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Peripheral expression of NPY1R-based heteroreceptor complexes reflects hippocampal neuroimmune status: Novel biomarkers for early Alzheimer's disease detection

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ABSTRACT

Alzheimer's disease (AD) evolves from a prodromal phase with subtle neuroimmune disturbances to overt cognitive decline and hippocampal neurodegeneration. Detecting these early changes non-invasively remains a critical unmet need. Here we show that the abundance of neuropeptide-Y receptor-type-1 (NPY1R)–based heteroreceptor complexes(NPY1R–GALR2 and NPY1R–TrkB), quantified by proximity ligation assay (PLA), constitutes a peripheral read-out of hippocampal status across two complementary rat models. In an acute Accell-siRNA model that selectively knocked down NPY1R in the cerebroventricular space (8 days post-injection), both heterocomplexes were markedly reduced in the dentate gyrus and in circulating white-blood cells (WBCs), yet hippocampal neurogenesis (doublecortin$^+$ cells) and object-in-place memory were still preserved, mimicking the pre-symptomatic/mild cognitive-impairment (MCI) stage of AD. Conversely, in a bilateral olfactory bulbectomy (OBX) model that reproduces chronic AD-like pathology (2 weeks post-surgery), the same heteroreceptor complexes were diminished centrally and peripherally, and this loss co-occurred with impaired object-in-place performance and a 25 % decrease in dentate gyrus neurogenesis. These findings demonstrate that peripheral NPY1R-based heteroreceptor levels mirror hippocampal neuroimmune alterations both before and after the emergence of cognitive and neurogenic deficits. A blood-based PLA assay targeting these complexes could therefore enable ultra-early AD screening, monitor disease progression, and guide the timely initiation of disease-modifying therapies.

1. Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD) are characterized by progressive neuronal loss and cognitive decline, often accompanied by deficits in hippocampal synaptic plasticity and adult neurogenesis. Accumulating evidence links impaired hippocampal neurogenesis to memory dysfunction in AD: the aberrant accumulation of misfolded proteins in AD brains is associated with a gradual reduction in neurogenesis in the dentate gyrus, contributing to memory loss and dementia [37,47]. While adult hippocampal neurogenesis persists even in older individuals and is thought to support learning and memory throughout life, it becomes attenuated in neurodegenerative conditions

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[11,38,51,59,78]. Current pharmacological treatments for dementia (e.g., acetylcholinesterase inhibitors and memantine) provide only transient symptomatic relief and do not halt disease progression [45,75,76,81]. This limitation highlights an urgent need to elucidate the multifaceted pathophysiology of AD and identify novel targets that can modify disease processes [12,29]. Neuropeptide systems, which are richly distributed in brain regions critical for memory [42,43,55,84], have emerged as promising modulators of hippocampal neurogenesis and cognitive function, and thus represent potential avenues for innovative therapeutic and biomarker development, especially when assessed across both prodromal and fully symptomatic stages of the disease.

Neuropeptide Y (NPY), one of the most abundant neuropeptides in the brain, exemplifies such a modulatory system. NPY is widely expressed in the hippocampus and other limbic regions and exerts diverse effects on stress resilience, mood, and cognition [26,72]. In the hippocampal formation, NPY primarily signals through the NPY1 receptor (NPY1R), which plays a pivotal role in learning and memory processes. Activation of NPY Y1R has been shown to promote dentate gyrus neural progenitor proliferation and neuroplasticity [10,13]. Consistent with a pro-cognitive role, NPY expression correlates with memory capacity: hippocampal NPY levels are upregulated following spatial learning tasks in rodents [29], whereas aged rats exhibit decreased hippocampal NPY expression concomitant with impaired memory performance and reduced neurogenesis [12]. In humans, AD patients show significantly lower NPY levels in the brain, cerebrospinal fluid, and plasma, along with a loss of hippocampal Y1R density compared to healthy individuals [28,50,66,69]. Conversely, enhancing NPY–NPY1R signaling can ameliorate cognitive deficits and stimulate neurogenesis. For example, intracerebroventricular administration of NPY or a NPY1R-selective agonist improves spatial memory in rodent models of AD-like pathology [70]. Likewise, exogenous NPY administration induces robust proliferation of dentate gyrus progenitor cells and increases new neuron formation via Y1R-dependent mechanisms [22,27]. These findings highlight the NPY–NPY1R system as a critical regulator of hippocampal plasticity and cognitive function, supporting its exploration as a therapeutic target in age-related cognitive decline and dementia.

Galanin (GAL) is another neuropeptide broadly distributed in the central nervous system that influences cognitive processes. Galanin modulates learning and memory in part by regulating classical neurotransmitter systems [44,77]. Dysregulation of the galanin system has been implicated in AD and related disorders: post-mortem studies report galanin hyperinnervation in the brains of AD patients and elevated anti-galanin autoantibody levels in cerebrospinal fluid correlate with greater neurodegeneration and cognitive impairment in AD [17,18]. The effects of galanin on cognition are complex and appear to depend on receptor subtype, dose, and regional context [8]. Notably, activation of the galanin *receptor 2* (GALR2) has been associated with neuroprotective and pro-cognitive outcomes. In a rat model of AD-like neurodegeneration, GALR2 stimulation protected against hippocampal toxicity and improved memory performance [46]. Galanin also supports neurogenic processes: administration of a GALR2/3 agonist (e.g. galanin (2–11)) increases proliferation of hippocampal precursor cells, leading to an enhanced generation of new granule neurons in the dentate gyrus [1]. These data suggest that GAL, particularly via GALR2, can positively modulate hippocampal neurogenesis and cognitive function. Importantly, NPY and galanin systems do not operate in isolation; emerging evidence indicates significant cross-talk between these neuropeptidergic pathways in the brain. Our group have described multiple interactions between GAL and NPY receptor signaling in limbic regions [14,15,24,56,62,63,65], including direct heteroreceptor complex formation between GALR2 and Y1R in the hippocampus [9,73]. These GALR2–Y1R heteroreceptor complexes were shown to facilitate synergistic effects, as GALR2 and NPY1R co-activation enhanced spatial memory and neuroplastic responses in vivo. Such findings highlight a general principle that G-protein coupled receptors can form heteromeric complexes to integrate their signaling pathways, yielding functional outcomes (e.g. neurogenesis, cognition, mood regulation) that exceed those achieved by single-receptor activation alone [64].

Beyond their central actions, NPY and galanin are also key players in neuroimmune communication. Both peptides are expressed not only in neurons but also in immune cells, where they modulate inflammatory responses [16,41,68]. NPY is a well-documented immunomodulator that can act directly on immune cells to influence their function. For instance, NPY signaling can bias macrophage polarization and cytokine release profiles, shifting the balance between pro- and anti-inflammatory states [16]. Similarly, galanin and its receptors (including GALR2) are expressed in peripheral immune cells such as monocytes and macrophages, and exogenous galanin profoundly alters cytokine and chemokine production depending on the activation state of these cells [41,68]. Through such mechanisms, neuropeptides like NPY and GAL serve as bi-directional messengers between the nervous and immune systems, coordinating neuroimmune homeostasis [85]. This cross-talk is increasingly recognized as a contributor to the pathogenesis of neurodegenerative diseases – for example, chronic peripheral inflammation and CNS immune activation are linked to accelerated cognitive decline in disorders like AD [35]. However, a major challenge in the field is the lack of easily accessible biomarkers that reflect these central neuroimmune and neuroplastic changes early in disease [31]. Standard AD biomarkers (e.g., amyloid-β or phospho-tau in cerebrospinal fluid and PET imaging) are either invasive or expensive, and they primarily capture established neuropathology rather than early functional disturbances [30]. There is thus a pressing need for minimally invasive biomarkers that can monitor brain-related molecular changes (such as altered neuropeptide signaling or neuroinflammation) before significant cognitive deterioration occurs. To explore this concept across the AD continuum, we combined an acute NPY1R-silencing paradigm that mimics mild-cognitive-impairment (MCI) with a chronic olfactory-bulbectomy (OBX) model that reproduces established AD-like pathology [48,58].

In this context, circulating immune cells offer a translational opportunity. White blood cells (WBCs) share various signaling receptors with central neurons and glia, including those for neuropeptides and growth factors, and can be readily obtained from patients. We hypothesized that the heteroreceptor complexes formed by neuropeptide receptors in the brain might also be detectable in peripheral blood cells, providing a proxy measure of central neuroimmune interactions. Notably, NPY is known to interact with the brain-derived neurotrophic factor (BDNF) system [2,19,71], and we demonstrated that NPY's NPY1R may physically associate with the BDNF receptor TrkB in a heteroreceptor configuration [5,6]. The present study set out to determine whether heteroreceptor complexes formed by NPY1R with GALR2 or TrkB are (i) detectable in the rat hippocampus, (ii) mirrored in circulating white-blood cells (WBCs), and (iii) informative of functional status along the AD continuum. To this end we adopted a **two-tier approach**. First, an acute intracerebroventricular Accell-siRNA protocol was used to knock down NPY1R and model a **pre-symptomatic / mild-cognitive-impairment (MCI) stage**, allowing us to test whether an isolated loss of NPY1R is sufficient to lower heterocomplex density in both brain and blood before overt dysfunction emerges. Second, the same endpoints were examined in the **bilateral olfactory-bulbectomy (OBX) model**, which recapitulates established AD-like pathology and cognitive decline. Across both models we quantified hippocampal and peripheral heteroreceptor complexes by proximity ligation assay (PLA), scored hippocampal neurogenesis by doublecortin (DCX) immunostaining, and evaluated spatial memory with the object-in-place task. We hypothesised that (i) peripheral levels of NPY1R-GALR2 and NPY1R-TrkB complexes track hippocampal levels, and (ii) their decline marks the transition from an MCI-like state (receptor loss without functional impairment) to full AD-like pathology (receptor loss accompanied by neurogenic and cognitive deficits). Demonstrating such a

correspondence would provide proof-of-concept for a minimally invasive, blood-based biomarker that links peripheral immune read-outs to central neuroimmune and neuroplastic alterations throughout disease progression.

2. Materials and methods

2.1. Animal care, housing, and experimental design

Adult male Sprague-Dawley rats (6–8 weeks old; 200–250 g) obtained from Janvier (Madrid, Spain) were kept in groups of six per cage. Cages contained paper bedding and cardboard tunnels to encourage burrowing and exploratory activity, thereby supporting social interaction and reducing stress-related variability. Animals were maintained on a 12 h light/12 h dark schedule under controlled temperature (22 ± 2 °C) and humidity (55–60 %). All behavioural testing was carried out between 09:00 and 14:00 h. Procedures conformed to EU Directive 2010/63/EU and Spanish regulations (Real Decreto 53/2013) and were authorised by the University of Málaga Ethics Committee (CEUMA 45–2022-A).

Animals were allocated within each experimental model (i.c.v. siRNA or OBX) by a simple draw-of-lots randomisation. Group identities were coded so that investigators responsible for data acquisition and analysis were blind to treatment. This blinding minimised observer bias during behavioural scoring and histological quantification.

2.2. Stereotaxic cannulation

Rats were anaesthetised with isoflurane (5 % for induction; 1.5–2.5 % for maintenance; ISOFLO®, Zoetis). A 22-gauge stainless-steel guide cannula (Plastics One) was implanted into the right lateral ventricle using the following stereotaxic coordinates [67]: 1.4 mm lateral, 1.0 mm posterior to bregma, and 3.6 mm below the skull surface. After surgery, animals recovered individually for 24 h before being returned to their home cages. The cannulation protocol follows procedures established in our previous studies [15,56,62,63,65].

2.3. Generation of NPY1R-knock-down rats

To down-regulate NPY1R, Accell™ SmartPool siRNA (0.35 nmol in Accell Delivery Medium; Dharmacon) was infused intracerebroventricularly (i.c.v.) through the implanted cannula. Control rats received a non-targeting Accell™ siRNA pool. Animals were sacrificed 6, 8, or 10 days post-infusion to capture the time course of receptor knock-down. Deep anaesthesia was achieved with pentobarbital (100 mg kg^{-1}, i.p.), followed by transcardial perfusion with ice-cold 4 % paraformaldehyde (PFA). These parameters mirror our previously published GALR2-siRNA approach [63].

2.4. Peripheral blood collection and peripheral white-blood cells (WBCs) isolation

For both siRNA and OBX cohorts, blood was collected immediately before transcardial perfusión, whole blood was withdrawn via cardiopuncture and layered onto Ficoll-Paque™ Plus (1.077 g mL^{-1}; GE Healthcare). After density-gradient centrifugation, the buffy coat containing peripheral white-blood cells (WBCs) was collected, washed, and processed for downstream analyses [40].

2.5. Brain sectioning

Post-fixation brains were cryoprotected in 30 % sucrose and sectioned coronally at 30 μm through the dorsal hippocampus (–1.60 to –5.30 mm relative to bregma) [67], using a HM-550 cryostat (Microm). Sections were stored in antifreeze solution at –20 °C until use.

2.6. Immunohistochemical Quantification of NPY1R

Free-floating hippocampal sections underwent antigen retrieval (10 mM sodium citrate, pH 6.0; 65 °C, 90 min) and endogenous peroxidase quenching (0.6 % H_2O_2, 30 min). Slices were incubated overnight at 4 °C with mouse anti-NPY1R antibody (1:200; sc-393192, Santa Cruz) diluted in 2.5 % donkey serum. After PBS washes, sections were exposed to biotinylated donkey anti-mouse IgG (1:200; Sigma) for 90 min, followed by ExtrAvidin-peroxidase (1:100; Sigma) for 60 min in the dark. Bound antibody was visualised with 0.05 % diaminobenzidine (DAB) and 0.03 % H_2O_2. Slides were mounted, dehydrated, and coverslipped with DePeX (Merck). NPY1R-positive profiles in the dentate gyrus were quantified stereologically with the optical fractionator method on an Olympus BX51 microscope, as previously described [14,56,63].

2.7. Analysis of heteroreceptor complexes by in situ proximity-ligation assay

To visualise NPY1R–GALR2 and NPY1R–TrkB heteroreceptor complexes in the dorsal dentate gyrus (DG), we performed an **in situ** proximity-ligation assay (PLA) on free-floating sections, following the protocols of Borroto-Escuela et al. [15] and Narváez et al. [64]([Bo15, 64]). Sections were pre-equilibrated for 60 min at 37 °C in blocking buffer within a humidified chamber, then incubated overnight at 4 °C with optimally titrated primary antibodies. The antibody pairs were: mouse anti-NPY1R (1: 200, Santa Cruz Biotechnology) plus rabbit anti-TrkB (1: 200, ZRB1281, Sigma-Aldrich), and mouse anti-NPY1R (1: 200, Santa Cruz Biotechnology) plus rabbit anti-GALR2 (1: 100, Sigma-Aldrich).

After three PBS washes, sections were exposed to a mixture of NaveniFlex goat- and rabbit-specific secondary probes (Navinci, Sweden) for 1 h at 37 °C. Subsequent incubations with Enzyme A and Enzyme B were carried out at 37 °C for 60 min and 30 min, respectively. Following additional washes, rolling-circle amplification was initiated by Enzyme C/Tex615 for 90 min at 37 °C. Slices were mounted in DAPI-containing antifade medium (Abcam, ab104139), stored at −20 °C, and imaged on a confocal microscope as described previously [14,24,61].

For white-blood cells (WBCs) analyses, 1×10^7 cells were seeded onto poly-L-lysine–coated coverslips (10-min coating at room temperature), allowed to adhere for 24 h (37 °C, 5 % CO_2), and fixed in 4 % paraformaldehyde for 10 min at 4 °C. Cells were permeabilised in 0.1 % Triton X-100 (2 min, RT), blocked for 20 min in 0.5 % BSA, and incubated overnight (4 °C) with the same primary-antibody pairs used for brain tissue. PLA detection on WBCs followed the manufacturer's instructions (Navinci) and published adaptations for non-adherent cells [21,23,79].

2.8. Quantification of doublecortin-positive (DCX^+) neuroblasts

To evaluate treatment-induced changes in neuronal differentiation, DCX immunohistochemistry was conducted on dorsal DG sections [74, 80]. Antigen retrieval was performed for 90 min at 65 °C in 10 mM sodium-citrate buffer (pH 6.0), followed by quenching of endogenous peroxidase with 0.6 % H_2O_2 (30 min, RT). Sections were incubated overnight at room temperature with rabbit anti-DCX (1: 1500, ab18723, Abcam) diluted in 2.5 % donkey serum. After PBS washes, biotinylated anti-rabbit IgG (1: 200, B8895, Sigma-Aldrich) was applied for 90 min, and signal amplification was achieved using ExtrAvidin-peroxidase (1: 100, 60 min, dark). Immunoreactivity was visualised with 0.05 % diaminobenzidine/0.03 % H_2O_2, then sections were dehydrated, cleared, and coverslipped with DePeX (Merck). DCX^+ cells were quantified with the optical-fractionator method under an Olympus BX51 stereology workstation, as detailed previously [3,56,63].

2.9. Bilateral olfactory bulbectomy (OBX)

The OBX procedure was performed as previously described [20,48,58] with minor modifications. Adult male Sprague–Dawley rats were anaesthetised with isoflurane (induction 4–5 %; maintenance 2.5 %) in oxygen (1 L min^{-1}) and secured in a stereotaxic frame (Kopf Instruments). Core temperature was maintained at 37 ± 0.5 °C with a feedback-controlled heating pad. After shaving and disinfecting the scalp, a midline incision (~20 mm) exposed the skull from the frontal to the parietal sutures. Two 2-mm burr holes were drilled 7.0 mm anterior to bregma and 2.0 mm lateral to the midline, according to Paxinos & Watson [67]. The olfactory bulbs were aspirated bilaterally with a blunt 18-gauge needle attached to a low-pressure vacuum pump, taking care to spare the frontal cortex. Hemostasis was achieved with hemostatic sponges and the cavities were packed with the same material. The incision was closed with 4–0 absorbable sutures. Sham-operated rats underwent identical procedures except that the dura was left intact and no bulb tissue was removed.

All animals received post-operative analgesia (carprofen 5 mg kg^{-1}, s.c.) and 1 mL of warm sterile saline (0.9 % NaCl, s.c.) to prevent dehydration. Rats were monitored continuously until full recovery from anaesthesia, housed singly and sutures were inspected daily. A 14-day recovery period was allowed before behavioural testing (n = 5 Sham, n = 5 OBX). Only animals in which complete bilateral bulb removal and an intact frontal cortex were confirmed histologically post-mortem were included in subsequent analyses. Proximity ligation assays (PLA), doublecortin expression in hippocampal sections and peripheral blood mononuclear cells were carried out as described above. For the Object-in-Place (OiP) task, discrimination performance during the first minute of testing was used for analysis [25], because is unaffected by the hyperactivity sometimes observed in OBX rats.

2.10. Behavioural evaluation

2.10.1. Object-in-place (OiP) spatial-memory task

Spatial memory dependent on hippocampal integrity was assessed with the OiP paradigm [82]. The advantage of this task over the Morris water maze task is its lesser stress on the rodents, which can interfere with learning and memory performance [32].

The object-in-place task trials were structured into three phases: habituation, training, and testing [4,7,14,56].

All trials were conducted in a PVC open-field arena (100 × 100 × 60 cm) under dim lighting.

- **Habituation**: Rats were handled for two consecutive days and then allowed to freely explore the empty arena for 10 min.
- **Training**: Twenty-four hours later, four distinct objects (similar size/weight; differing in shape and colour) were positioned 10 cm from each corner. Each rat explored for 3 min. Objects were cleansed with 5 % ethanol between animals to eliminate olfactory cues.
- **Test**: After a 24 h retention interval, two objects swapped positions. Exploration was recorded for 3 min. Exploration time directed toward moved objects (C) versus stationary objects (S) was used to compute the discrimination index (DI = [C − S] / [C + S]). A positive DI denotes intact OiP memory. Object locations and sequence were counterbalanced across animals; arenas and objects were sanitised with 5 % ethanol between sessions. Behaviour was video-tracked and analysed offline using RATON Time 1.0 (Fixma, Spain) by observers blinded to treatment. Total exploration during training and testing, as well as locomotor activity, were monitored to rule out nonspecific performance effects.

2.11. Statistical procedures

All statistical analyses considered the individual rat as the experimental unit. Data are presented as mean ± SEM; sample sizes are specified in figure legends. Statistical evaluation was performed in GraphPad PRISM 8.0 (GraphPad Software, USA). One-way ANOVA followed by Newman–Keuls post hoc tests, or unpaired Student's t-tests where appropriate, were employed. Significance thresholds were set at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

3. Results

3.1. Temporal profile of NPY1R immunoreactivity in the dorsal dentate gyrus

Densitometric stereology of NPY1R-immunostained sections revealed a clear time-dependent down-regulation of receptor protein after intracerebroventricular (i.c.v.) delivery of the Accell-siRNA cocktail. A one-way ANOVA applied to the quantitative counts (four groups: Vehicle, 6 d, 8 d, 10 d; $n = 5$ per group) yielded a robust main effect of treatment ($F_{3,16} = 6.70$, $\mathbf{p} < 0.01$; Fig. 1a–c). Pair-wise Newman–Keuls comparisons demonstrated that the 8-day cohort displayed the deepest loss of NPY1R immunoreactivity: receptor density at 8 d was significantly lower than Vehicle (**$p < 0.01$) and was also reduced relative to both the 6 d and 10 d groups ($p < 0.05$ for each contrast). No statistical difference emerged between 6 d and 10 d animals (ns). Thus, maximal knock-down of NPY1R protein is achieved eight days after siRNA administration.

3.2. Impact of NPY1R silencing on GALR2- and TrkB-containing heteroreceptor complexes in the hippocampus

Proximity-ligation assays (PLA) performed in the dorsal dentate gyrus (subgranular and polymorphic layers) showed discrete red puncta indicative of heteromeric NPY1R partnerships. Quantification of NPY1R–GALR2 PLA signal (Fig. 2a–d) revealed a significant treatment effect (one-way ANOVA, $F_{3,16} = 3.65$, $p < 0.05$). Post-hoc Newman–Keuls testing confirmed that the 8 d group presented a markedly diminished cluster density versus Vehicle and versus both the 6 d and 10 d cohorts ($p < 0.05$ for all three comparisons).

A parallel analysis of NPY1R–TrkB complexes (Fig. 3) yielded an even stronger statistical outcome: the ANOVA was significant ($F_{3,16} = 5.20$, $p < 0.05$), with Newman–Keuls tests indicating that animals examined 8 d after siRNA injection had significantly fewer heteromers than Vehicle (**$p < 0.01$) and fewer than the 6 d and 10 d groups ($p < 0.05$ each). Collectively, these findings demonstrate that loss of NPY1R not only lowers receptor abundance but also compromises its ability to assemble functional complexes with GALR2 and TrkB within hippocampal circuitry.

3.3. Peripheral mirror of central changes: heteroreceptor complexes in circulating white blood cells

Using the same PLA methodology on Ficoll-isolated peripheral blood mononuclear cells, we observed bright puncta corresponding to NPY1R-based heteromers (Fig. 4 and Fig. 5). For NPY1R–GALR2 assemblies (Fig. 4a–d), a one-way ANOVA again identified a significant effect of siRNA exposure ($F_{3,16} = 4.21$, $p < 0.05$). Post-hoc evaluation showed that the 8 d animals exhibited significantly fewer complexes than Vehicle, 6 d, and 10 d subjects ($p < 0.05$ for each).

Analysis of NPY1R–TrkB complexes in white blood cells produced comparable results (Fig. 5): the ANOVA was highly significant ($F_{3,16} = 7.47$, $\mathbf{p} < 0.01$). Newman–Keuls tests demonstrated a pronounced reduction at 8 d relative to Vehicle (**$p < 0.01$) and to both 6 d and 10 d groups ($p < 0.05$).

These peripheral data recapitulate the hippocampal profile, indicating that circulating immune cells faithfully reflect central alterations in NPY1R-dependent heteroreceptor signalling.

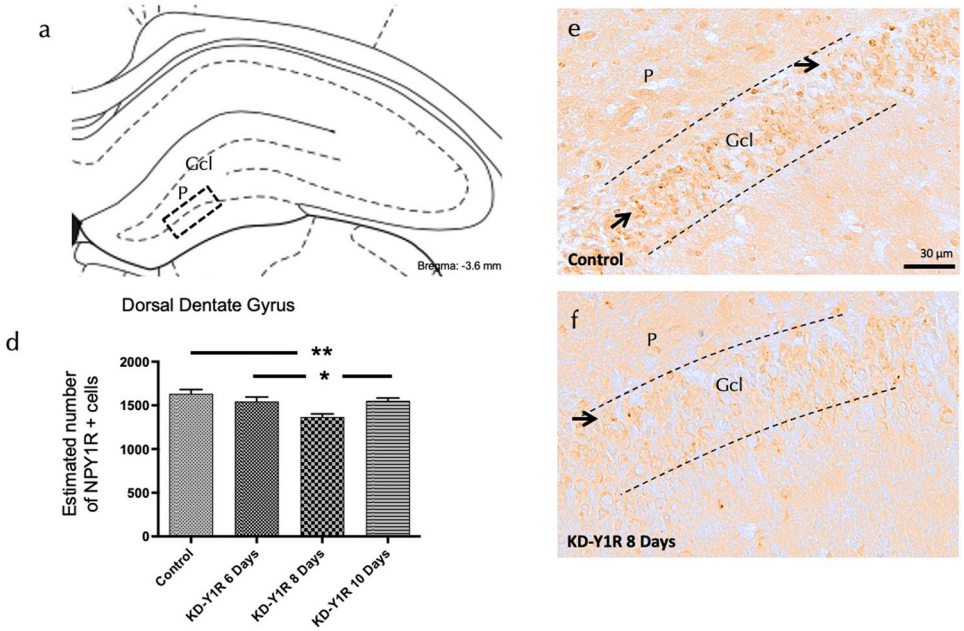

Fig. 1. NPY1R expression in the dorsal dentate gyrus after intracerebroventricular NPY1R knock-down. (a) Representative photomicrograph illustrating NPY1R-immunoreactive (NPY1R-IR) neurons in the granular cell layer (GCL) of the dorsal dentate gyrus (DG). Scattered labelled cells are also visible in the polymorphic layer (P). The section corresponds to the dorsal hippocampal level at approximately –3.6 mm from bregma according to Paxinos and Watson. (b) Stereological quantification of total NPY1R-IR cells in the dorsal DG 6, 8 or 10 days after intracerebroventricular (icv) delivery of NPY1R siRNA. A significant reduction is observed at 8 days compared with control (one-way ANOVA followed by Newman–Keuls post-hoc test, $P < 0.01$) and 6 and 10 days groups (one-way ANOVA followed by Newman–Keuls post-hoc test, $P < 0.05$). Data are mean ± SEM (n = 5 per group). (c, d) High-magnification micrographs showing the marked loss of NPY1R immunolabelling in the dorsal DG 8 days after siRNA treatment (d) relative to the vehicle control (c). Arrows indicate NPY1R-positive neurons; dashed lines delineate the GCL. Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

3.4. NPY1R knock-down spares neuroblast density in the dorsal dentate gyrus

To determine whether the transient depletion of NPY1R influences ongoing neurogenesis, we stereologically quantified doublecortin-immunoreactive (DCX^+) neuroblasts in the dorsal dentate gyrus of animals killed 6, 8, or 10 days after siRNA infusion. The one-way ANOVA performed on DCX^+ cell estimates (Vehicle vs 6 d vs 8 d vs 10 d; $n = 5$ per group) produced no significant group effect ($F_{3,16} = 0.27$, $\mathbf{p} > 0.05$; Fig. 6a–d). Hence, despite the pronounced reduction in NPY1R protein and heteroreceptor assemblies observed at the 8-day time-point, the overall population of immature neurons remained unchanged throughout the examined interval.

3.5. Object-in-place memory remains intact after NPY1R knock-down

Cognitive consequences of the knock-down were probed with the object-in-place paradigm. Rats received siRNA or vehicle and were subjected to the three-phase protocol—10-min arena habituation (no objects), a 3-min sample trial with four distinct objects, and, 24 h later, a 3-min choice trial in which two objects swapped locations (Fig. 1a). Discrimination indices collected after siRNA administration were analyzed with a one-way ANOVA (Vehicle, 6 d, 8 d, 10 d; $n = 5$ per group). The analysis indicated no significant treatment effect ($F_{3,16} = 0.14$, $p > 0.05$; Fig. 7), demonstrating that spatial associative recognition memory was preserved.

Supplementary behavioural parameters supported this conclusion: total object exploration time and spontaneous locomotor activity during both sample and test sessions did not differ between groups (all ANOVA $p > 0.05$; data not shown). Consequently, acute knock-down of NPY1R and the concomitant disruption of its heteroreceptor complexes, does not impair either the generation of DCX^+ neuroblasts or object-in-place memory under the conditions tested.

3.6. OBX model reveals heteroreceptor loss with cognitive and neurogenic déficits

Fig. 8a shows that olfactory-bulbectomised (OBX) rats exhibited a marked impairment in object-in-place memory: the discrimination index (DI) was significantly lower than that of sham controls (unpaired t-test: $t(8) = 4.790$, $p < 0.01$). Consistent with this behavioural deficit, proximity ligation assays (PLA) revealed a robust reduction of NPY1R–GALR2 heteroreceptor complexes in the dentate gyrus of OBX animals ($t(8) = 4.763$, $p < 0.01$) and a parallel decrease in NPY1R–TrkB complexes ($t(8) = 3.920$, $p < 0.01$) (Fig. 8b). Strikingly, the same pattern was mirrored in peripheral blood mononuclear cells: OBX rats displayed lower densities of NPY1R–GALR2 ($t(8) = 4.910$, $p < 0.01$) and NPY1R–TrkB ($t(8) = 5.307$, $p < 0.001$) puncta compared with shams (Fig. 8c). Finally, neurogenesis in the dentate gyrus, assessed by doublecortin (DCX) immunoreactivity, was significantly reduced in the OBX group ($t(8) = 3.387$, $p < 0.01$) (Fig. 8d).

4. Discussion

4.1. Reduction of NPY1R expression in the hippocampus after siRNA knockdown

As expected, intracerebroventricular delivery of NPY1R-targeted siRNA produced a marked reduction of Y1R protein in the dorsal hippocampus, with receptor levels reaching a nadir approximately one week post-injection. This time course (maximal knockdown at 8 days) is consistent with prior siRNA knockdown experiments targeting related GPCRs in the rodent brain [63]. The effective but transient ablation of NPY1R in vivo provides a model to probe the consequences of acutely

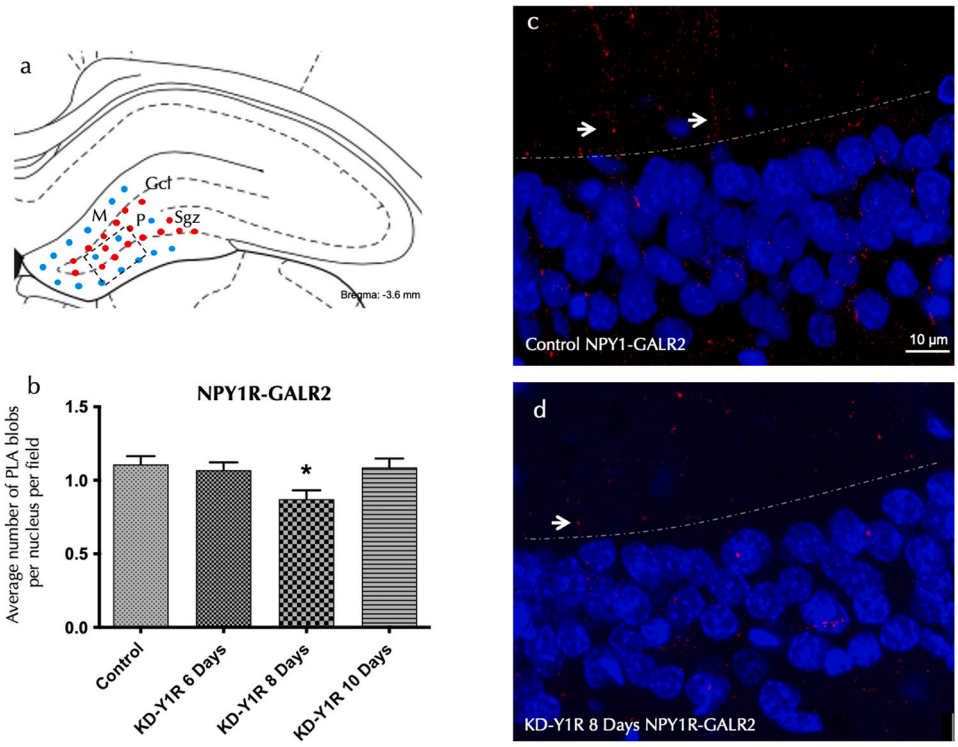

Fig. 2. NPY1R–GALR2 heteroreceptor complexes in the dorsal dentate gyrus. (a) Representative confocal image obtained with in-situ proximity ligation assay (PLA) showing NPY1R–GALR2 PLA-positive red puncta (arrows) distributed within the subgranular (SGZ) and polymorphic (P) layers of the dorsal dentate gyrus (DG). The section corresponds to –3.6 mm from bregma (Paxinos & Watson). (b) Quantitative stereological analysis of PLA signal density reveals a significant reduction in NPY1R–GALR2 complex density 8 days after intracerebroventricular NPY1R siRNA compared with control, 6-day and 10-day groups (one-way ANOVA, Newman–Keuls post-hoc, $P < 0.05$ for each comparison). Data are expressed as mean ± SEM (n = 5 per group). (c, d) High-magnification micrographs illustrating abundant PLA puncta in vehicle-treated animals (c) and the marked loss of complexes 8 days after siRNA treatment (d). Dashed lines outline the granular cell layer (GCL). Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

diminished NPY signaling in the adult hippocampus. Notably, a loss of hippocampal NPY1R is a hallmark observed in Alzheimer's disease patients, who exhibit significantly reduced NPY1R density alongside lower NPY levels in the brain and cerebrospinal fluid compared to healthy individuals [28,50,66]

By transiently mimicking this aspect of AD-related neuropeptidergic deficit in an otherwise healthy brain, our approach allowed us to dissect the immediate molecular consequences of Y1R loss while avoiding the confounds of developmental compensation observed in chronic genetic models. These results provide a robust foundation for understanding how transient receptor depletion may relate to the gradual neurochemical alterations observed in early AD pathology, setting the stage for future studies that address whether chronic Y1R deficiency exacerbates neurodegenerative progression [30,52].

4.2. Decrease in hippocampal NPY1R–GALR2 and NPY1R–TrkB heteroreceptor complexes

In the hippocampus, NPY1R knockdown led to a pronounced decrease in both NPY1R–GALR2 and NPY1R–TrkB heteroreceptor complexes, demonstrating that these assemblies depend on the availability of the NPY1R protomer. This finding aligns with our previous work showing that NPY1R forms heteromeric complexes with GALR2 in the dentate gyrus [73,9] and with TrkB receptors (the major BDNF receptor) in hippocampal neurons [5]. Notably, co-activation of NPY1R-GALR2 and NPY1R–TrkB produced greater enhancements in neuroplasticity and memory than activation of either receptor alone, an effect attributed to direct receptor–receptor coupling in heterocomplexes. In the context of neurodegeneration, this finding is significant: it suggests that early loss of NPY–NPY1R (as occurs in AD) not only deprives the brain of NPY's direct pro-cognitive influence, but also uncouples NPY signaling from other neuroprotective systems like galanin/GALR2 and BDNF/TrkB. The attenuation of both NPY1R-GALR2 and NPY1R–TrkB interactions observed here may thus represent a mechanism by which neuropeptide deficits translate into reduced neurotrophic support in the diseased hippocampus. This finding is particularly significant given that reduced heteromer formation may underlie the diminished neurotrophic support observed in AD [22,27]. More broadly, our data support the emerging principle that G-protein-coupled receptors can form functionally significant heteroreceptor complexes to coordinate neuromodulatory networks. The NPY1R–GALR2–TrkB axis in the dentate gyrus may be one such network, and the loss of these heteroreceptor assemblies may, therefore, contribute not only to the synaptic deficits but also to the neuroimmune dysregulation that characterizes early AD. Importantly, recent advances in multiplex immunoassays, highlight the potential clinical utility of detecting blood-based protein biomarkers associated with other biological processes for a comprehensive evaluation of AD status [36].

4.3. Parallel reduction in heteroreceptor complexes in peripheral white blood cells

A key novel finding of our study is that the reduction in NPY1R-based heteroreceptor complexes was not confined to the brain, the same pattern was observed peripherally in circulating white blood cells. Eight days after NPY1R knockdown, WBCs from the knockdown rats showed significantly decreased densities of NPY1R–GALR2 and NPY1R–TrkB complexes, mirroring the hippocampal changes. This parallel decline

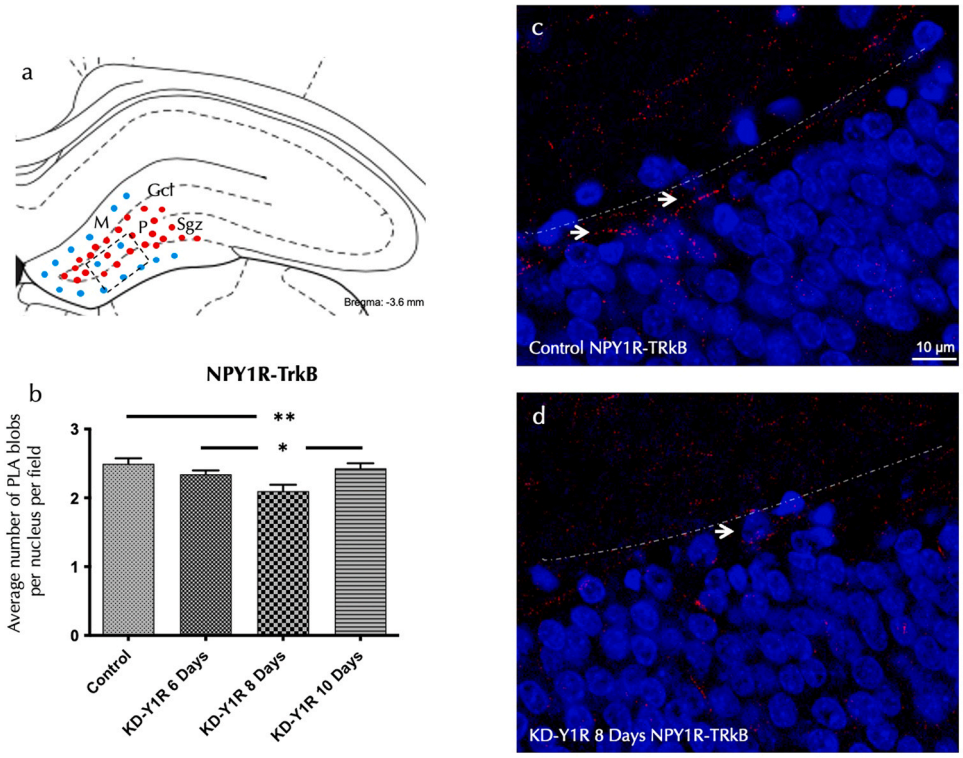

Fig. 3. NPY1R–TrkB heteroreceptor complexes in the dorsal dentate gyrus. (a) Representative confocal image illustrating PLA-positive red puncta that denote NPY1R–TrkB complexes. Signals are enriched in the subgranular zone (SGZ), the granule-cell layer (GCL) and the polymorphic layer (P) of the dorsal DG, whereas the molecular layer (M) is virtually devoid of PLA reactivity (blue DAPI counter-stain only). The section corresponds to –3.6 mm from bregma according to Paxinos & Watson. (b) Quantitative stereological evaluation (red puncta · nucleus^{-1}, observer blinded to treatment) shows a pronounced decline in complex density eight days after intracerebroventricular delivery of NPY1R siRNA compared with control, 6-day and 10-day cohorts (one-way ANOVA, Newman–Keuls post-hoc: *$P < 0.01$ vs control, $P < 0.05$ vs KD-Y1R 6 d and KD-Y1R 10 days). Data are mean ± SEM, n = 5 animals per group; horizontal bars above columns denote pairwise comparisons. (c, d) High-magnification micrographs reveal plentiful PLA clusters in vehicle-treated rats (c) and a conspicuous loss of complexes after eight days of NPY1R knockdown (d). White arrows highlight PLA blobs; dashed contours delineate the GCL. Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

provides compelling evidence that peripheral immune cells can reflect and mirror central receptor–receptor interactions, supporting our hypothesis that such heterocomplexes in blood might serve as proxies for brain neurochemical status. To our knowledge, this is the first demonstration that a GPCR heteroreceptor complex in the CNS is detectible in peripheral cells and dynamically tracks CNS alterations. One interpretation is that the central loss of NPY1R triggered systemic signaling changes (e.g., altered release of NPY or other factors) that secondarily downregulated NPY1R-containing complexes in immune cells. Alternatively, baseline expression of these receptor complexes in WBCs may be governed by similar regulatory mechanisms as in the brain, such that knocking down NPY1R in the CNS (and the resultant neuroimmune response) leads to a coordinated reduction peripherally. Regardless of the precise mechanism, the peripheral readout faithfully recapitulated the central receptor changes, emphasizing an intimate neuroimmune link.

The demonstration that NPY1R–GALR2 and NPY1R–TrkB complexes exist in WBCs, and are diminished by central NPY1R knockdown, suggests that these neuropeptide receptors might form functional heteromers in immune cells that coordinate neuroimmune signaling. A loss of NPY1R–heterocomplexes coupling in immune cells could conceivably tilt the peripheral immune balance toward a more pro-inflammatory profile (since NPY's restraining influence is reduced) a scenario consistent with the pro-inflammatory state often seen in early AD [16,41,68]. In fact, systemic inflammation has been linked to accelerated cognitive decline in AD patients [35,54,83]. Our results raise the possibility that one contributing factor to this neuroimmune activation in AD is the breakdown of homeostatic neuropeptide signaling in both brain *and* periphery.

From a biomarker standpoint, the peripheral mirroring of hippocampal heteroreceptor status is a breakthrough that could pave the way for novel blood-based diagnostics. There is a pressing need for minimally invasive biomarkers that reflect central neuropathological changes at early, preclinical stages of AD [34,39]. Current plasma biomarkers (e.g., amyloid-β, phosphorylated tau, neurofilament light) largely capture downstream neurodegeneration or protein aggregation, and they may not report on the brain's neurochemical and neuroimmune state. In contrast, quantifying NPY1R–GALR2 and NPY1R–TrkB complexes in peripheral blood cells would provide a window into neuroprotective signaling pathways and neuroimmune interactions that are actively engaged in the hippocampus. Our findings suggest that a drop in these heteroreceptor complexes in blood could serve as a red flag for a corresponding loss of neurotrophic support and immunoregulatory capacity in the brain's milieu, a translational biomarker of "hippocampal neuroimmune status".This approach, if validated clinically, could complement existing AD biomarkers by indicating a state of reduced NPY/galanin/BDNF signaling, which might herald cognitive decline even before massive neuron loss occurs. The notion that blood-borne heterocomplex levels represent an *ultra-early* biomarker is further strengthened by our new OBX data (see below), where identical peripheral reductions arise in the absence of any siRNA exposure.

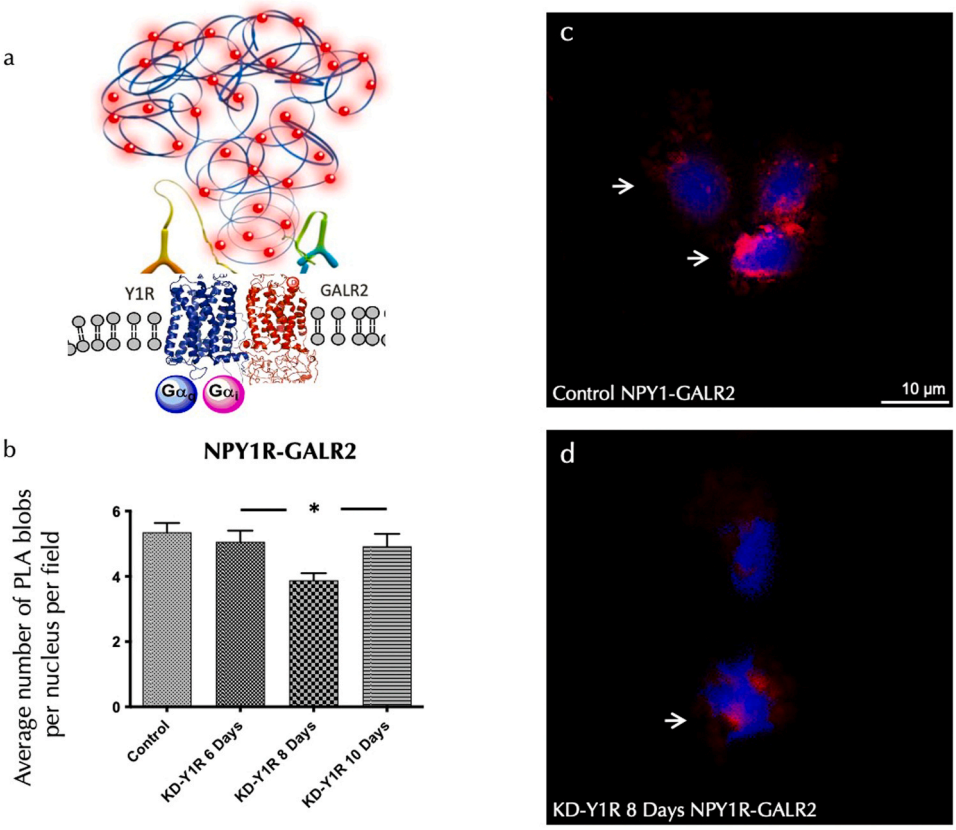

Fig. 4. NPY1R–GALR2 heteroreceptor complexes in peripheral white-blood cells. Identification of GALR2/NPY1R heteroreceptor complexes in freshly isolated rat white blood cells (WBCs) was carried out with the in-situ PLA, which allows direct visualisation of proteins that reside within < 40 nm of each other. Highly selective primary antibodies for GALR2 and NPY1R were employed (a). (b) Quantitative analysis of complex density (Average number of PLA blobs) performed by an observer blind to treatment demonstrates a significant fall in GALR2/NPY1R complexes 8 days after NPY1R-siRNA injection compared with control, 6-day and 10-day groups (one-way ANOVA, Newman–Keuls post-hoc: $P < 0.05$ vs control, KD-Y1R 6 d and KD-Y1R 10 d). Data are mean ± SEM (n = 5 rats per group); horizontal bars above columns indicate pairwise comparisons. (c, d) Representative confocal images showing PLA-positive signals (red puncta) distributed throughout the cytoplasm of DAPI-labelled leucocytes; nuclei appear blue. Micrographs illustrate abundant PLA clusters in vehicle-treated animals (c) and their marked reduction 8 days after knock-down (d). White arrows highlight PLA puncta. Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

4.4. OBX model reveals heteroreceptor loss with cognitive and neurogenic deficits

Olfactory bulbectomy produced a 25 % drop in dentate-gyrus neurogenesis and a robust object-in-place deficit, closely mirroring the cognitive and neurogenic decline observed in prodromal–mild AD patients [25,33,57]. Crucially, OBX rats showed the same pattern of NPY1R–GALR2 and NPY1R–TrkB loss in both hippocampus and WBCs that we observed after acute NPY1R silencing. This convergence across two mechanistically distinct models (transient knock-down vs. chronic neurodegeneration) demonstrates that Heteroreceptor decline precedes functional impairment (siRNA, Stage 1), but persists and correlates with overt deficits once pathology is established (OBX, Stage 2).

4.5. Specificity of the i.c.v. Accell-siRNA intervention

Intracerebroventricular (i.c.v.) delivery of self-penetrating Accell-siRNA has been shown to produce a neuron-selective knock-down that remains largely confined to the CNS: in adult rats a single 10-µg bolus achieved ≈ 97 % uptake in multiple brain regions while sparing glia and peripheral tissues [60]. Independent pharmacokinetic analyses of antisense oligonucleotides administered into the cerebrospinal fluid corroborate this compartmentalisation; for example, intrathecal nusinersen reaches CSF concentrations > 100-fold higher than plasma, with peripheral traces cleared rapidly by renal filtration [49]. Multimodal imaging in rodents further shows that oligonucleotides exit the CSF mainly via meningeal lymphatics to cervical nodes and kidneys, with minimal direct exposure of circulating leucocytes [53]. Taken together, these data indicate that the Accell-siRNA used here is highly unlikely to down-regulate NPY1R complexes in blood cells through direct pharmacological action; instead, the parallel loss of heteroreceptor complexes we observe in hippocampus and PBMCs most plausibly reflects secondary neuro-immune signalling triggered by central NPY1R depletion.

4.6. NPY1R knock-down spares neuroblast density and object-in-place memory

Interestingly, despite the pronounced molecular alterations induced by NPY1R knockdown, the number of DCX-positive neuroblasts in the hippocampus remained unchanged, and spatial memory performance in the object-in-place task was not impaired. This suggests that the acute reduction of NPY1R does not immediately translate into deficits in neurogenesis or explicit cognitive dysfunction. In our previous work using physiologically normal adult rats, we found that an NPY1R agonist on its own did **not**enhance neurogenesis in the dorsal hippocampus *unless* GALR2 or TrkB were co-activated [5,73,9]. The adult neurogenic niche in the dentate gyrus is regulated by a multitude of factors (growth

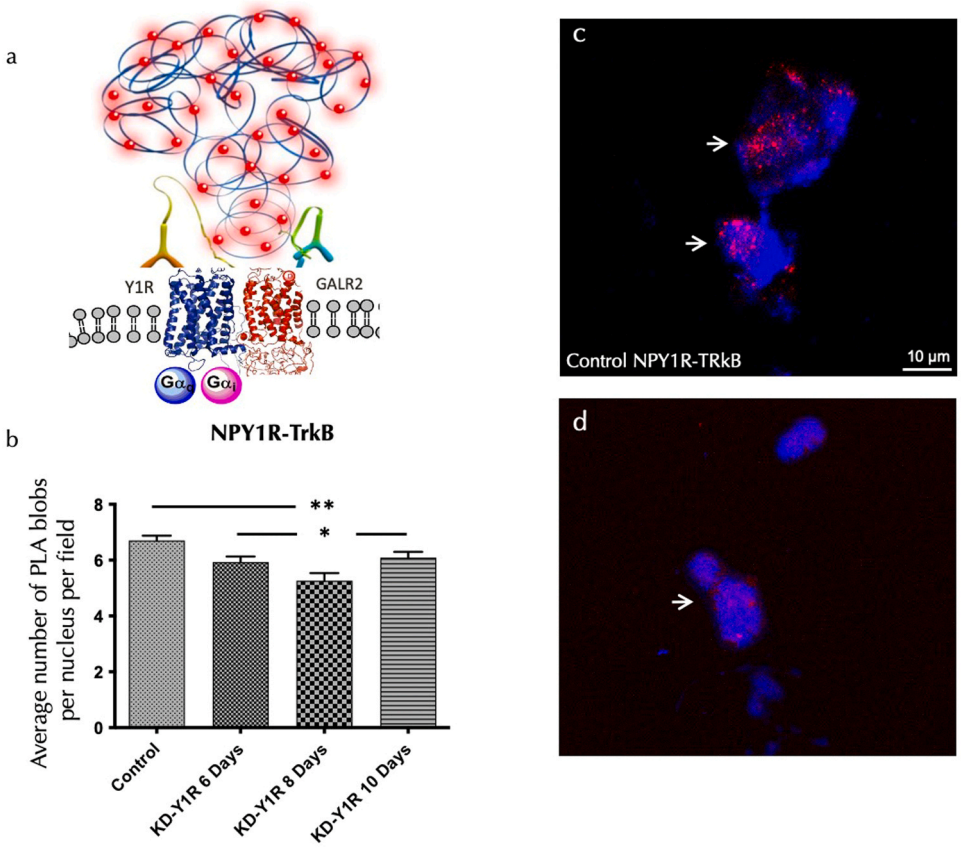

Fig. 5. NPY1R–TrkB heteroreceptor complexes in peripheral white-blood cells. NPY1R–TrkB heteroreceptor complexes were visualized in circulating leukocytes with the in-situ PLA, which reveals protein–protein interactions occurring within < 40 nm. Highly specific primary antibodies against NPY1R and TrkB were employed. (a) Representative diagram. Positive PLA signals appear as discrete red puncta distributed throughout the extracellular part of the cytoplasmatic membrane. (b) Quantitative analysis of PLA puncta (Average number of PLA blobs) performed by an investigator blinded to treatment. Eight days after intracerebroventricular administration of NPY1R siRNA, the density of NPY1R/TrkB complexes is significantly reduced compared with control, 6-day and 10-day groups (one-way ANOVA, Newman–Keuls post-hoc: $^{**}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. KD-Y1R 10 days). Data are mean ± SEM (n = 5 rats per group); horizontal bars indicate inter-group comparisons. (c, d) Representative micrographs illustrate numerous PLA clusters in WBCs from control animals (c) and their marked reduction 8 days after NPY1R knock-down (d). White arrows indicate PLA-positive clusters; nuclei are counter-stained with DAPI. Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

factors, other neurotransmitters, and peptides), and it appears that redundancy and compensation can buffer against short-term loss of any single promotor of neurogenesis. Notably, the absence of neurogenic decline in our NPY1R knockdown rats also confirms that the siRNA intervention was not overtly toxic to neural progenitors or their microenvironment. In a chronic neurodegenerative scenario, where NPY levels and NPY1R expression are persistently low (and accompanied by inflammation and other insults), one would expect a more pronounced reduction in neurogenesis over time [11,38,51,59,78]. Regarding spatial memory performance, our results suggest that an acute, transient reduction of NPY1R in the dorsal hippocampus is not sufficient to disrupt the complex neural processes underlying object-location associative memory. A mild perturbation restricted to dentate gyrus neuromodulation might be compensated by the integrity of other circuit components. The hippocampus of knockdown rats still had all its principal neurons intact and only a temporary loss of one modulatory input, while other neurochemical systems and spatial encoding mechanisms would remain largely functional [7].

Overall, peripheral detection and monitoring NPY1R-linked heteroreceptor loss, as we propose, could signal an ongoing neurochemical vulnerability long before cognitive symptoms appear, which is precisely the scenario for early intervention in disorders like prodromal AD.

4.7. Biomarker implications along the AD continuum

Taken together, the Stage-1 and Stage-2 datasets outline a temporal biomarker trajectory: peripheral NPY1R-based heterocomplexes drop first, signalling hippocampal vulnerability while neurogenesis and memory are still intact; with continued pathology (OBX stage), the same peripheral read-out now co-occurs with neurogenic failure and cognitive decline. Clinically, this two-point calibration suggests that a blood test quantifying NPY1R–GALR2/TrkB heteromers could (i) flag high-risk individuals at a “mild-cognitive-impairment–plus” stage and (ii) track progression or therapeutic response once deficits emerge.

5. Limitations

Despite the promising results, several limitations must be addressed. First, our knockdown of Y1R was acute and performed in young, healthy rats, which may not fully recapitulate the chronic receptor deficits seen in AD, although incorporation of the chronic OBX model partially mitigates this caveat by providing a symptomatic comparator. Long-term studies in aged or genetic AD-model animals are necessary to determine whether sustained reductions in Y1R and its associated heteroreceptor complexes lead to impairments in neurogenesis and cognition. Second, while the PLA method provides evidence for proximal associations between receptors, it does not definitively prove functional

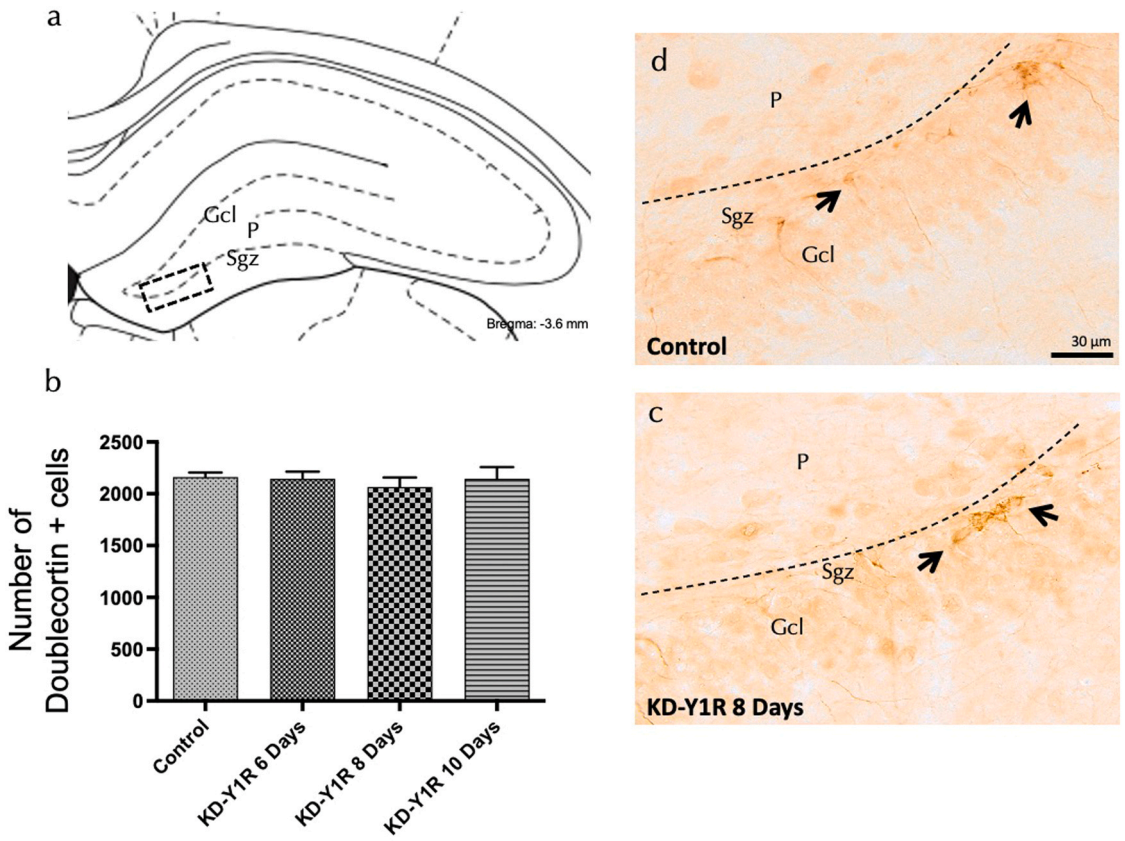

Fig. 6. Doublecortin (DCX) immunoreactivity in the dorsal dentate gyrus after acute NPY1R knock-down. Doublecortin-positive (DCX⁺) cells—an index of immature neurons—were examined in the dorsal dentate gyrus 6, 8 and 10 days after the SiRNA icv treatments. (a, d) DCX⁺ somata are confined mainly to the subgranular zone (SGZ) at the border of the granule-cell layer (GCL) and the polymorphic layer (P). Images correspond to Bregma −3.6 mm in the Paxinos & Watson rat atlas [67]. (b) Stereological quantification of total DCX-immunoreactive cells revealed no significant differences among groups (one-way ANOVA, n.s.). (c, d) Representative confocal micrographs from the Control group (c) and the KD-Y1R 8 Days group (d) illustrate the similar density and distribution of DCX⁺ cells across conditions. Arrows indicate DCX-positive neuroblasts; dashed lines outline the GCL. Data are mean ± SEM (n = 5 rats per group). Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

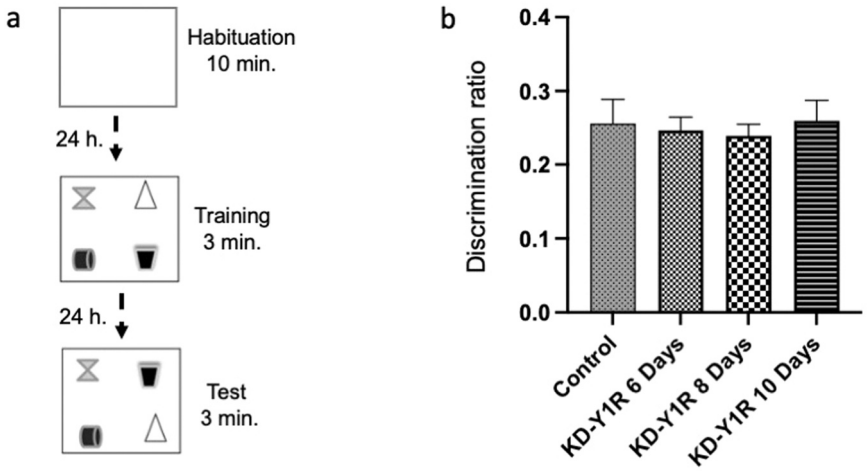

Fig. 7. Object-in-place memory after NPY1R knock-down. (a) Schematic timeline of the object-in-place protocol. Rats underwent a 10-min habituation session in an empty arena, followed 24 h later by a 3-min training session with four distinct, fixed objects. After a further 24 h, two objects were transposed and animals were given a 3-min test session to evaluate spatial recognition. (b) Discrimination index (DI) during the test phase. Neither transient silencing of NPY1R for 6, 8 nor 10 days (KD-Y1R 6 d, 8 d, 10 d) altered the ability of rats to distinguish the displaced objects when compared with vehicle controls. One-way ANOVA revealed no significant group effect (n.s.), and post-hoc Newman–Keuls comparisons confirmed the absence of pair-wise differences. Data are expressed as mean ± SEM (n = 5 animals per group). Abbreviations used: Control (Accell Non-targeting Pool diluted in siRNA Delivery Media); KD-Y1R 6 Days (Accell Smart pool NPY1R siRNA for 6 days); KD-Y1R 8 Days (Accell Smart pool NPY1R siRNA for 8 days); KD-Y1R 10 Days (Accell Smart pool NPY1R siRNA for 10 days).

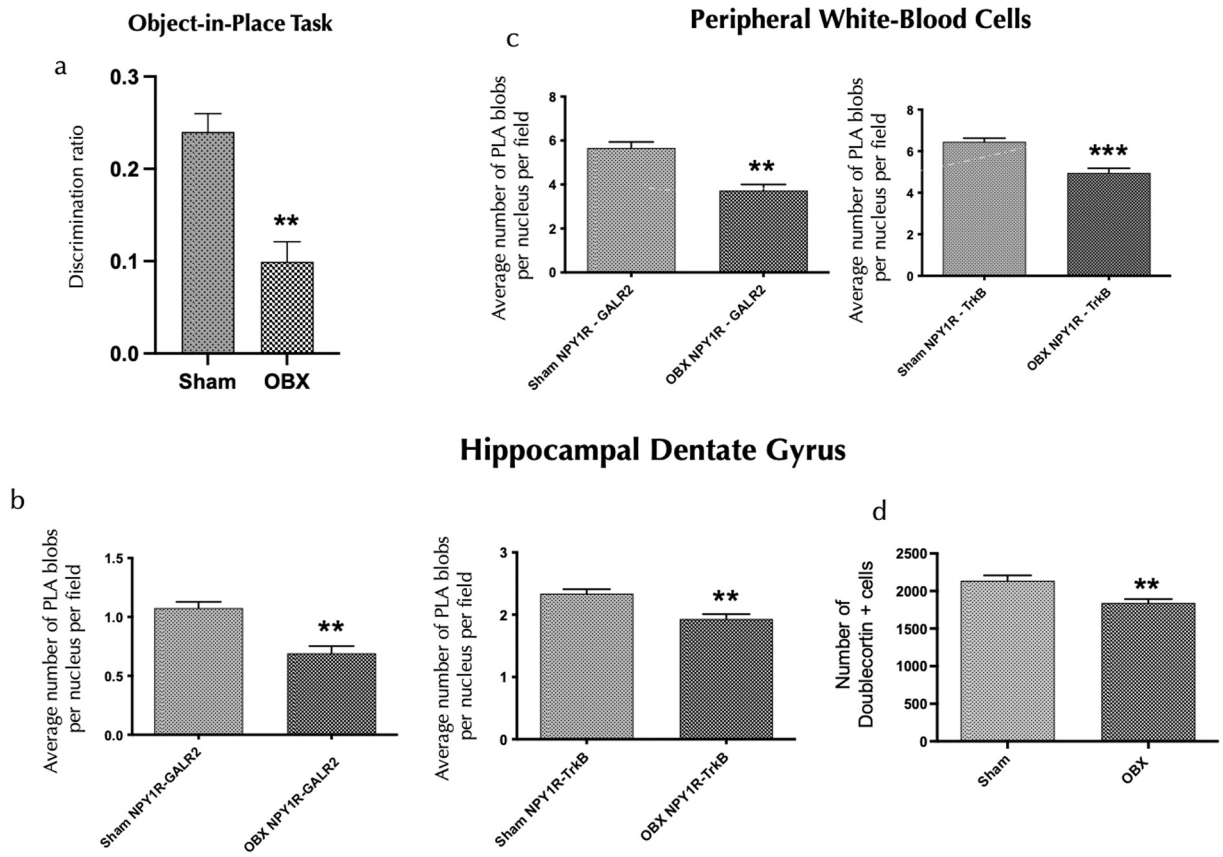

Fig. 8. Bilateral olfactory bulbectomy (OBX) reproduces hippocampal–peripheral loss of NPY1R heteroreceptor complexes and impairs object-in-place memory. *(a)* Object-in-place task. The discrimination index (DI) calculated from the first 60 s of the test session is markedly reduced in OBX rats compared with sham-operated controls. *(b)* Hippocampal dentate gyrus. Proximity ligation assay (PLA) reveals fewer NPY1R–GALR2 and NPY1R–TrkB heteroreceptor puncta per nucleus in OBX animals. *(c)* Peripheral white-blood cells (PBMCs) show an identical reduction in both heteroreceptor complexes, indicating that the peripheral read-out mirrors the central deficit. *(d)* Neurogenesis is also compromised in OBX rats, as shown by the lower number of doublecortin-positive (DCX⁺) immature neurons in the dentate gyrus. Bars represent mean ± SEM (n = 5 per group). Unpaired two-tailed Student's *t*-test: **p < 0.01, ***p < 0.001 vs. Sham. Abbreviations used: OBX (Bilateral olfactory bulbectomy); PLA (Proximity ligation assay); NPY1R (neuropeptide-Y type-1 receptor); GALR2 (Galanin receptor-2); TrkB (Tropomyosin receptor kinase B.

interaction. Complementary techniques (e.g., co-immunoprecipitation or FRET) are needed to validate these heteromeric interactions more rigorously. Third, the translational potential of our peripheral marker findings remains to be validated in human subjects, where additional confounds such as systemic inflammation, medication use, and genetic heterogeneity might affect receptor expression on immune cells [31].

Future directions

Validate the heterocomplex assay in longitudinal human cohorts spanning subjective cognitive decline, MCI and manifest AD, to test its stage-sensitivity predicted here. Future research should extend these findings to clinical samples by exploring whether patients with mild cognitive impairment or early AD exhibit similar reductions in peripheral NPY1R–GALR2 and NPY1R–TrkB complexes. Longitudinal studies in human cohorts, ideally combined with neuroimaging and CSF biomarker analyses, are essential to validate these peripheral markers as predictors of central neuroimmune status and cognitive decline. In addition, future preclinical work should employ chronic receptor knockdown models in aged or transgenic AD animals to assess the cumulative effects on neurogenesis, synaptic plasticity, and behavior. Mechanistic studies are needed to elucidate the pathways by which central neuropeptide receptor alterations are communicated to the periphery—examining whether this communication occurs via endocrine factors, cytokines, or direct neural-immune pathways. Finally, given the feasibility and scalability of blood-based assays, it will be important to develop high-throughput methods (such as optimized flow cytometry or multiplexed immunoassays) to quantify receptor heterocomplexes reliably, thus paving the way for their adoption as non-invasive biomarkers in clinical settings

In summary, our two-stage approach demonstrates that NPY1R-based heteroreceptor complexes in blood faithfully track hippocampal neuroimmune status from an MCI-like prodrome (siRNA) to AD-like dysfunction (OBX). These findings represent an important step toward developing minimally invasive diagnostic tools for early detection and monitoring of Alzheimer's disease, with significant potential for improving therapeutic interventions and patient outcomes.

CRediT authorship contribution statement

Jose Antonio Reyes-Bueno: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. **Jesús Romero-Imbroda:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Miguel Angel Barbancho-Fernández:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Amelia García-Casares:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation. **Carlos Arrabal-Gómez:** Investigation, Methodology. **Narvaez Pelaez Manuel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Isabel**

Moreno-Madrid: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Estela Díaz-Sanchez:** Investigation, Methodology. **Kjell Fuxe:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Data curation, Conceptualization. **Pedro Serrano-Castro:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Funding acquisition, Conceptualization. **Dasiel Borroto-Escuela:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118332.

Data availability statement

The data that support the findings of this study are openly available in Institutional repository of the University of Malaga (RIUMA) and from the corresponding author upon reasonable request.

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