, respectively. The in vitro transcription reaction was performed by using dig-UTP RNA labelling mix (Roche, Mannheim, Germany) and SP6 or T7 RNA polymerase (Roche), following the manufacturer's instructions.

2.4.2 *In situ* hybridization

In situ hybridization was performed on coronal sections of adult mouse hindbrain as previously described (Carulli et al., 2006). Mice were deeply sedated by 3% isoflurane inhalation before cervical dislocation. Brains were removed, rapidly frozen on dry ice and stored at -80°C. Fourteen µm thick sections were cut on a cryostat, collected on Superfrost slides, and air dried at room temperature for 20–30 minutes. Then, sections were fixed in 4% paraformaldehyde for 10 minutes, permeabilized for 10 minutes in PBS with 0.5% Triton X-100, and acetylated by 10 minutes of incubation in a solution made of 250 ml water with 3.5 ml triethanolamine and 625 µl acetic anhydride added dropwise. Prehybridization was performed in hybridization buffer made of 50% formamide, 5x SSC, and 2% blocking reagent (Roche) for 3 hours at room temperature. Hybridization with dig-labelled probes (400 ng/ml) was performed in the same buffer overnight at 64°C. Stringency washing was performed in 0.2xSSC for 1 hour at 64°C. For the detection of dig-labelled hybrids, the slides were equilibrated in maleic acid buffer (0.1 M maleic acid and 0.15 M

NaCl, pH 7.5) and incubated for 1 hour at room temperature with 1% blocking reagent made in maleic acid buffer (blocking buffer) and then over night with alkaline phosphatase conjugated anti-dig antibodies (Roche) diluted 1:5000 in blocking buffer. The slides were washed twice for 30 minutes each in maleic acid buffer and incubated overnight in colour development buffer [2.4 mg levamisole (Sigma), 200 µl NBT/BCIP mix (Sigma72091) corresponding to 3.30 mg/10mi of 4-nitroblue tetrazolium and 1.75 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 10 ml of a buffer made of 0.1 M Trizma base, 0.1 M NaCl, and 0.005 M MgCl2, pH 9.5]. The colour development solution was changed twice and stopped after approximately 16 hours in neutralizing buffer (0.01 M Trizma base and 0.001 M EDTA, pH 8), and the sections were mounted in Moviol, coverslipped and analysed under a Zeiss light microscope equipped with a Leica DFC 320 digital camera.

For each animal, six to ten sections spanning from -4.36 and -4.72 mm relative to mouse Bregma for the dorsal raphe nucleus and from -4.96 and -5.20 mm relative to Bregma for the dorsal raphe caudal and intermediate part were used to measure *Npy1r* mRNA relative levels.

The analysis was performed with ImageJ software (NIH).The area of interest of clearly distinguishable nuclei was defined following the boundaries of the labeled regions [(DRC/DRI) dorsal raphe caudal part and inferior part, [DR(DRD-DRV) dorsal raphe, dorsal and ventral part]. Optical densities were measured and averaged after a rodboard calibration. Background was measured by averaging three to five spots [optical density (OD) unit] in the surrounding blank region of the nucleus to be evaluated, then subtracted from the correspondent nucleus value.

2.5 Treatment

An independent cohort of mice was injected i.p. with either escitalopram (Sigma, 1 mg/kg of body weight in a volume of 10 ml/kg saline) (Brown et al., 2012) or saline 30 minutes before the MWM reversal task on day 1 and perfused 2 hours after the end of the task.

2.6 Data Analysis

Quantitative results were analysed by two-way ANOVA for repeated measures and two-way ANOVA followed by Newman-Keuls post-hoc test for multiple comparisons as indicated. Planned

comparisons of mutant and control mice at the moment of platform change in MWM were evaluated by t tests. Because the c-Fos data contained only a single treatment dose, planned comparisons of mutant and control mice in the saline and escitalopram-treated group and same-genotype comparisons across the two treatment groups were analysed by t tests. All data are expressed as mean ± SEM, and the level of statistical significance was set at $p<0.05$ (* for post hoc comparison; # for main effect).

3. Results

3.1 Characterization of the behavioural phenotype of Npy1r$^{Y5R-/-}$ mice

3.1.1 Npy1r$^{Y5R-/-}$ mice displayed decreased behavioural flexibility in reversal tasks.

In the MWM, Npy1r$^{Y5R-/-}$ mice showed similar decline of latency to find the hidden platform over 4 days of training (Fig. 1A) but a significant stronger preference for the target zone (day 5) compared to the controls, as previously demonstrated (Longo et al., 2014) (Fig.1B).

To investigate whether the higher persistence of Npy1r$^{Y5R-/-}$ mice for the target zone during the probe trial of MWM might be related to lower behavioural flexibility, control mice and conditional mutants underwent the MWM reversal task. The reversal MWM was performed by changing the platform location on day 6 (SW, new target zone). Npy1r$^{Y5R-/-}$ mice showed an increased latency in finding the newly located platform, compared to control mice, indicating lack of the flexibility required to choose the right solution. In particular, Npy1r$^{Y5R-/-}$ mice took more time to reach the platform on the 1st day of the reversal task, whereas no difference was observed between Npy1r$^{Y5R-/-}$ and Npy1r^{2lox} mice on the 2nd day (Fig.1C).

In the WTM reversal task, Npy1r$^{Y5R-/-}$ mice displayed similar acquisition learning (% of correct arm choices) of a submerged escape-platform location compared to control littermates and, twenty-four hours later (test phase), both groups reached the criterion to proceed to reversal phase (Fig. 2A). However, when the escape platform location was switched to the opposing arm, mutant animals demonstrated significantly impaired learning of the new platform location. The criterion (4/5) was

achieved by 75% of Npy1r^{2lox} mice. In contrast, 52% of Npy1r$^{Y5R-/-}$ mice persistently swam toward the previously trained arm choice, exhibiting behavioural inflexibility (Fig. 2B). Npy1r$^{Y5R-/-}$ mice made a significantly greater number of errors during the reversal session (Fig. 2C) and required an additional 8 trials to complete the task (Fig. 2D).

3.1.2 Npy1r$^{Y5R-/-}$ mice displayed normal working memory, sociability and social memory and repetitive behaviours.

A T-maze forced alternation task was used to analyse Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice working memory. Both genotypes displayed a similar performance during the 5 days of training (25 trials) since no significant differences were observed in the % of correct arm choice (Npy1r^{2lox} mice = 71.20 ± 15.34; Npy1r$^{Y5R-/-}$ mice = 75 ± 12.12; n =5).

A three-chamber social interaction test was performed to assess sociability and social memory of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. Both control mice and conditional mutants demonstrated similar sociability in terms of time spent and distance travelled in the mouse chamber compared to the object chamber (Fig. 3A) and a similar social memory in terms of time spent and distance travelled in the novel mouse chamber compared to the familiar mouse chamber (Fig. 3B).

No significant differences between Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice were observed in the marble burying test or in the time spent in twirling or biting repetitive behaviours (Supplemental Table 1).

3.2 Npy1r$^{Y5R-/-}$ mice displayed a decrease of serotonin immunoreactivity in the orbitofrontal cortex and of *Npy1r* mRNA expression in the dorsal raphe.

To examine the effect of conditional inactivation of Y1R in Y5R containing neurons on OFC serotoninergic system, we analysed 5-HT immunoreactive staining in brain coronal sections of Npy1r$^{Y5R-/-}$ and Npy1r^{2lox} mice. As shown in Fig.4A, the 5-HT immunoreactivity was significantly decreased in the OFC of Npy1r$^{Y5R-/-}$ mice compared to their control littermates. In addition, *in situ* hybridization of brain coronal sections revealed a significant decrease of *Npy1r* mRNA in the dorsal raphe of Npy1r$^{Y5R-/-}$ mice compared to Npy1r^{2lox} mice (Fig.4B).

3.3 Npy1r$^{Y5R-/-}$ mice displayed increased neuronal activity in the orbitofrontal cortex.

We evaluated the difference in baseline activity of OFC between Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice by assessing expression of c-Fos, an established marker for neural activity (Kovacs, 2008; Marrone et al., 2008; Nonkes et al., 2010). We found that c-Fos was upregulated in the OFC of Npy1r$^{Y5R-/-}$ mice compared to their control littermates (Fig. 5A).

To analyse c-Fos or Y1R and Y5R expression in GABAergic interneurons and in principal cells of the OFC, we next examined co-localization of parvalbumin-immunoreactivity (IR) (GABA) and CamKII-IR (glutamate) with c-Fos-IR or with Venus fluorescence (*Npy1r* gene promoter) and Cre recombinase-IR (*Npy5r* promoter) in coronal brain sections of Npy1r$^{Y5R-/-}$ mice, respectively.

Y1R positive neurons were observed in layers II and III, but not in layer V of OFC. Analysis of sections from different animals (8-12 per mouse) suggested that ≈60% of c-Fos positive neurons (Fig. 5B) and ≈80% of Y1R/Y5R positive neurons (coexpressing Venus fluorescence and Cre recombinase) (Fig. 5C upper panels) colocalize with pyramidal neurons (=CamKII-positive cells) in layers II and III of OFC. However, parvalbumin positive cells did not coexpress Venus or Cre immunoreactivities (Fig. 5C lower panels).

Immunohistochemical staining of Y1R positive neurons in brain coronal sections revealed a significant decrease of the receptor protein in layers II and III of the OFC of Npy1r$^{Y5R-/-}$ mice compared to their control littermates (Supplemental Fig. 1).

3.3 Escitalopram treatment rescued the behavioural flexibility and decreased cFos-IR in the OFC of Npy1r$^{Y5R-/-}$ mice.

To test the effect of SSRI on behavioural inflexibility and on OFC activation, Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice were treated with saline or escitalopram (1mg/kg, i.p.) 30 minutes before the MWM reversal task (Brown et al., 2012) and c-Fos immunocytochemical staining was measured two hours after the end of the 1st day of the reversal task when the choice behaviour activation of OFC has been reported to return to basal levels (Brigman et al., 2013).

As shown in Fig.6, saline treated Npy1r$^{Y5R-/-}$ mice display behavioural inflexibility that is associated with an increase of OFC basal neuronal activity compared to Npy1r^{2lox} mice. Escitalopram

treatment enhanced reversal learning performance (Fig. 6A) and significantly decreased the density of c-Fos–positive neurons in OFC of Npy1r$^{Y5R-/-}$ mice (Fig. 6B) compared to saline treated Npy1r$^{Y5R-/-}$ mice.

Escitalopram treatment did not affect either MWM reversal task performance or density of c-Fos expressing neurons in the OFC of Npy1r^{2lox} mice.

Moreover, no difference in c-Fos-IR was observed in the dorsomedial striatum of saline or escitalopram treated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice (Supplemental Fig. 2).

4. Discussion

The Y1 and Y5 receptors for NPY have overlapping functions in regulating anxiety (Bertocchi et al., 2011; Eva et al., 2006; Sørensen et al., 2004). By using a target-reversal design, in the present study we investigated whether the conditional ablation of Y1R in Y5R expressing neurons decreases behavioural flexibility of Npy1r$^{Y5R-/-}$ mice, which would be consistent with their anxiety profile, compared to their control littermates (Bondi et al., 2008; Longo et al., 2014; Salomons et al., 2012). Our data demonstrate that Npy1r$^{Y5R-/-}$ mice display reversal learning impairment in the early phase of both MWM and WTM reversal tasks, as shown by the longer latency (MWM) and the increased number of trials (WTM) required to learn the new platform location, compared to their control littermates. Furthermore, Npy1r$^{Y5R-/-}$ male mice display increased baseline c-Fos-IR in the layers II and III of the OFC, where ≈80% of Y1R/Y5R positive neurons colocalize with pyramidal neurons.

Reversal learning is a form of cognitive flexibility that enables behavioural adaptation to changing internal states and external environmental circumstances (Izquierdo et al., 2016). Behavioural flexibility strongly depends on the functional integrity of the OFC (Hamilton and Brigman, 2015; Ragozzino, 2007; Rudebeck et al., 2013; Schoenbaum et al., 2009). Strikingly, it appears that both OFC lesions and OFC hyperactivity impair cognitive flexibility. OFC damage in many species causes severe reversal deficits while preserving a normal ability to learn initial contingencies

(Boulougouris et al., 2007; McAlonan and Brown, 2003). On the other hand, an increased activity in OFC is correlated with worse behavioural performance during reversal learning in mice and in rats, suggesting that an optimal change in firing rate is required for OFC function (Bissonette et al., 2015; Brigman et al., 2013; Schoenbaum et al., 2009). In addition, NMDA receptor-mediated synaptic plasticity in the OFC has been reported to play an important role during reversal learning (Brigman et al., 2013; van Wingerden et al., 2012).

Y1Rs are Gi-coupled receptors mainly located post-synaptically that exert potent anti-excitatory actions by decreasing glutamate release in several brain regions, including the neocortex (Stanić et al., 2011; Vollmer et al., 2016). The analysis of Venus, Cre recombinase and α-CamKII coexpression in the OFC of Npy1r$^{Y5R-/-}$ mice suggests that the inactivation of *Npy1r* gene mainly occurs in the pyramidal neurons of layers II-III, that project intra-cortically to regulate striatal pyramidal projecting neurons of layer V. These results suggest that the decrease of the inhibitory NPYergic tone in OFC of Npy1r$^{Y5R-/-}$ mice might increase excitability of glutamatergic neurons and, in turn, drive inflexible behaviour. This hypothesis is in line with previous studies showing that an elevated baseline firing rate of pyramidal neurons in the OFC, resulting from a decrease in the number of GABAergic interneurons, is associated with reversal learning impairment (Bissonette et al., 2015).

On the other hand, evidence from studies in rodents and primates demonstrate that 5-HT is implicated in the modulation of reversal learning in the OFC. Electrophysiological studies have shown that 5-HT inhibits pyramidal cell firing in the OFC (Rueter et al., 2000; Wallace et al., 2014). The depletion of brain 5-HT or chronic intermittent stress, which reduces frontal cortex 5-HT release, impairs the ability of animals to adapt their response to changes in reward contingencies in the environment, resulting in perseverative behaviour (Bondi et al., 2008; Clarke et al., 2007; Man et al., 2012). Furthermore, increasing serotonergic tone with acute or chronic SSRI treatments has been reported to enhance cognitive flexibility and to decrease perseverative behaviours (Bari et al., 2010; Furr et al., 2012).

Previous studies have shown that germinal knockout of the *Npy1r* gene decreases the expression of the 5-HT synthesis-limiting enzyme tryptophan hydroxylase in the dorsal raphe nucleus (Karl et al., 2004). Here we demonstrated that conditional inactivation of Y1R in Y5R positive neurons significantly reduces *Npy1r* mRNA in the dorsal raphe nucleus, where 5-HT neurons projecting to OFC originates (Waselus et al., 2011). Moreover, Npy1r$^{Y5R-/-}$ mice display a significant decrease of 5-HT immunoreactive fibres in the OFC.

The observation that the acute treatment of Npy1r$^{Y5R-/-}$ mice with escitalopram reverses the inflexible phenotype and decreases c-Fos expression in the OFC suggests that the dysregulation of serotonin observed in the OFC of Npy1r$^{Y5R-/-}$ mice might be responsible for the reversal impairment and the increased OFC neuronal activity observed in these conditional mutant mice.

Although we cannot exclude that other brain regions may contribute to reversal learning impairment of Npy1r$^{Y5R-/-}$ mice, our results suggest that, in the corticostriatal circuit, this phenotype is specifically associated to increased neuronal activity in the OFC since no significant changes in c-Fos-IR were observed in the dorsomedial striatum of saline or escitalopram treated mutant mice. Interestingly, previous studies showed that OFC and dorsomedial striatum enable behavioural flexibility by supporting distinct but complementary functions, the former one facilitating the initial shift away from a previous strategy, and the latter one supporting the maintenance of a new strategy once selected (Ragozzino, 2007).

Reversal learning impairment observed in Npy1r$^{Y5R-/-}$ mice is associated with an anxiety-like behaviour (Longo et al. 2014) but it appears to be otherwise highly specific and not associated with changes in spatial learning memory, stereotypic-repetitive and compulsive behaviours, sociability and social memory.

Accordingly, several lines of evidence have shown that anxiety and cognition are closely associated and interacting processes (Grupe and Nitschke, 2013; Likhtik and Paz, 2015). Moreover, cognitive impairments associated with frontal lobe executive function (i.e. cognitive set-shifting, behavioural

flexibility and perseveration) are common in humans suffering from anxiety disorders, and also in OCD (Ferreri et al., 2011; Milad and Rauch, 2007).

OCD is characterized by persistent intrusive thoughts (obsessions), repetitive actions (compulsions), excessive anxiety and impaired executive functions, including cognitive inflexibility (Chamberlain et al., 2006). Multiple layers of evidence link OCD with dysregulation of fronto-striatal neuro-circuitry and associated monoamine systems (Szechtman et al., 2017). Imaging studies investigating the neural mechanism underlying OCD symptoms highlight the role of overactive frontostriatal pathways in mediating obsessions and reversal learning impairment (Lagemann et al., 2012; Simon et al., 2010). SSRIs are actually the pharmacologic first-line treatment of OCD in humans and successful treatments are associated with reductions in OFC hyperactivity (Pauls et al., 2014).

This study has demonstrated that targeted deletion of *Npy1r* gene in Y5R coexpressing neurons leads to behavioural inflexibility, OFC baseline hyperactivity, decrease of serotonin in the OFC and it is sensitive to SSRI, suggesting that neurons containing both Y1Rs and Y5Rs are essential for normal functioning of corticostriatal synapses. Although Npy1r$^{Y5R-/-}$ mice do not recapitulate the wide range of obsessive-compulsive symptoms, we suggest that they may provide a new neurobehavioural mouse model of the cognitive inflexibility endophenotypic trait within OCD with a degree of predictive, as well as construct and face validity (Fineberg et al., 2011).

ACKNOWLEDGMENTS

Grants: PRIN 2008PLKP3E_003 from MIUR; Fondazione CRT, Torino to CE; Progetto di ateneo San Paolo S1315_RIC14_L2_EVC_01 to CE. We thank Dr J. Urban for polyclonal antibody and protocols for Y1R immunohistochemistry.

CONFLICT OF INTEREST

The authors report no biomedical financial interests or potential conflicts of interest.

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FIGURE LEGENDS

Fig.1.

Spatial learning, memory retention and reversal learning in the Morris Water Maze. **(A)** Acquisition phase. No significant difference in escape time was observed between Npy1r$^{Y5R-/-}$ and Npy1r^{2lox} mice each day. Data are mean ± SEM; n = 9-16 from 5 litters. Two-way ANOVA for repeated measures revealed a significant effect of days [F(3,69)=93.75, p = 0.0000]. **(B)** Probe trial. After training, the platform was removed from the north east quadrant (Target) where it was originally placed. The y axis indicates the percentage of distance travelled in one specific quadrant. Data are mean ± SEM; n = 9–16 from 5 litters. Two-way ANOVA revealed a significant effect of quadrants [F(3,92) = 62.06, p = 0.0000] and of interaction between genotype and quadrants [F(3,92)= 8.235, p = 0.00006]. ***p<0.001 versus percentage of total distance travelled by Npy1r^{2lox} mice in the Target, by Newman-Keuls. SE, south east; SW, south west; NW, north west. **(C)** Reversal learning. Twenty-four hours after the probe trial, the platform was moved in the opposite quadrant (SW, new target zone). Npy1r$^{Y5R-/-}$ mice showed increased escape latency in finding the new located platform compared to Npy1r^{2lox} mice in the 1st day of the reversal task. Data are mean ± SEM; n = 9–16 from 5 litters. Two-way ANOVA for repeated measures revealed a significant effect of time [F(1,23)= 10.33, p = 0.0038]. Student t test revealed a significant effect of genotype in the 1st day of the reversal task [t(1,23) = 2.099, p = 0.047]. #p < 0.05 versus Npy1r^{2lox} mice.

Fig. 2. Acquisition phase and reversal learning in water T-maze. **(A)** Training blocks. No significant difference in the percentage of correct arm choice was observed between Npy1r$^{Y5R-/-}$and Npy1r^{2lox} mice. Test block. Mice of both groups reached the criterion (4/5 trials) to proceed to the reversal task. Data are mean ± SEM; n = 19-20 from 12 to 13 litters. Two-way ANOVA for repeated measures revealed a significant effect of training blocks [F(3,111)= 16.426, p= 0.0000]. **(B)** Reversal learning. Twenty-four hours after the training, the escape platform was switched to the opposing arm. Npy1r$^{Y5R-/-}$ mice showed a slower increase of correct arm choice compared to

Npy1r^{2lox} mice. Data are mean ± SEM; n = 19-20 from 12 to 13 litters. Two-way ANOVA for repeated measures revealed a significant effect of blocks [F(5,185)=50.96, p =0.0000] and of genotype [F(1,37)= 4.77, p = 0.035]. (**C**) Total number of errors (n) made during the 1-day reversal learning session. Npy1r$^{Y5R-/-}$ mice made a greater number of errors compared to Npy1r^{2lox} mice. Data are mean ± SEM; n = 19-20 from 12 to 13 litters. Student t test revealed a significant effect of genotype for the total number of errors [t(1,37) = -2.920, p = 0.006]. ## p<0.01 versus Npy1r^{2lox} mice. (**D)** Total number of trials (n) to complete the task [(total number of trials to achieve the criterion (4/5) or to complete the task when the criterion was not achieved (30 trials)]. Npy1r$^{Y5R-/-}$ mice made a greater number of trials compared to Npy1r^{2lox} mice. Data are mean ± SEM; n = 19-20 from 12 to 13 litters. Student t test revealed a significant effect of genotype for the total number of trials [t(1,37) = -3.5, p = 0.00124]. ## p<0.01 versus Npy1r^{2lox} mice.

Fig. 3. Sociability and social memory in the three chamber test. **(A)** Discrimination between Mouse and Object. No significant difference in the % of distance travelled and time spent in the Object and in the Mouse chambers was observed between control and mutant mice. Data are mean ± SEM; n = 8 from 3 to 4 litters. Left panel: Two-way ANOVA revealed a significant difference in the chamber choice [F(1,31)=5.797, p=0.023]. Right panel. Two-way ANOVA revealed a significant difference in the chamber choice [F(1,31)=13.326, p =0.001]. **(B)** Discrimination between Familiar and Novel mouse. No significant difference in the % of distance travelled and of time spent in the Familiar and the Novel mouse chambers was observed between control and mutant mice. Data are mean ± SEM; n = 8 from 3 to 4 litters. Left panel: Two-way ANOVA revealed a significant difference in the chamber choice [F(1,31)=15.87, p<0.001]. Right panel: Two-way ANOVA revealed a significant difference in the chamber choice [F(1,31)=11.2, p=0.002].

Fig. 4. Serotonin immunoreactivity in the orbitofrontal cortex and *Npy1r* mRNA expression in the dorsal raphe of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. (**A**) Left panel: Student t test revealed a significant decrease of the density of 5-HT immunoreactive fibres in the OFC of Npy1r$^{Y5R-/-}$ mice compared to

their control littermates. Data are expressed as the mean fractional area ± SEM, n = 4–5 from two litters. [t(1,7)=3.119, p=0.017]. # p<0.05 versus Npy1r^{2lox} mice. Right panel**:** representative pictures of brain coronal sections showing 5-HT immunoreactivity (IR) in the OFC of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice (scale bar: 150 µm). (**B)** Expression of *Npy1r* messenger RNA (mRNA) in the dorsal raphe of Npy1r^{2lox} control and Npy1r$^{Y5R-/-}$ conditional mutants. Left panel: data are expressed as relative optical density (OD) and are the mean ± SEM; n=5 from two litters. Student t test revealed a significant decrease of *Npy1r* mRNA expression in the dorsal raphe, dorsal and ventral part [DR (DRD-DRV)] and in dorsal raphe, caudal part and inferior part (DRC/DRI) of Npy1r$^{Y5R-/-}$ mice compared with control Npy1r^{2lox} littermates. [t (1,8)=2.996, p=0.017] and [t(1,8)=3.306, p=0.011], respectively. # p<0.05 versus Npy1r^{2lox} mice. Right panel: representative picture of non-radioactive *in situ* hybridization of *Npy1r* mRNA on brain coronal sections from Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ male mice (Scale bars: 100 µm).

Fig. 5. Expression pattern of Y1R, Y5R, α-calcium/calmodulin-dependent protein kinase, parvalbumin and c-Fos in the OFC. **(A)** Left panel: baseline c-Fos-IR in Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. Data are mean ±SEM; n = 4-5 from 2 litters. Student t test revealed a significant increase in the density of c-Fos-labelled neurons in the OFC of Npy1r$^{Y5R-/-}$ mice compared to Npy1r^{2lox} mice [t(1,7)=2.789, p=0.027]. Right panel: representative pictures of brain coronal sections showing baseline c-Fos immunoreactive neurons in the OFC of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice (Scale bar = 100 µm). **(B)** Confocal images showed the distribution of Venus fluorescence, α-calcium/calmodulin-dependent protein kinase (α-CamKII) and c-Fos immunostaining in the OFC of Npy1r$^{Y5R-/-}$ mice. Merged images showed the degree of colocalization between Venus, α-CamKII and c-Fos (Scale bar = 100 µm). **(C)** Confocal images of Venus fluorescence, Cre recombinase and α-CamKII or parvalbumin (PaV) immunostaining in the OFC of Npy1r$^{Y5R-/-}$ mice (Scale bar = 100 µm). Upper panels: colocalization of Venus, Cre and α-CamKII is indicated by arrows in the

enlarged merged image (Scale bar = 25 μm). Lower panels: no colocalization was observed between Venus, Cre and PaV.

Fig. 6. Effect of escitalopram treatment on behavioural inflexibility and OFC neuronal activation of Npy1r$^{Y5R-/-}$ mice. **(A)** MWM reversal task performance of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice 30 minutes after acute treatment with saline (sal) or 1 mg/kg ip escitalopram (esc). Left panel: escitalopram treated Npy1r$^{Y5R-/-}$ mice showed a reduced escape latency (s) across the 4 trials on the first day of the reversal task compared to saline treated Npy1r$^{Y5R-/-}$ mice. Data are mean ± SEM; n = 8-12 from 5-8 litters. Two-way ANOVA for repeated measures revealed a significant effect of genotype [$F(1,37)=7.054$, $p=0.012$], of time [$F(1,111)=9.341$, $p=0.000$] and of genotype and treatment interaction [$F(1,37)=6.364$, $p=0.016$]. Right panel: mean escape latency (s) of escitalopram or saline treated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice in the first day of MWM reversal task. Data are mean ± SEM; n = 8-12 from 5-8 litters. **$p<0.01$ and *$p<0.05$ versus saline treated Npy1r$^{Y5R-/-}$ mice, by Newman-Keuls. No significant differences were observed between saline and escitalopram treated Npy1r^{2lox} mice ($p=0.144$), saline treated Npy1r$^{Y5R-/-}$ mice and escitalopram treated Npy1r^{2lox} mice ($p=0.090$), escitalopram treated Npy1r$^{Y5R-/-}$ mice and saline treated Npy1r^{2lox} mice ($p=0.263$) and escitalopram treated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice ($p=0.925$). **(B)** Density of c-Fos immunoreactive neurons in the OFC of saline or escitalopram treated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice 2 hours after the end of the first day of the MWM reversal task. Left panel: escitalopram treatment reverted the increase of c-Fos positive neurons in the OFC of Npy1r$^{Y5R-/-}$ mice. Data are mean ± SEM; n = 5-6 from 3 to 5 litters. Two-way ANOVA revealed a significant effect of genotype [$F(1,21)= 6.056$, $p= 0.024$] and of treatment [$F(1,21)= 6.874$, $p= 0.017$]. #$p<0.05$ versus saline treated Npy1r^{2lox} mice [$t(1,9)=-2.306$] and versus escitalopram treated Npy1r$^{Y5R-/-}$ mice [$t(1,8)=2.554$] and ## $p<0.01$ versus escitalopram treated Npy1r^{2lox} mice, by Student t test. No significant differences were observed between saline and escitalopram treated Npy1r^{2lox} mice [$t(1,10)=1.113$; $p=0.292$], escitalopram treated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice [$t(1,9)=-1.059$; $p= 0.317$] and escitalopram treated Npy1r$^{Y5R-/-}$

mice and saline treated Npy1r^{2lox} mice [(t(1,9)=0.141, p=0.891)]. Right panel: representative pictures of brain coronal sections showing c-Fos immunoreactive neurons in the OFC of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice after acute treatment with saline or escitalopram (Scale bar = 100 µm).

Figure 1

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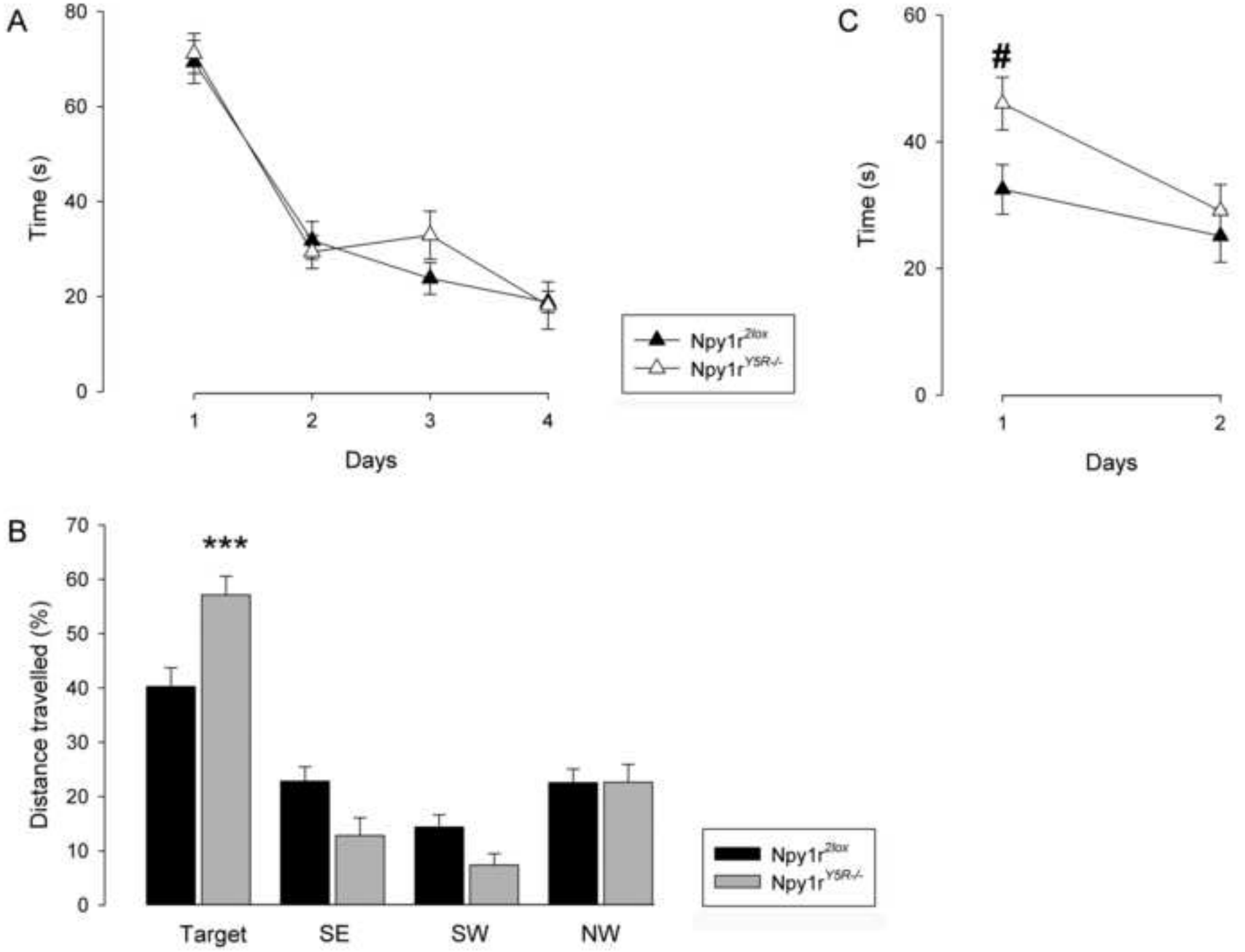

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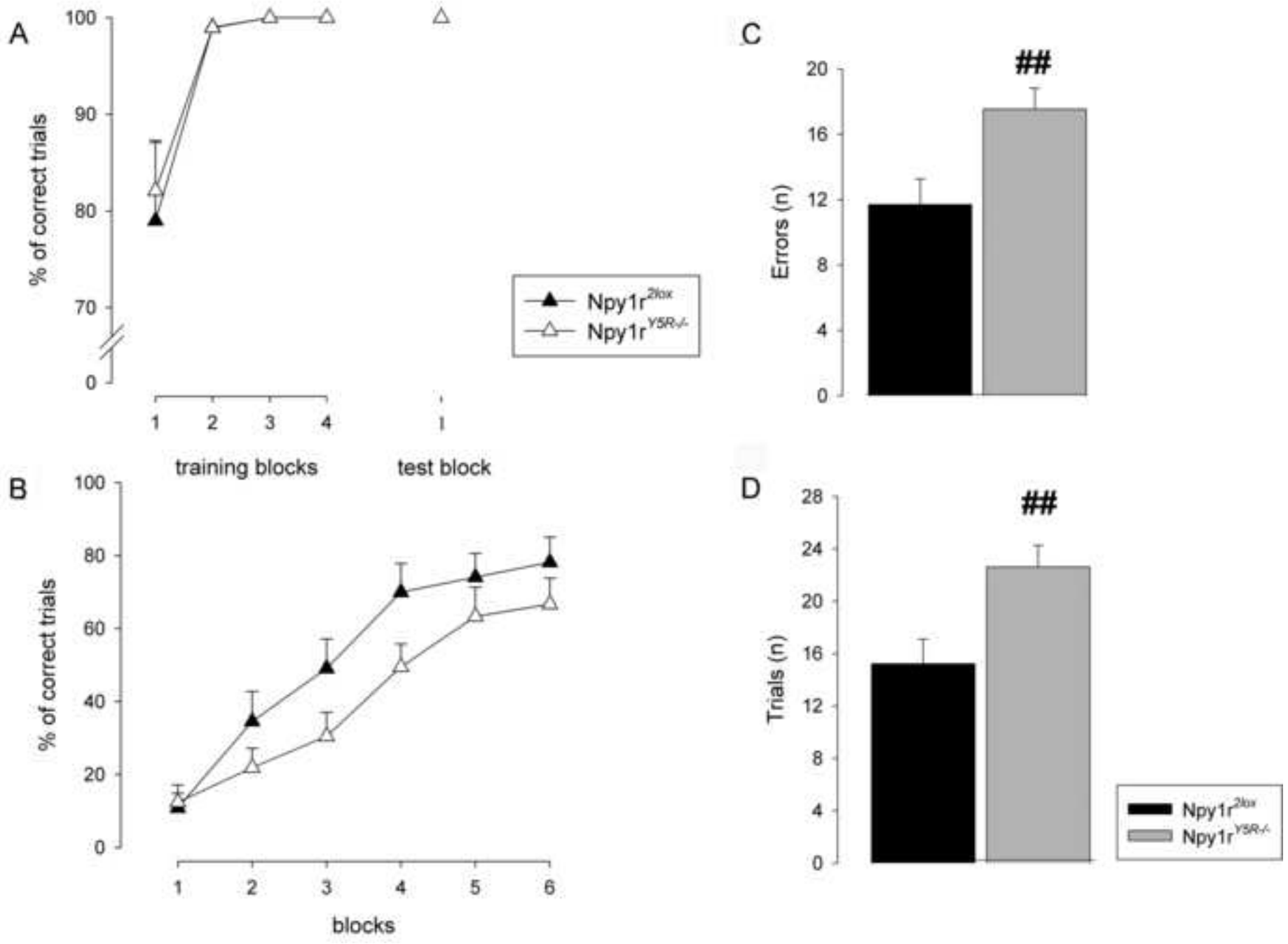

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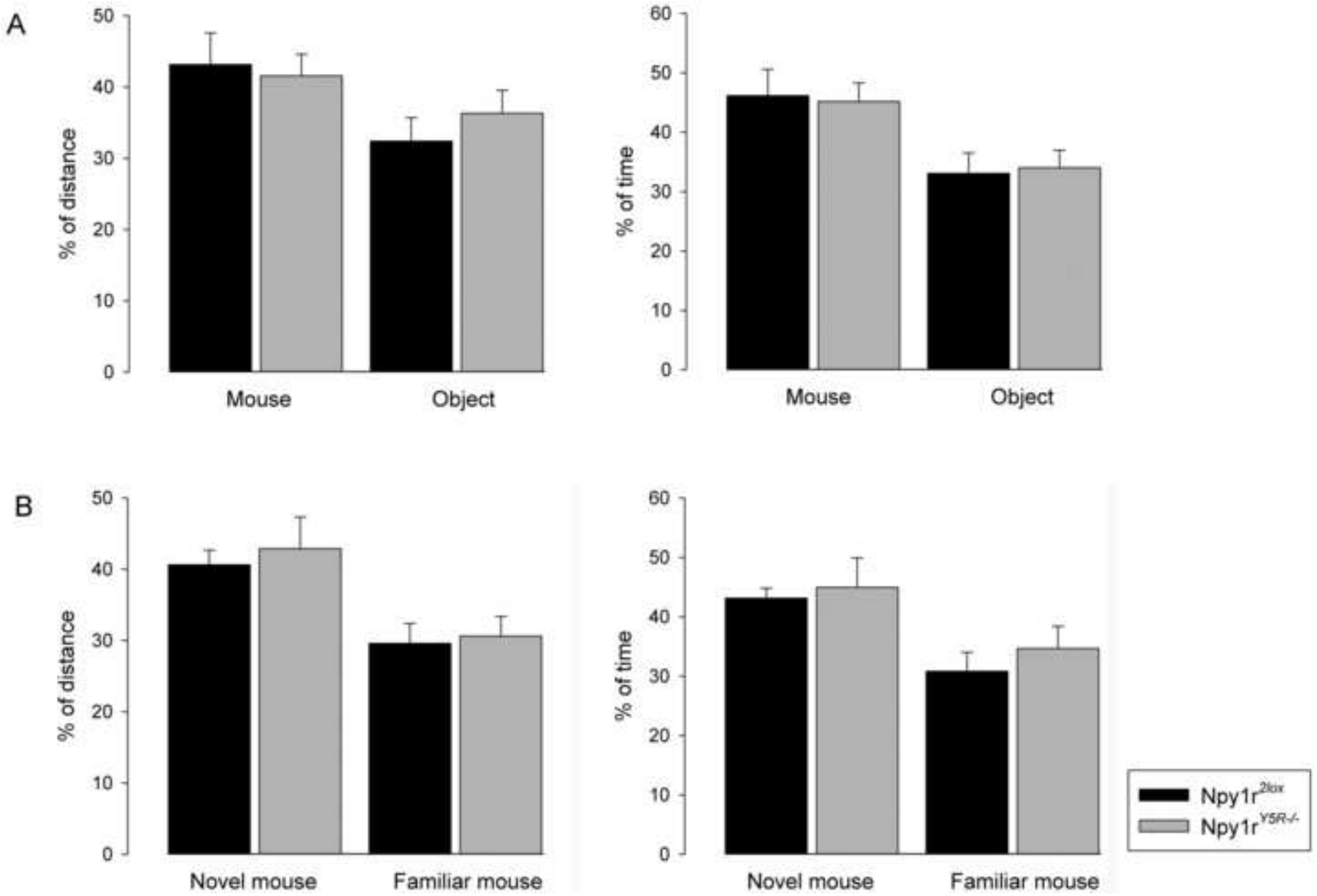

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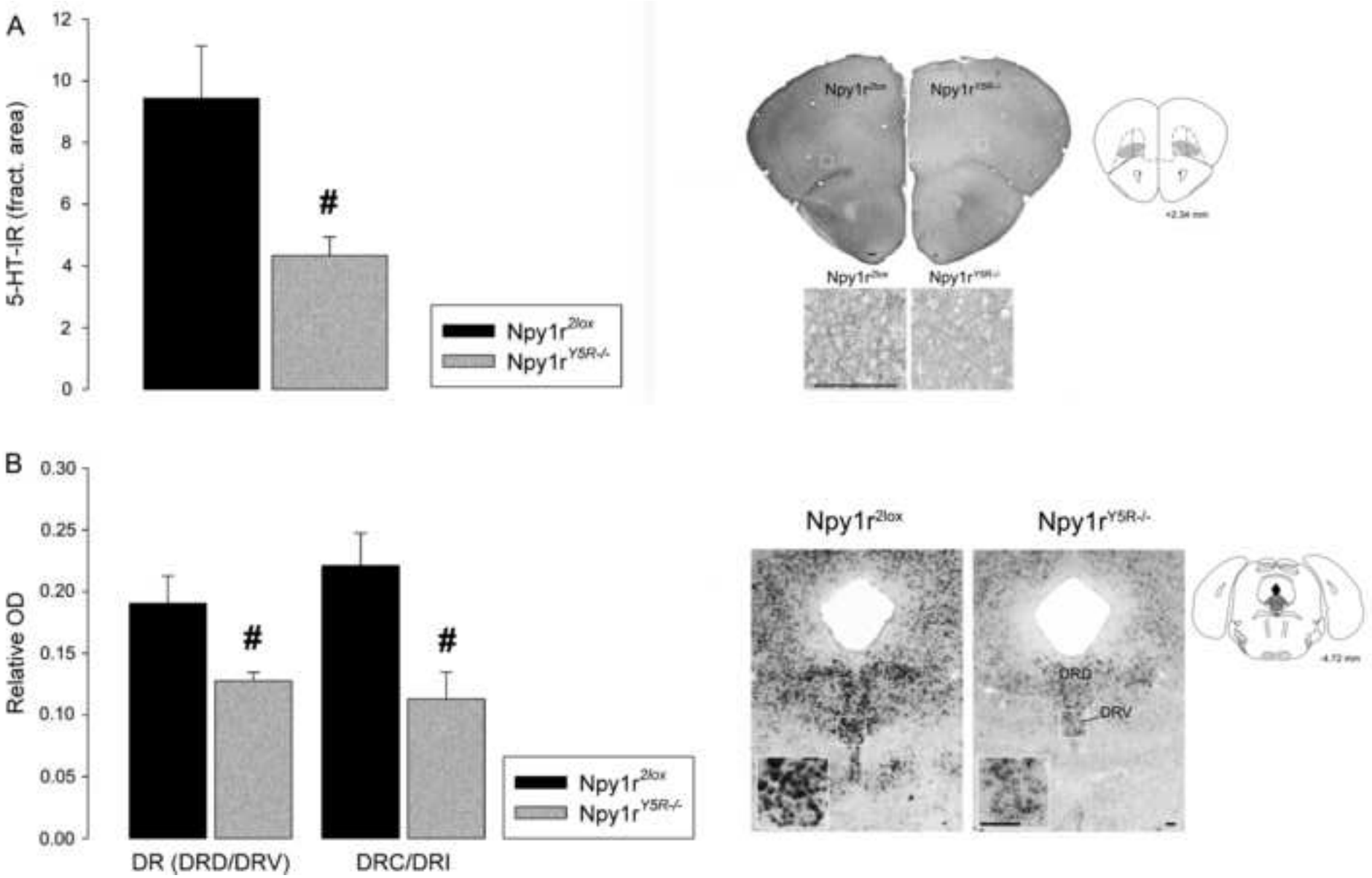

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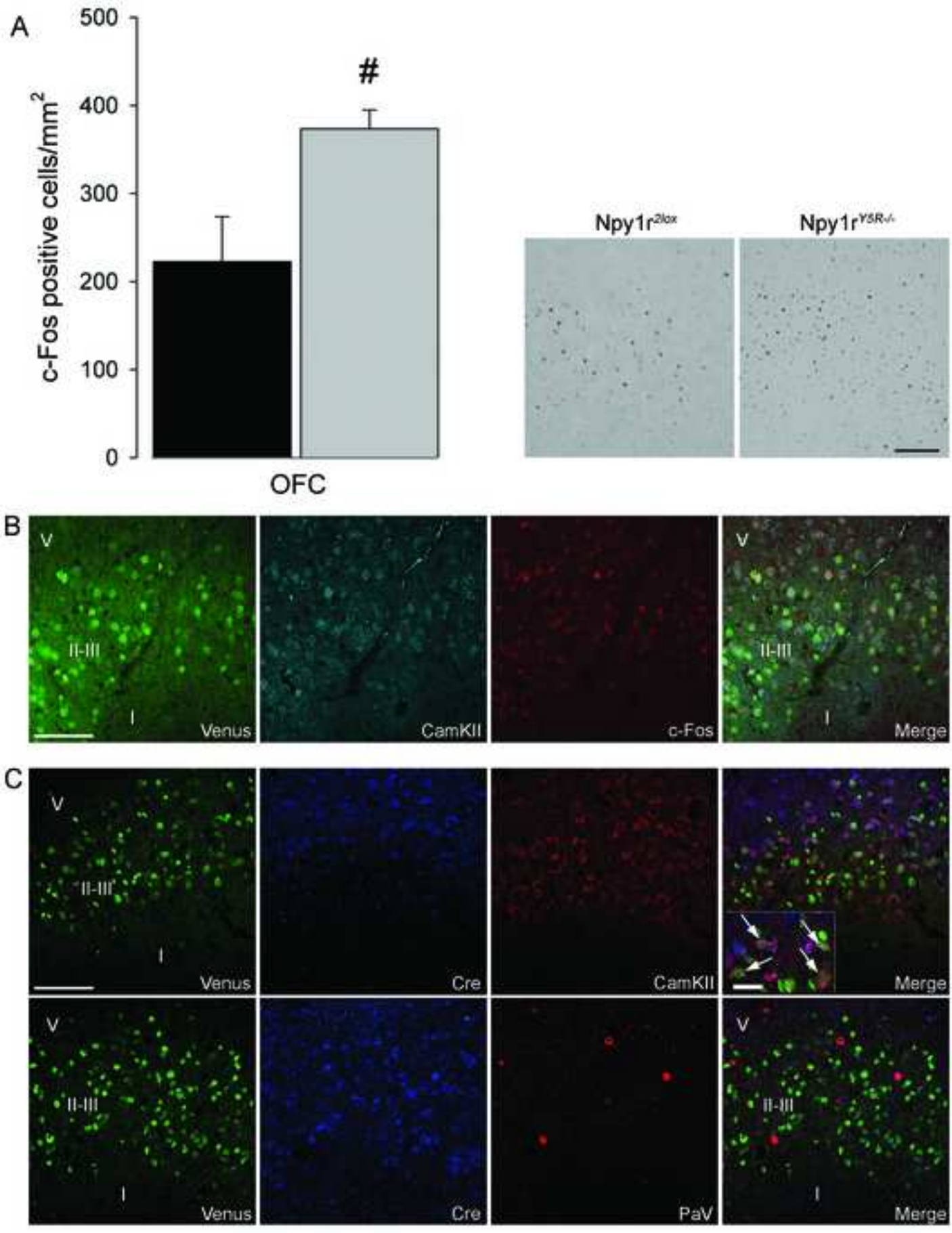

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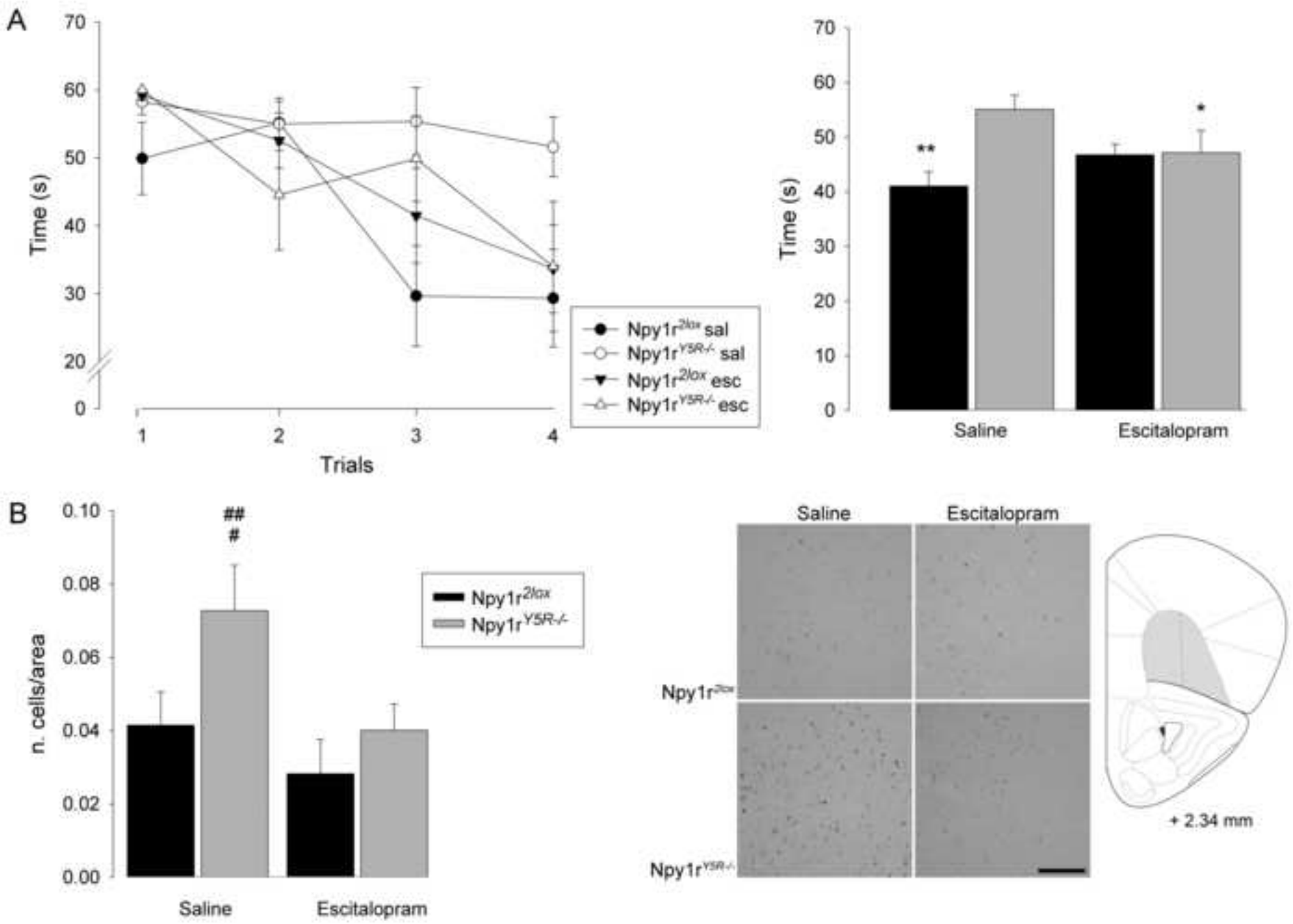

Supplementary Material

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