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Experimental Research Article

The effect of Neuropeptide Y Y2 receptor blockade on memory impairment and autophagy in a rat model of Alzheimer's disease

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ABSTRACT

Introduction: While the involvement of neuropeptide Y (NPY) in learning and memory, as well as the role of the Y1 receptor, are well established, the function of the Y2 receptor remains a topic of debate. While the involvement of neuropeptide Y (NPY) in learning and memory, as well as the role of the Y1 receptor, are well established, the function of the Y2 receptor remains a topic of debate. Some studies suggest that NPY may also play a role in autophagy. In our investigation, we aimed to explore whether NPY and its Y2 receptor inhibitor could influence memory modulation or affect Beclin-1 expression in a rat model of Alzheimer's disease (AD). NPY may also have a role in autophagy, according to some studies.

Methods: Intracerebroventricular (i.c.v) injections of amyloid-beta (Aβ1-42, 2µg/µl/side) were used to establish an animal model of AD. NPY (10 ng/µl, 10 µl, i.c.v) was administered 30 minutes before the retrieval. Y2 antagonist BIIE-0246 was injected 15 minutes before NPY administration in the targeted groups. BIIE-0246 was used at three different concentrations (20 nM, 200 nM, and 2 µM). Passive avoidance memory and novel object recognition were both evaluated. Subsequently, Beclin-1 protein expression in the hippocampus was determined using western blot analysis.

Results: It was found that NPY administration improved passive avoidance and cognitive memory in animals treated with Aβ. Injecting BIIE-0246 before NPY did not reverse the improving effect of NPY on passive avoidance and Novel Object Recognition memories. Furthermore, compared to sham-operated animals, Aβ treatment significantly reduced the hippocampal expression of Beclin-1 protein ($P \leq 0.05$), and neither NPY nor NPY Y2 receptor inhibitors affected Beclin-1.

Conclusion: In Aβ-induced memory impairment, it is thought that NPY can improve both aversive and cognitive memory. Blocking NPY Y2 receptors with BIIE-0246 did not alter NPY's memory-enhancing effect.

Keywords:
Neuropeptide Y
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Introduction

Alzheimer's disease (AD) is the most common age-related disorder that lacks an affective cure due to its multifaceted pathogenesis. Characterized by amyloid precursor protein (APP) dysfunction, which progresses to amyloid-β and tau hyperphosphorylation deposits, AD ultimately leads to neuronal demise (Letra and Santana 2017). Evidence suggests that metabolic dysfunction exacerbates or even initiates central damaging signaling pathways in neurodegenerative disorders, as shown by various clinical and experimental studies (Cai et al., 2012).

Neuropeptide Y (NPY), a 36-amino acid peptide, plays a crucial role in regulating energy homeostasis (Zhang et al., 2011). It is offen found in mamalian nervous system. High levels of NPY have been detected in the hippocampus, amygdala, olfactory bulb, striatum, and cerebral cortex of rodents (Duarte-Neves et al., 2016b). NPY influences diverse functions such as food intake, cardiovascular control, learning, memory (Borbély et al., 2013), nociception (Gupta et al., 2018), aging (Botelho and Cavadas 2015), nerve neurogenesis (Spencer et al., 2016), Huntington's disease (van Wamelen et al., 2013), and epilepsy (Decressac and Barker 2012). Furthermore, decreased NPY levels have been found in the CNS of AD patients (Spencer et al., 2016).

NPY interacts with six receptors (Y1, Y2, Y3, Y4, Y5, and Y6), which are coupled with G-protein and either modulate protein kinase A (PKA) activity (Michel et al., 1998) or activate other signaling pathways including phosphatidylinositol-3- kinase (PI3-K), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), or protein kinase C (PKC) (Persaud and Bewick 2014). Many *in vivo* and *in vitro* investigations on distinct excitotoxic degeneration models have suggested a neuroprotective role for NPY (Domin 2021). NPY has neuroprotective effects on amyloid-β-neurotoxicity, which is believed to be mediated by ERK and Akt signaling through Y1 and Y2 receptors (Spencer et al., 2016). A decline in NPY and its receptors in the hippocampus of patients with AD has been revealed (Martel et al., 1990). The decline of NPY and its receptors in the hippocampus of AD patients, as well as reduced plasma NPY levels, are associated with AD onset (Minthon et al., 1996). Animal studies showed that NPY can ameliorate depressive-like behavior and spatial memory deficits (dos Santos et al., 2013). Although Y1 receptors have been shown to inhibit fear learning in certain brain regions such as the amygdala and hippocampus, the role of Y2 receptor remains controversial (Hörmer et al., 2018). It has been shown that administration of Y2 receptor agonists, prior to or simultaneously with neurotoxic substances prevented neuronal cell death caused by AMPA and kainate in rat hippocampal slice cultures (Silva et al., 2003). This neuroprotective effect was blocked by BIIE-0246, a selective Y2 receptor antagonist (Silva et al., 2003; Xapelli et al., 2007).

Autophagy plays a critical role in maintaining cellular homeostasis by degrading aged peptides and organelles. Additionally, it has a key role in clearing Aβ aggregation (Pickford et al., 2008). Beclin-1, a key protein in the autophagy pathway, orchestrates the autophagy-inducing action of Vps34 and aids in recruiting membranes to shape autophagosomes (Jaeger and Wyss-Coray 2010; Komatsu et al., 2007). Reduced Beclin-1 levels have been observed in the brains of AD patients (Rohn et al., 2011; Wei et al., 2008). Furthermore, recent studies suggest that NPY may induce autophagy in various neurodegenerative disease models, potentially yielding neuroprotective effects (Aveleira et al., 2015; Croce et al., 2012). However, more studies are required to support this theory. So, in this study, we evaluated whether the administration of NPY and the inhibition of the Y2 receptor by BIIE-0246 could modulate memory and Beclin-1 expression in the hippocampus of rats.

Materials and Methods

Animals

Adult male Wistar rats (250–300 g) were housed four per cage in the animal facility of Guilan University of Medical Sciences, maintained at a controlled temperature of 22±2 ℃. Rats were subjected to a 12-hour light-dark cycle and provided ad libitum access to food and water. All efforts were made to minimize animal suffering and adhere to ethical guidelines. The study protocol was approved by the "Ethical Committee of the School of Medicine (IR.GUMS.REC.1397.152)", following the principles outlined in the "NIH Guide for the Care and Use of Laboratory Animals."

Experimental procedure

A total of 56 animals were randomly divided into seven experimental groups (n= 8): Control, Aβ, NPY, Aβ+NPY, and Aβ+BIIE+NPY (three groups). BIIE-

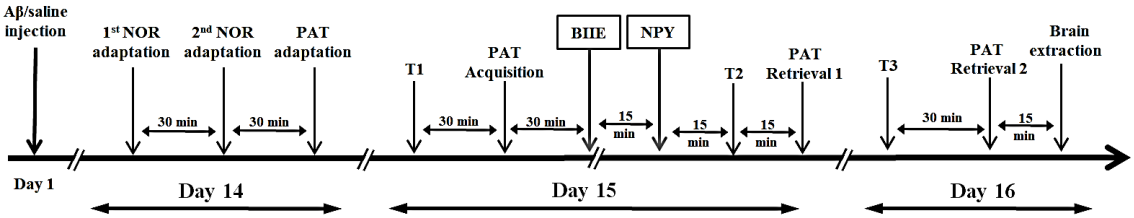

FIGURE 1. Schematic timeline of the experimental procedure. Time intervals between different phases of novel NOR and PAT are shown. T1, T2, and T3 indicate three consecutive phases of the NOR test. NOR: novel object recognition; PAT: passive avoidance task; NPY: Neuropeptide Y; BIIE-0246: NPY Y2 inhibitor.

0246 was administrated at three distinct doses: 20 nM, 200 nM, and 2 µM. The drug was administrated in a volume of 10 µl into the lateral cerebral ventricles (5 µl each side). On the first day, control animals received a bilateral intracerebroventricular (i.c.v) injection of saline, while the Aβ group received the same volume of 2µg/µl/side i.c.v. Aβ (Ashourpour et al., 2020). NPY (10 ng/µl, 10 µl, i.c.v) was administered 30 minutes before the retrieval phase of Passive Avoidance Test (PAT). BIIE-0246 as a Y2 antagonist, was injected 15 minutes before NPY injection in the targeted groups.

Figure 1 depicts the experimental protocol for behavioral tests and medication injections.

Stereotaxic Surgery

A mix of ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) was used to anesthetize the rats. Animals were then placed into a stereotaxic instrument (Stoelting, Chicago, IL, USA). Bilateral cannulation (23-gauge guide cannulae) was performed according to ventricular coordinates: anterioposterior: -0.8, lateral: ±1.6 mm from midline, and DV: −3 mm from the skull surface, based on the atlas of Paxinos and Watson (Paxinos and Watson 2006). ICV injections were adminstrated via a guide cannula using injection needles (30-gauge) connected by polyethylene tubing to a 10 µl Hamilton microsyringe. The injection process lasted for 2 minutes, and the injection needles (extending 1 mm from the end of the guide cannula) were left in place for an additional minute before being slowly withdrawn.

Behavioral test

Novel object recognition (NOR) test

In this experiment, we divided an empty white box (50x50 cm) into 16 equal halves. The NOR test consisted of a habituation, training, and testing phase, conducted over days 14-16, as described elsewhere (Rashtiani et al., 2021b). During habituation, the rats were placed in the white box without any objects for two consecutive five-minute intervals. Twenty-four hours later, training commenced. Two identical items were located at two different places within the cage, and the rats were given five minutes to explore them (T1). Exploration time was recorded for each object, including touching, kissing, licking, chewing, and when the animal's nose was within one centimeter of the object. Retention was assessed at 30 minutes for short-term memory (T2) and 24 hours for long-term memory (T3). During these retention phases, rats explored the objects for 5 minutes in the presence of one familiar (F) and one novel object (N). The times spent exploring familiar and novel objects were recorded separately. Then, the Differentiation Index (DI) was calculated using the formula DI = N / (N + F) *100, where N and F represent the time spent recognizing the novel and familiar objects, respectively.

Passive avoidance task

The step-through passive avoidance apparatus consisted of two chambers (30cm×20cm×20cm each): one transparent plastic room and the other a dark chamber with opaque plastic walls and ceiling. A rectangular space (8cm×8cm), between the two chambers, could be closed with an opaque guillotine door. Stainless steel bars (2 mm in diameter), placed one centimeter apart, formed the floor of both rooms, with the floor of the dark room being electrifiable. To assess memory based on negative reinforcement, the step-through passive avoidance task was employed, as previously described (Rashtiani et al., 2021a).

On day 14, rats were placed in the illuminated room and allowed to freely explore both the dim and bright rooms for 5 minutes to acclimate to the apparatus. The

habituation process was repeated after 30 minutes.

On the 15th day, following shuttle box habituation, rats were placed in the illuminated room, and after a 10-second delay, the guillotine door was raised, and the crossover latency was recorded. When a rat entered the dark compartment, the door was closed behind it, and a shock (50Hz, 1mA, 3s duration) was applied (Mazrooie et al., 2017). Immediate memory was assessed after 5 minutes of training. Retention tests were conducted 1 and 24 hours post-training to evaluate short- and long-term memory. Rats were placed in the illuminated room, and after a 10-second delay, the guillotine door was opened. Step-through latency (STL) and time spent in the dark compartment (TDC) were measured for up to 300 seconds. No electric shocks were administered during this session.

Western Blotting

After completing the behavioral assessments, the animals were sacrificed by a guillotine, and their hippocampi were dissected and processed for western blotting. Tissues from the brain were lysed and homogenized using a lysis buffer containing Tris-HCl, NaCl, sodium deoxycholate, SDS, EDTA, Triton x-100, and a cocktail protease inhibitor (pH 8.0). The protein concentration was determined using the Bradford method with bovine serum albumin as the calibration standard. For SDS-PAGE, total proteins were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The separated protein bands were then transferred to PVDF membranes using western blotting equipment (Amersham Bioscience; USA) and probed with specific antibodies. Beclin-1 expression in rat hippocampi was assessed using an antibody against Beclin-1 (diluted at 1:1000, mouse monoclonal antibody, SC-8038, Santa Cruz Biotechnology, Inc). As a positive control, Beta-actin was used (diluted: 1:1000, mouse monoclonal, SC-47778, Santa Cruz Biotechnology, Inc). Discovery of the primary antibodies was performed using a horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, Razi, Tehran, Iran). Chemiluminescence was used to detect immunoreactive polypeptides (Amersham Biosciences, USA) followed by autoradiography. Band densities were quantified using densitometric scans with Image J software (NIH, USA).

Statistical analysis

The data were analyzed using Prism 9 software and are presented as mean ± standard error of the mean (SEM). The Kruskal-Wallis test with the Dunn test was employed when the Shapiro-Wilk test confirmed that the distribution was not normal. Alternatively, one-way analysis of variance (ANOVA) was utilized, and significance was assessed using Tukey's post hoc test. A significance level of $P \leq 0.05$ was considered for all comparisons to determine statistical significance.

Results

Passive avoidance memory

The results revealed a significant difference in STL and TDC among groups in the early retrieval test ($P < 0.05$, Fig. 2A & B). As shown, the injection of 10 µl NPY (10 ng/µl) into the lateral ventricles increased STL from 6.75± 0.72 sec in Aβ treated rats to 246.8±23.24 sec in the Aβ+NPY group and decreased TDC from 216.4±19.27 to 20±10.79 sec, indicating a significant memory improvement ($P \leq 0.05$). Also, NPY injection into intact animals increased STL ($P \leq 0.01$) and decreased TDC ($P \leq 0.001$) compared to the Aβ group. In addition, the injection of BIIE-0246, a potent selective non-peptide NPY Y2 receptor antagonist, did not significantly change STL or TDC in the early retrieval test (Fig. 2A & B).

Also, twenty-four hours later, STL and TDC exhibited a significant difference between groups ($P<0.05$, Fig. 2C & D). Although memory improvement was observed as STL increased and TDC decreased, the late retrieval scores were not statistically significant (Fig.2C, D). Also, STL increased and TDC decreased significantly in the NPY group compared to the Aβ group ($P \leq 0.05$). In addition, the injection of BIIE-0246 did not significantly change STL or TDC in the late retrieval test.

Novel Object Recognition

The results indicated a significant difference in the differentiation index (DI) among the various groups ($P<0.05$; Fig. 3). The administration of NPY significantly increased the DI ($P \leq 0.05$) from 44.6 ± 4.85 in Aβ-treated rats to 64.17 ± 4.98 in Aβ+NPY treated rats. Additionally, the DI was significantly increased in the NPY group compared to the Aβ group ($P \leq 0.05$). However, the DI did not change significantly after BIIE-0246 treatments.

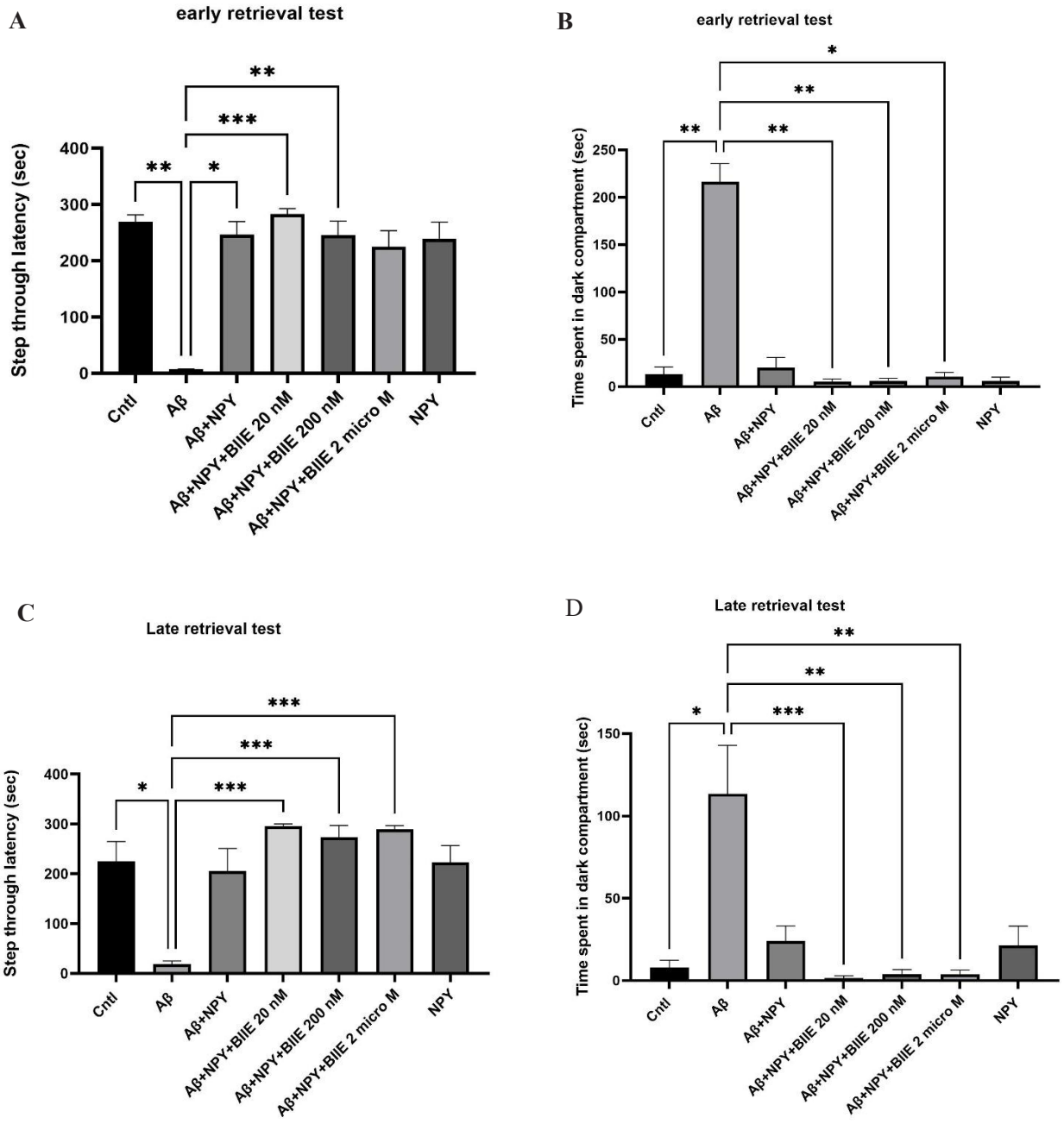

FIGURE 2. Effect of NPY and BIIE-0246 on passive avoidance memory in early and late retrieval test. Changes in the step-through latency (A) and total time in the dark compartment (B) one hour after acquisition. Changes in the step-through latency (C) and total time in the dark compartment (D), 24 hours after acquisition. Data are presented as mean ± SEM ***P<0.001, **P<0.01, **P<0.05, *P<0.05, (Kruskal-Wallis; Dunn test, n =8).

Western blot analysis of Beclin-1 hippocampal expression

As depicted in Fig. 4, the expression level of Beclin-1 in hippocampal tissue significantly varied among groups ($P < 0.05$). The analysis revealed that Aβ treatment notably suppressed the relative hippocampal expression of Beclin-1 protein compared to the sham-operated animals ($P \leq 0.05$). However, the administration of NPY to Aβ-treated rats did not induce significant changes in Beclin-1 expression ($P \leq 0.05$). Additionally, i.c.v. injection of different doses of BIIE-0246 did not affect Beclin-1 expression levels.

Discussion

The results of this study demonstrated that i.c.v. injection of Aβ1-42 reduced passive avoidance and rec-

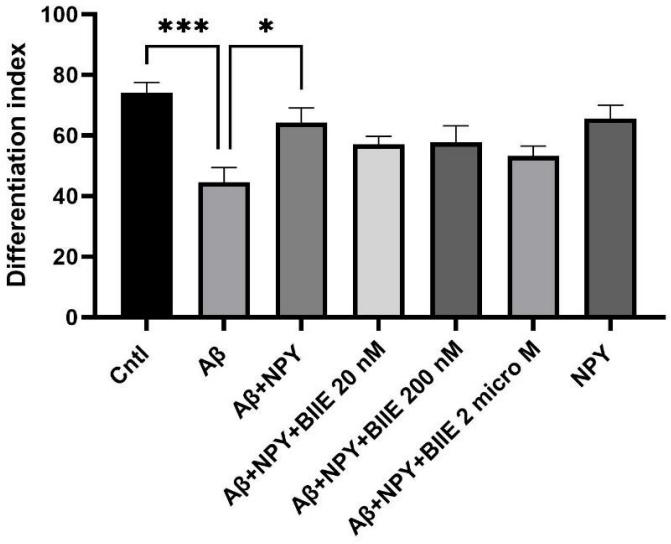

FIGURE 3. Effect of NPY and BIIE-0246 on the Differentiation Index (DI) in the novel object recognition test. Data are presented as mean ± SEM ***P<0.01,*P<0.05, (ANOVA one way; tukey-hoc test, n = 8).

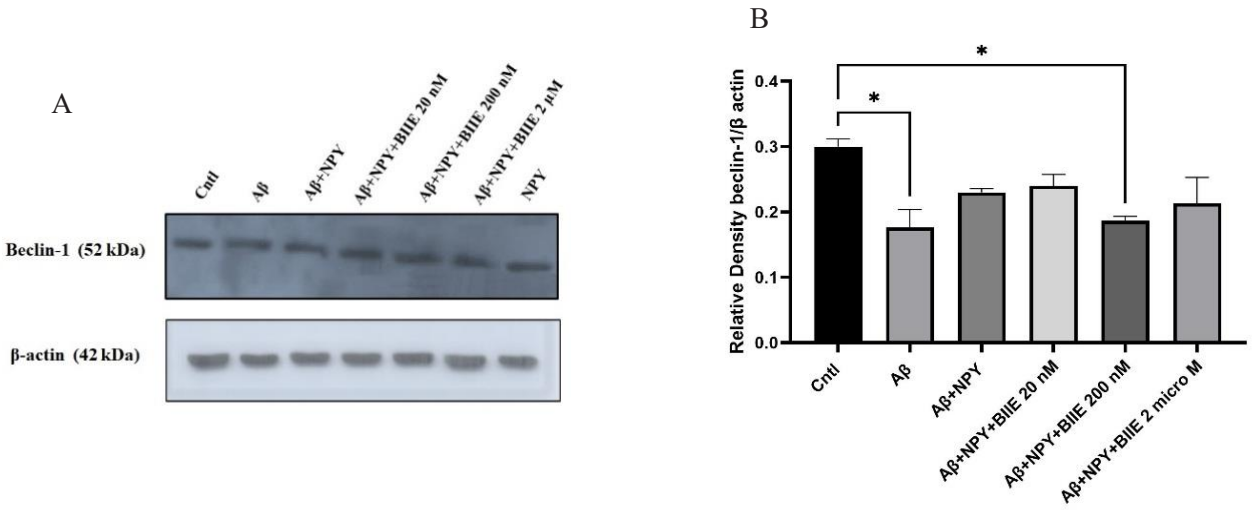

FIGURE 4. Altered Beclin-1 expression in the hippocampus of the rat. Representative western blot analysis comparing the expression of Beclin-1 in different groups (A). Densitometric comparison of the average expression of Beclin-1(B). Beta-actin was used for the normalization of protein loading. (ANOVA, tukey-hoc test; *P<0.05, n=4).

ognition memory, as well as the expression of Beclin-1 protein. Furthermore, NPY successfully corrected the cognitive deficits caused by Aβ1-42, but this restoration was not reversed when the Y2 receptor was inhibited.

Accumulation of Aß1-42 in the brain is known to lead to the formation of senile plaque, which damages synapses and causes cognitive impairment (Sadigh-Eteghad et al., 2015). Additionally, this peptide disrupts mitochondrial function, calcium homeostasis, induces oxidative stress, and promotes apoptosis (Carrillo-Mora et al., 2014). Furthermore, i.c.v. administration of Aβ1-42 leads to an increase in endoplasmic reticulum (ER) stress and associated proteins, such as glucose-regulated protein-78 (GRP78) and eukaryotic translation initiation factor-2 (eIF2), along with choline acetyltransferase (ChAT) activity (Goswami et al., 2020). Previous reports have indicated that Aβ1-42 impairs the structural integrity and degradative activity of autophagic vesicles, prompting our investigation into the role of NPY and its Y2 receptor in the dementia process by directly injecting Aβ1-42 into the cerebral ventricles.

Our study found that NPY treatment could mitigate the cognitive and aversive memory deficits induced by i.c.v. Aβ injection. Prior research has suggested that

exogenous NPY may exert a neuroprotective effect in animal models of neurodegenerative disorders such as Parkinson's disease (Cannizzaro et al., 2003) and AD (Nilsson et al., 2001). In a neuroblastoma cell line, Croce et al. found that NPY inhibited Aβ protein (fragment 25–35)-induced cell loss (Croce et al., 2011). They also discovered that NPY could reverse the effects of Aβ on intracellular nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Croce et al., 2012). Smialowska et al. earlier reported the neuroprotective function of NPY, demonstrating that various NPY receptors inhibited kainate/AMPA-induced neuronal cell death in the hippocampus. In addition, intrahippocampal NPY injection reduced the severity of damage caused by intrahippocampal kainate injection (Smiałowska et al., 2003).

In mammals, NPY is predominantly found in brain regions most affected by the pathological mechanisms of AD, including the cerebral cortex and hippocampus. Additionally, Y1, Y2, and Y5 receptors are expressed in these brain regions (Duarte-Neves et al., 2016a).The role of Y1 and Y5 receptors in memory improvement has been demonstrated in various studies (Bertocchi et al., 2021; Borroto-Escuela et al., 2022; Rangani et al., 2012). Therefore, we utilized BIIE-0246, a potent and selective Y2 receptor antagonist, to investigate the involvement of Y2 receptors in mediating the memory-improving effect of NPY. Our data revealed that blocking the Y2 receptor did not diminish the memory-improving effect of NPY. However, the outcomes of Y2 receptor studies have been conflicting. For example, Smialowska et al. demonstrated that intrahippocampal administration of NPY Y2 and Y5 agonists provided neuroprotection even 1 hour after kainate injection (Śmiałowska et al., 2009). Silva et al. found that activation of the Y2 receptor alone was sufficient to protect CA1 pyramidal cells from AMPA-induced neurodegeneration (Silva et al., 2003). This suggests that hippocampal Y2 receptors may be involved in NPY's memory-enhancing function. However, it should be noted that BIIE-0246 has modest antagonist activity due to its high molecular weight, low solubility, and partial receptor occupancy (Mittapalli and Roberts 2014). Despite the findings by Fendt et al. that Y2 receptor activation can prevent fear memory consolidation (Fendt et al., 2009), Verma et al. in 2012 discovered that NPY knockout mice showed a significant acceleration in Pavlovian fear conditioning, while Y2 receptor deletion had no effect on fear learning (Verma et al., 2012). Additionally, Hormer et al. confirmed that deleting the Y2 receptor enhanced contextual fear memory and improved working and spatial memory (Hörmer et al., 2018), which aligns with our findings indicating that blocking NPY Y2 receptors improves cognitive and aversive memory. However, other studies found no differences in Y2 receptor-deficient mice's conditioned fear memory (Pickens et al., 2009), working memory, or reference memory (Karl et al., 2010).

In the current study, NPY/BIIE was administered before the retrieval test and demonstrated the ability to enhance retrieval memory. The effects of NPY on learning and memory appear to be biphasic, showing distinct anatomical and temporal characteristics, which support a regulatory role for NPY in maintaining systemic homeostasis (Gøtzsche and Woldbye 2016). According to reports, NPY weakens acquisition but improves consolidation and retention in various animal models of memory deficit (Gøtzsche and Woldbye 2016; Ishida et al., 2007). Additionally, NPY or Y1 receptor knockout mice showed enhanced acquisition of cued fear conditioning, whereas Y2 receptor deficiency did not affect it (Akanmu et al., 2006). Moreover, Y2 knockout mice exhibited no deficits in acquisition or memory retention in the light/dark passive avoidance test (Karl et al., 2010).

Presynaptic Y2 receptors are predominantly found in regions such as the frontal cortex, hippocampus, lateral septum, and lateral hypothalamus (Shende and Desai 2020). The memory-enhancing effect of BIIE-0246 could be attributed to hippocampal presynaptic Y2 receptors, which act as autoreceptors and exert a negative modulatory role on neurotransmitter release by regulating potassium channel opening and calcium channel closing (Stanić et al., 2011). Méndez-Couz et al. demonstrated that blocking Y2 receptors improves spatial memory acquisition in a water maze task and enhances probe test efficiency. They also noted that Y2 receptor antagonism led to an increase in hippocampal Y2 receptor expression while decreasing it in the prefrontal cortex (Méndez-Couz et al., 2021). Recently, Kornhuber et al. reported that NPY extends non-social memory, with Y1 receptors mediating memory-enhancing effects in the dorsolateral septum, while Y2 receptors improve memory in the medial amygdala (Kornhuber and Zoicas 2020). However, BIIE-0246 has been shown to have off-target effects at alpha-1A adrenergic recep-

tors (Brothers et al., 2010), which positively modulate both short- and long-term synaptic plasticity (Doze et al., 2011) and exhibit cognitive-enhancing effects (Perez 2020).

A Western blot analysis revealed a decrease in Beclin-1 expression in the hippocampus of Aβ-treated rats, indicating a reduction in autophagic processes in the Alzheimer's model, which aligns with previous reports suggesting autophagy impairment as a pathophysiological mechanism in neurodegenerative disorders (Komatsu et al., 2006). Pickford et al. also observed a decrease in brain expression of Beclin-1 in AD patients, as well as a reduction in Beclin-1 expression in transgenic mice expressing human amyloid precursor protein (APP) (Pickford et al., 2008). Although i.c.v. injection of NPY increased hippocampal Beclin-1 compared to Aβ-treated rats, there was no significant difference observed when comparing Aβ+NPY treated rats. According to Aveleira et al., NPY stimulates autophagy in rat-differentiated hypothalamic neural cells and in a mouse hypothalamic neuronal cell line, in addition to increasing LC3II and lowering p62. They also stated that activation of Y1 or Y5 receptors, but not Y2 receptor, exerts this effect (Aveleira et al., 2015). However, changes in Beclin-1 expression alone may not be sufficient evidence, as phosphorylation of Bcl-2 at Thr 69, Ser 70, and Ser 87 residues promotes its separation from Beclin-1 and triggers autophagy, according to prior studies (Wei et al., 2008). Similarly, phosphorylation of Beclin-1 at the BH3 domain's Thr 119 promotes autophagy, whereas phosphorylation of this peptide at the Thr108 residue by a pro-apoptotic kinase (Mst1) improves the association between Beclin-1 and Bcl-2/Bcl-xl. This procedure stabilizes the Beclin-1 homodimer, inhibits autophagy, and lowers PI3K kinase activity (Xu and Qin 2019). On the other hand, NPYhas various neuroprotective properties, such as reducing neuroinflammation through nerve growth factor production, promoting neurogenesis, and reducing excitotoxicity (Aveleira et al., 2015). Consequently, further studies are required to fully understand the role of NPY in autophagy. Additionally, BIIE-0246 did not alter memory retrieval in the present study, suggesting that its practical applicability may be limited due to its high molecular weight and low brain penetration. However, intranasal administration of peptide compounds has been reported to affect brain tissue (Fatoba et al., 2018). Other Y2 receptor antagonists, such as JNJ-5207787 or SF-11, can reach the brain and be delivered through various peripheral routes.

Taken all together, we found that NPY can improve both aversive and cognitive memory. The blockade of Y2 receptor by BIIE-0246 had no effect on NPY's memory-improving effect. Despite the fact that Aβ therapy reduced hippocampal Beclin-1 expression, NPY was unable to compensate for it. In this model of dementia, the memory-improving characteristic of NPY could not be linked to autophagy regulation. Further investigation is needed to clarify the presynaptic involvement of Y2 receptor in the regulation of memory processes in various dementia models.

Acknowledgements

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Conflict of interest

There is no conflict of interest for any of the contributing authors. The authors alone are responsible for the content and writing of the paper.

Ethics approval

The guidelines of "institutional Guide for Care and Use of Laboratory Animals" were followed in all the experiments on animals. Furthermore, our experiments were approved by the Ethics Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1397.152)

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