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The action of neuropeptide AF on passive avoidance learning. Involvement of neurotransmitters

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ABSTRACT

Neuropeptide AF (NPAF) is an amidated octadecapeptide, which is member of the RFamide peptide family. NPAF is encoded by the farp-1 gene and acts through the G protein coupled NPFF-1 and NPFF-2 receptors. NPAF is involved in several physiological functions of the central nervous system, however we have little evidence about the involvement of NPAF in learning and memory. Therefore, the aim of the present study was to investigate the action of NPAF on consolidation of memory in a passive avoidance learning paradigm in mice. We have also investigated the underlying neurotransmissions and the action of NPAF on β-amyloid-induced memory impairment. Accordingly, mice were pretreated with a nonselective muscarinic acetylcholine receptor antagonist, atropine, a non-selective 5-HT2 serotonergic receptor antagonist, cyproheptadine, a mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide, a D2, D3, D4 dopamine receptor antagonist, haloperidol, a non-selective opioid receptor antagonist, naloxone, a nitric oxide synthase inhibitor, nitro-L-arginine, a $\alpha_1/\alpha_{2\beta}$-adrenergic receptor antagonist, prazosin, a nonselective β-adrenergic receptor antagonist, propranolol or β-amyloid 25–35 in combination with NPAF administration. Our results demonstrate for the first time that NPAF improves the consolidation of passive avoidance learning. This effect is mediated through muscarinic cholinergic, 5HT1- and 5HT2-serotoninergic, dopaminergic, nitrergic and α- and β-adrenergic neurotransmissions, but not by opioid transmission, since atropine, cyproheptadine, methysergide, haloperidol, nitro-L-arginine, prazosin and propranolol reversed the action of NPAF, whereas naloxone was ineffective. The present study also shows that NPAF reverses the β-amyloid 25–35-induced memory impairment.

1. Introduction

Neuropeptide AF (NPAF, A18F amide, AGEGLSSPFWSLAAPQRFamide) is an amidated octadecapeptide, which was isolated together with neuropeptide FF (NPFF, F8F amide, FLFQPQRFamide) from bovine brain (Yang, Fratta, Majane, & Costa, 1985). Subsequently, another RFamide peptide, the neuropeptide SF (NPSF, SLAAPQRFamide), was isolated from rodent spinal cord (Bonnard et al., 2001). NPAF, NPFF and NPSF signal through two $G_{i/o}$-protein coupled receptors (GPCRs), known as NPFF-2 (GPR74, NPGPR, HLWAR77) and NPFF-1 (GPR147, OT7T022) (Fukusumi, Fujii, & Hinuma, 2006). These peptides play role in several physiological functions, including the regulation of nociception (Kavaliers, 1990; Yang et al., 1985; Yudin, Tamarova, & Krishtal, 2006), insulin and somatostatin release (Fehmann et al., 1990), food and water intake (Newmyer & Cline, 2009; Newmyer, Siegel, & Cline, 2010), adipocyte metabolism (van Harmelen et al., 2010), motility of colon (Raffa & Jacoby, 1989), body temperature (Desprat & Zajac, 1997), blood pressure (Roth, Disimone, Majane, & Yang, 1987), locomotion and releases of CRF, ACTH and corticosterone (Jaszberenyi, Bagosi, Csabafi, Palotai, & Telegdy, 2014; Jaszberenyi et al., 2009), anxiety and depression (Palotai, Telegdy, Tanaka, Bagosi, & Jaszberenyi, 2014). There is a growing base of evidence revealing the involvement of RFamide peptides in learning and memory as well. In 1993, Kavaliers and Colwell (1993) were the first who reported that a lower dose (1 μg) of NPFF improves, whereas a higher dose (10 μg) impairs long-term spatial memory acquisition in Morris water maze test (Kavaliers & Colwell, 1993). In 2010, Betourne et al. demonstrated that the non-selective NPFF receptor agonist 1DMe (D-Tyr1(NMe)Phe3]NPFF) impairs both the short-term retention in object location task and the long-term spatial memory in Morris water maze test (Betourne et al., 2010).

The expression and distribution of RFamide peptides and their receptors have been described particularly in the central nervous

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system (CNS). NPAF and NPFF are proteolytic products of their neuropeptide precursor encoded by the farp-1 gene, whereas NPFF-2 and NPFF-1 receptors are encoded by the frf-3 gene (Fukusumi et al., 2006). NPAF and NPFF are expressed in the hypothalamus and in the nucleus of the solitary tract. Immunoreactive fibers and terminals were found in several brain regions, such as the lateral septum, amygdala, hypothalamus, neurohypophysis, thalamus, periaqueductal gray, and several medullary nuclei (Kivipelto, Majane, Yang, & Panula, 1989). Expression of NPFF-1 and NPFF-2 receptors have been identified in brain sites, which directly or indirectly influences behavior, cognition and memory. In particular, NPFF receptors were identified in the septal nucleus, bed nucleus of stria terminalis (BNST), posteromedial cortical amygdaloid nucleus, parafascicular thalamic nucleus, medial mammillary nucleus, CA3 region of the ventral hippocampus (Bonini et al., 2000) and in distinct cortical areas (Gouarderes, Puget, & Zajac, 2004). Taking into consideration all the behavioral and autoradiographic data, we can assume that the RFamide peptides can be involved in learning and memory processes. Although the effect of NPFF on memory and the distribution of NPFF receptors have been investigated particularly, the action of NPAF on learning and memory remains to be elucidated.

Most neuropeptides are co-expressed with at least one classic neurotransmitter in the CNS. Generally, neuropeptides behave as neuromodulators exerting multiple actions on physiological brain functions and, consequently, on behavior. Their effects involve changes in membrane excitability, gene transcription, receptor affinity and in modulation of neurotransmitter release (Ogren, Kuteeva, Elvander-Tottie, & Hokfelt, 2010). It has been demonstrated that NPFF controls the release of serotonin, glutamate and GABA in the medial prefrontal cortex (mPFC) (Chen, Li, Liang, & Huang, 1999), whereas NPAF stimulates the release of dopamine in the amygdala and the striatum (Jaszberenyi et al., 2009). Another study also revealed that activation of NPFF receptors interferes with dopaminergic, serotoninergic and opioid transmissions (Huang, Li, Wong, Tan, & Chen, 2002). However, the involvement of distinct neurotransmissions in the action of NPAF on memory formation remains to be clarified.

β-amyloid plays a key role in the pathology of Alzheimer's disease, which is the most common form of dementia (Kanekiyo & Bu, 2014). Accordingly, behavioral investigations, using rodent models, revealed the inhibitory action of β-amyloid on memory consolidation (Chen, Wright, & Barnes, 1996; Telegdy, Tanaka, & Schally, 2009). The β-amyloid-induced neurotoxicity has been associated with oxidative stress, apoptosis, impaired mitochondrial function and receptor mediated effects (Bossy-Wetzel, Schwarzenbacher, & Lipton, 2004). A recently published study suggested that kisspeptin – which is also a member of the RFamide peptide family – can prevent the β-amyloid-induced neurotoxicity (Milton, Chilumuri, Rocha-Ferreira, Nercessian, & Ashioti, 2012). However, there is no published data about the action of NPAF on β-amyloid-induced memory impairment.

The aim of the present study was to clarify the involvement of NPAF in learning and memory. Therefore, we investigated the action of NPAF on memory consolidation in a passive avoidance learning paradigm in mice. We also investigated the underlying neurotransmissions and the action of NPAF on the β-amyloid-induced memory impairment. Accordingly, mice were pretreated with a nonselective muscarinic acetylcholine receptor antagonist, atropine, a non-selective 5-HT2 serotonergic receptor antagonist, cyproheptadine, a mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide, a D2, D3, D4 dopamine receptor antagonist, haloperidol, a non-selective opioid receptor antagonist, naloxone, a nitric oxide synthase inhibitor, nitro-L-arginine, a selective α_1-adrenergic receptor antagonist, prazosin, a nonselective β-adrenergic receptor antagonist, propranolol or β-amyloid 25–35 in combination with NPAF administration.

2. Methods and materials

2.1. Experimental animals and ethics statement

Male CFLP mice (Mus musculus, Bioplan Isaszeg, Hungary), weighing 25–28 g were used. The animals were maintained and treated during the experiments in accordance with the instructions of the Ethical Committee for the Protection of Animals in Research of the University of Szeged (Szeged, Hungary), which specifically approved this study. The mice were kept in their home cages at a constant temperature (23 °C) on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6:00 AM). Commercial food and tap water were available ad libitum. To minimize the effects of nonspecific stress, the mice were handled daily. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

2.2. Surgery

For intracerebroventricular (i.c.v.) administration, the mice were implanted with a 10 mm long stainless steel Luer canulla (prepared from a hypodermic Luer needle of 20 G × 1.5 in., Henke-Sass Wolf, Tuttlingen, Germany) aimed at the right lateral cerebral ventricle under sodium pentobarbital (Nembutal, 35 mg/kg, intraperitoneally, i.p.) anesthesia. The stereotaxic coordinates were 0.2 mm posterior; 0.2 mm lateral to the bregma; 2.0 mm deep from the dural surface. Cannulas were secured to the skull with dental cement and acrylate. The i.c.v. treatment was applied via the cannula in 2 μl bolus injection. Post-operative care was provided in order to ensure the full recovery of the animals. To monitor anesthetic recovery, someone was always present with the animals recovering from anesthesia until the mice were ambulatory. Nursing support was also provided including quiet, darkened resting place, timely wound maintenance, increased ambient warmth, a soft resting surface, rehydration with oral fluids, and a return to normal feeding through the use of highly palatable foods. The mice were used after a recovery period of 5 days.

After the experiments, methylene blue was injected into the lateral ventricle, then the animals were decapitated and the brains were dissected to verify the permeability of the cannulas. Only animals with correctly located cannulas were used for statistical evaluation.

2.3. Treatments

NPAF (purchased from Bachem Inc., Switzerland) was applied via the i.c.v. cannula in a dose of 0.5 or 1.0 or 2.0 μg/animal. For combined treatment, only 1.0 μg NPAF was used. NPAF was administered after the learning trial.

Receptor blockers were applied immediately after the learning trial, followed 30 min later by NPAF administration. The following receptor blockers were used: atropine sulfate (EGYS, Budapest, Hungary), 2 mg/kg i.p.; cyproheptadine hydrochloride (Tocris, Bristol, UK), 5 mg/kg i.p.; methysergide hydrogen maleate (Sandoz, Cologne, Germany), 5 mg/kg i.p.; haloperidol (G. Richter, Budapest, Hungary) 10 μg/kg i.p.; naloxone hydrochloride (Endo Labs, Wilmington, USA), 0.3 mg/kg i.p.; nitro-L-arginine methylester hydrochloride (Sigma–Aldrich Inc., St. Louis, USA), 10 μg/2 μl i.c.v.; prazosin hydrochloride (Tocris, Köln, Germany), 62.5 μg/kg i.p. and propranolol hydrochloride (ICI Ltd., Macclesfield, UK), 5 mg/kg i.p. The doses of the receptor blockers were selected on the basis

of our earlier experience as being effective when administered with other neuropeptides, but not affecting the paradigm per se (Telegdy & Adamik, 2002, 2013).

β-amyloid 25–35 (obtained from Bachem Inc., Switzerland) was applied via the i.c.v. cannula in a dose of 1.0 μg/animal. β-amyloid 25–35 was administered simultaneously with NPAF after the learning trial. The dose selection of β-amyloid 25–35 is based on previous experiments (Telegdy et al., 2009).

The control animals received 2 μl saline i.c.v. and 2 ml saline i.p.

2.4. Behavioral testing

2.4.1. Passive avoidance test

One-trial learning, step-through passive avoidance behavior was measured according to Ader, Weijnen, & Moleman (1972). The apparatus consists of two separate chambers connected through a guillotine door. One of the chambers was illuminated, while the other was dark. On Day 1 of the experiment, mice were placed on the illuminated platform and allowed to enter the dark compartment (learning trial). Since mice prefer dark to light, they normally entered within 5 s. On Day 2 (24 h later), two additional learning trials were delivered. The intertrial interval was 5 min. After the second trial, unavoidable mild electric footshocks (0.75 mA, 2 s) were delivered through the grid floor. The guillotine door was closed immediately after the mouse entered the dark chamber and the animals could not escape the footshock. After this single trial, the mice were immediately removed from the apparatus and were treated. The consolidation of passive avoidance behavior was tested 24 h later (Day 3). For consolidation, the animals were treated with NPAF following the learning trial. In the series of experiments with the receptor blockers, animals were treated first with the receptor antagonist and 30 min later with NPAF. In the experiments with β-amyloid 25–35, animals were treated with β-amyloid 25–35 simultaneously with NPAF following the learning trial. In the 24 h testing each mouse was placed on the platform and the latency to enter the dark compartment was measured up to a maximum of 300 s.

2.5. Statistical analysis

Statistical analyses of the experiments with different receptor antagonists were performed using SigmaPlot (version 11.0, Systat Software, Inc.). One-way analysis of variance (ANOVA) and Tukey's post hoc comparison test were used if the data showed normal distribution (by Kolmogorov–Smirnov test) and equal variance. Kruskal–Wallis ANOVA and Dunn's post hoc comparison test were used if the data were not normally distributed and/or the variances were not equal. In the figure captions, values are presented as means ± standard error of the mean (SEM). A probability level of 0.05 or less was accepted as indicating a statistically significant difference.

3. Results

NPAF improved significantly the consolidation of passive avoidance learning in a dose of 1.0 μg/2 μl i.c.v., whereas the 0.5 μg/2 μl and the 2.0 μg/2 μl doses were ineffective [One-way ANOVA: $F(3,35) = 9.53$; $p < 0.01$] (Tukey's post hoc test: $p < 0.05$ for 1.0 μg NPAF vs. control) (Fig. 1). In the series of experiments with atropine, NPAF facilitated the consolidation of passive avoidance learning.

Atropine (2 mg/kg i.p.) alone had no action, while atropine pretreatment fully blocked the effect of NPAF on memory consolidation [One-way ANOVA: $F(3,54) = 7.41$; $p < 0.001$] (Tukey's post hoc test: $p < 0.01$ for NPAF vs. control and $p < 0.05$ for combined vs. NPAF) (Fig. 2).

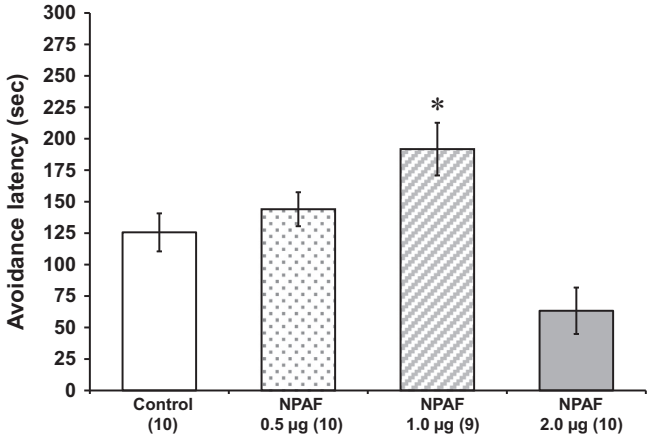

Fig. 1. The effects of different doses of NPAF on the consolidation of passive avoidance learning. NPAF (0.5 μg/2 μl, i.c.v.), NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, NPAF (2.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

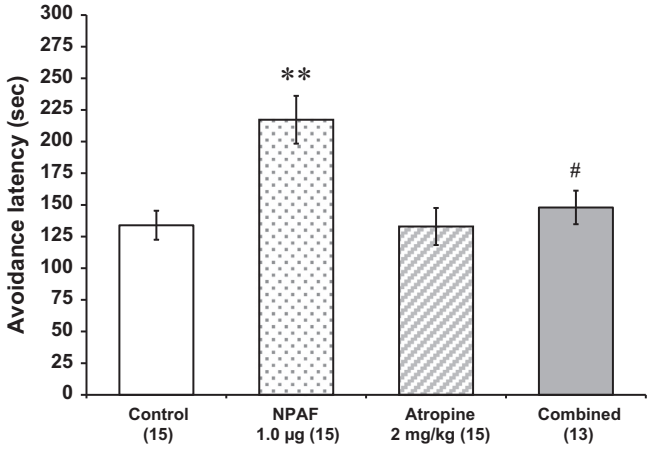

Fig. 2. The effects of the non-selective muscarinic cholinergic receptor antagonist, atropine on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{**}p < 0.01$ vs. control, atropine (2 mg/kg, i.p.), combined (atropine 2 mg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

In the experiments with cyproheptadine, NPAF improved the consolidation of passive avoidance learning. Cyproheptadine (5 mg/kg i.p.) itself had no action, while pretreatment with cyproheptadine completely reversed the NPAF-induced memory consolidation [One-way ANOVA: $F(3,16) = 9.44$; $p < 0.001$] (Tukey's post hoc test: $p < 0.01$ for NPAF vs. control and $p < 0.01$ for combined vs. NPAF) (Fig. 3).

In the experiments with methysergide, NPAF increased the avoidance latency. Methysergide (5 mg/kg i.p.) alone had no significant action, but methysergide pretreatment reversed the effect of NPAF on memory consolidation [One-way ANOVA: $F(3,32) = 8.33$; $p < 0.001$] (Tukey's post hoc test: $p < 0.05$ for NPAF vs. control, $p < 0.01$ for combined vs. NPAF and $p < 0.05$ for combined vs. methysergide) (Fig. 4).

In the series of experiments with nitro-L-arginine, NPAF facilitated the consolidation of passive avoidance learning. Nitro-L-arginine (10 μg/2 μl i.c.v.) itself had no action, while pretreatment with nitro-L-arginine fully blocked the action of NPAF on consolidation of memory [One-way ANOVA: $F(3,16) = 6.4$; $p < 0.01$] (Tukey's post hoc test: $p < 0.01$ for NPAF vs. control and $p < 0.05$ for combined vs. NPAF) (Fig. 5).

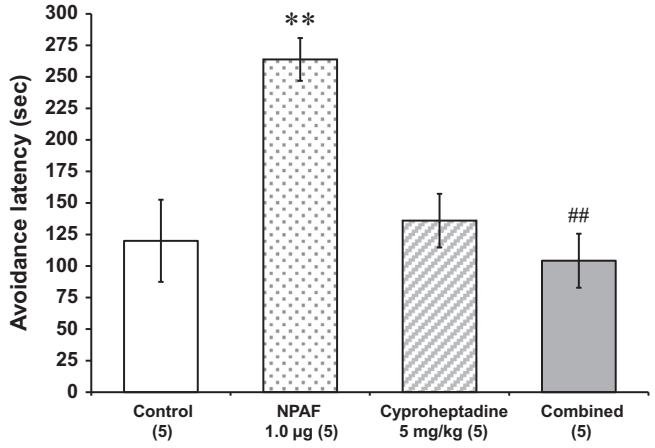

Fig. 3. The effects of the non-selective 5-HT2 serotonergic receptor antagonist, cyproheptadine on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{**}p < 0.01$ vs. control, cyproheptadine (5 mg/kg, i.p.), combined (cyproheptadine 5 mg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#\#}p < 0.01$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

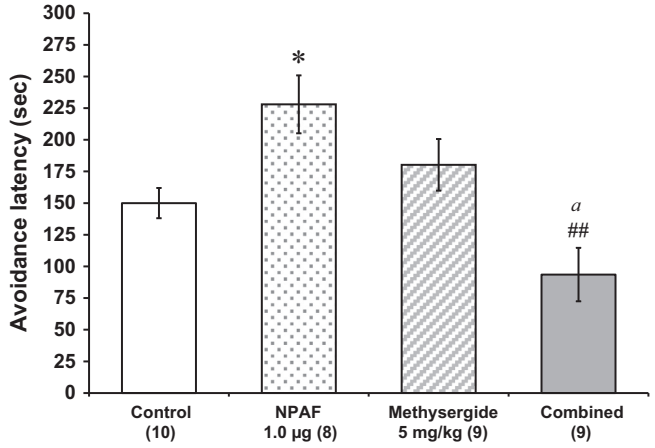

Fig. 4. The effects of the mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, methysergide (5 mg/kg, i.p.), combined (methysergide 5 mg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#\#}p < 0.01$ vs. NPAF (1.0 μg/2 μl, i.c.v.) and $^{a}p < 0.05$ vs. methysergide (5 mg/kg, i.p.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

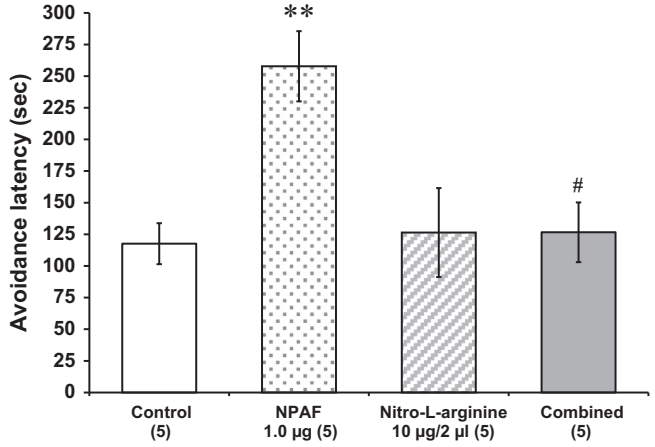

Fig. 5. The effects of the non-specific nitric oxide synthase (NOS) inhibitor, nitro-L-arginine on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{**}p < 0.01$ vs. control, nitro-L-arginine (10 μg/2 μl, i.c.v.), combined (nitro-L-arginine 10 μg/2 μl, i.c.v. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

In the series of experiments with prazosin, NPAF improved the consolidation of passive avoidance learning. Prazosin (62.5 μg/kg i.p.) alone had no significant effect, while prazosin pretreatment completely reversed the NPAF-induced memory consolidation [One-way ANOVA: $F(3,16) = 5.43$; $p < 0.01$] (Tukey's post hoc test: $p < 0.05$ for NPAF vs. control and $p < 0.05$ for combined vs. NPAF) (Fig. 6).

In the experiments with propranolol, NPAF increased the avoidance latency. Propranolol (5 mg/kg i.p.) itself had no action, while propranolol pretreatment blocked the action of NPAF on consolidation of memory [Kruskal–Wallis ANOVA: $H(3,36) = 19.26$; $p < 0.001$] (Dunn's post hoc test: $p < 0.05$ for NPAF vs. control, $p < 0.05$ for NPAF vs. propranolol and $p < 0.05$ for combined vs. NPAF) (Fig. 7).

In the series of experiments with haloperidol, NPAF facilitated the consolidation of passive avoidance learning. Although this effect did not reach statistical significance ($p > 0.05$) compared to the control group, pretreatment with haloperidol reversed significantly the effect of NPAF on memory consolidation [Kruskal–Wallis ANOVA: $H(3,56) = 9.25$; $p < 0.05$] (Dunn's post hoc test: $p < 0.05$ for combined vs. NPAF) (Fig. 8). Haloperidol (10 μg/kg i.p.) itself had no significant effect.

In the experiments with naloxone, NPAF improved the consolidation of passive avoidance learning. Naloxone (0.3 mg/kg i.p.) alone had no action and pretreatment with naloxone did not reverse the action of NPAF on memory consolidation [One-way ANOVA: $F(3,35) = 4.35$; $p < 0.05$] (Tukey's post hoc test: $p < 0.05$ for NPAF vs. control) (Fig. 9).

β-amyloid 25–35 (1 μg/2 μl i.c.v.) impaired significantly the consolidation of passive avoidance learning [One-way ANOVA: $F(3,26) = 19.53$; $p < 0.001$]. NPAF reversed fully the β-amyloid 25–35-induced impairment of memory (Tukey's post hoc test: $p < 0.05$ for NPAF vs. control, $p < 0.05$ for β-amyloid 25–35 vs. control, $p < 0.05$ for combined vs. NPAF and $p < 0.05$ for combined vs. β-amyloid 25–35) (Fig. 10).

4. Discussion

There is a growing evidence base revealing the involvement of RFamide neuropeptides in learning and memory. Autoradiographic studies showed that NPFF receptors are present in the memory related brain regions, such as the amygdala, hippocampus, BNST and cortical regions (Bonini et al., 2000; Gouarderes et al., 2004). It has been also shown that NPFF and NPFF receptors are involved

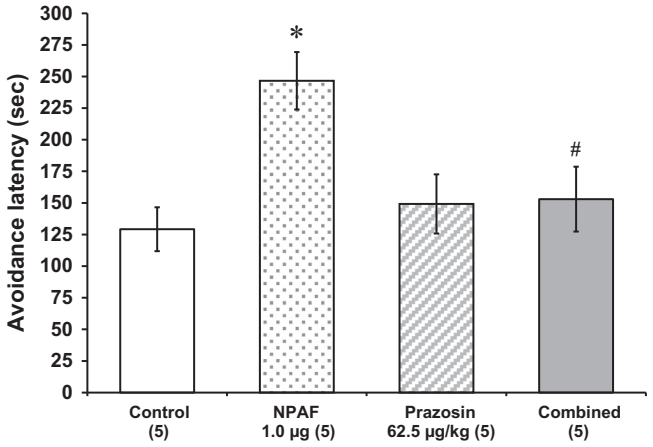

Fig. 6. The effects of the $\alpha_1/\alpha_{2\beta}$-adrenergic receptor antagonist, prazosin on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, prazosin (62.5 μg/kg, i.p.), combined (prazosin 62.5 μg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

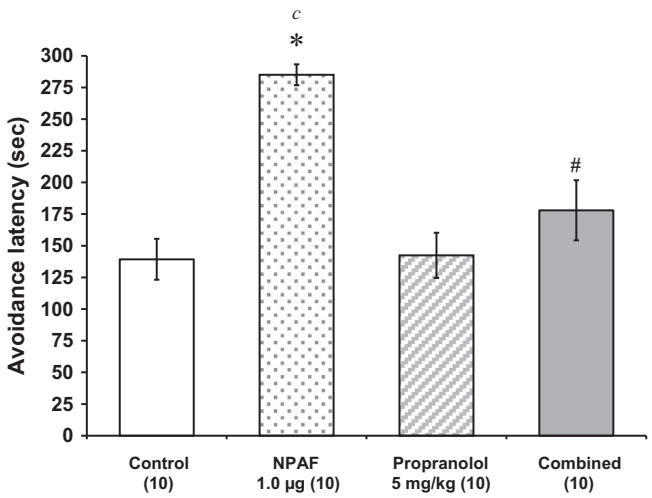

Fig. 7. The effects of the non-selective β-adrenergic receptor antagonist, propranolol on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control and $^{c}p < 0.05$ vs. propranolol, propranolol (5 mg/kg, i.p.), combined (propranolol 5 mg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

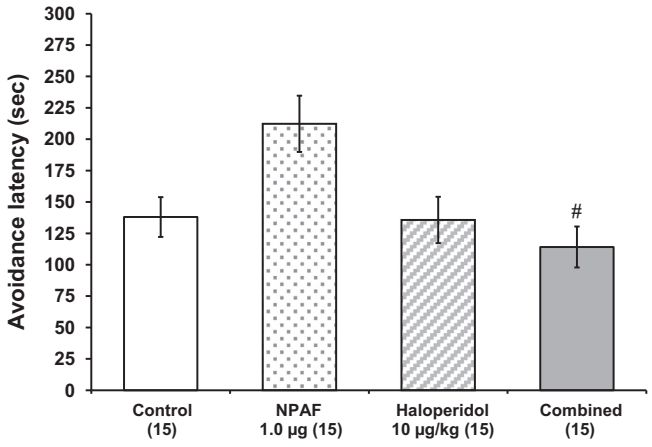

Fig. 8. The effects of the D2, D3, D4 dopamine receptor antagonist, haloperidol on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.), haloperidol (10 μg/kg, i.p.), combined (haloperidol 10 μg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

in learning and memory (Betourne et al., 2010; Kavaliers & Colwell, 1993). The present study demonstrates for the first time that NPAF improves consolidation of memory. It is interesting that both NPAF and NPFF may exert a dose-dependent action on learning and memory, since lower doses (1 μg) improve, whereas higher doses might impair memory consolidation. (In our study, a higher dose (2 μg) of NPAF showed only a tendency to decrease avoidance latency.) A possible mechanism could be the homologous desensitization of the receptor by GPCR kinases, which phosphorylate the already activated receptors and, consequently, decrease the responsiveness of the cell specifically to the ligand(s) of the given receptor (Freedman & Lefkowitz, 1996). Postsynaptic down-regulation of the receptor or activation of other, less-specific inhibitory receptors at higher concentrations may also explain the observed dose–effect relationship. These hypotheses need further verification.

Several neurotransmitter systems, including the cholinergic (Wallace & Bertrand, 2013), the serotoninergic (Meneses, 2013),

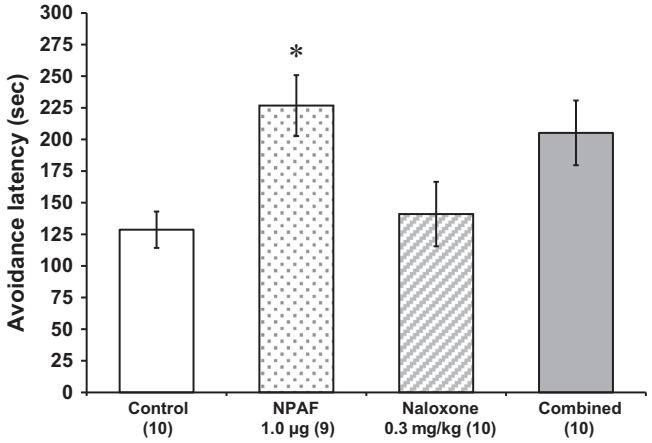

Fig. 9. The effects of the non-selective opioid receptor antagonist, naloxone on NPAF-induced memory consolidation. NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, naloxone (0.3 mg/kg, i.p.), combined (naloxone 0.3 mg/kg, i.p. +NPAF 1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

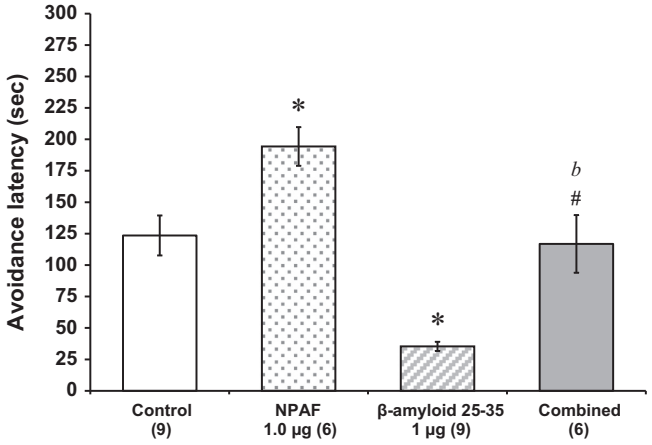

Fig. 10. The effect of NPAF on β-amyloid 25–35-induced impairment of consolidation of passive avoidance learning. NPAF (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, β-amyloid 25–35 (1.0 μg/2 μl, i.c.v.) $^{*}p < 0.05$ vs. control, combined (β-amyloid 25–35 1.0 μg/2 μl, i.c.v. +NPAF 1.0 μg/2 μl, i.c.v.) $^{\#}p < 0.05$ vs. NPAF (1.0 μg/2 μl, i.c.v.) and $^{b}p < 0.05$ vs. β-amyloid 25–35 (1.0 μg/2 μl, i.c.v.). The mean and S.E. are shown. Numbers in brackets denote the numbers of animals used.

the adrenergic (Berridge & Waterhouse, 2003), the dopaminergic (Pierce & Kumaresan, 2006), the nitrergic (Moroz & Kohn, 2011), and the opioid (Gholizadeh et al., 2013) transmissions play a role in learning and memory. Previous studies raised the hypothesis that the actions of RFamide peptides can be mediated – at least in part – through neurotransmitter systems (Chen et al., 1999; Huang et al., 2002; Jaszberenyi et al., 2009). Our previous studies demonstrated that the 13 amino acid endogenous isoform of kisspeptin (KP-13) facilitates consolidation of memory through α- and β-adrenergic, muscarinic cholinergic, GABA-A-ergic, nitrergic, 5HT1- and 5HT2-serotoninergic neurotransmissions (Telegdy & Adamik, 2013) and that the NPAF-induced anti-depressant-like behavior is mediated through muscarinic cholinergic and 5HT2-serotonergic transmissions (Palotai et al., 2014). Based on these findings, one can presume that the effect of NPAF on memory is also mediated through the stimulation of different transmitter systems. To test this hypothesis, we attempt to antagonize the NPAF-induced memory improvement by different transmitter receptor blockers. Our results show for the first time that muscarinic cholinergic, 5HT1- and 5HT2-serotoninergic, α- and β-adrenergic, D2-, D3-, D4-dopaminergic and nitrergic

neurotransmissions are all involved in the NPAF-induced memory consolidation, whereas opioid transmission may not be implicated.

The cholinergic system of the CNS arises from the basal forebrain structures, including the nucleus basalis, the substantia innominata and the medial septum-diagonal band complex and innervates numerous subcortical and cortical regions (Van der Zee & Keijser, 2011). The muscarinic cholinergic neurotransmissions in the memory-related brain sites, such the amygdala (Muller, Mascagni, Zaric, & McDonald, 2013), hippocampus (Mitsushima, Sano, & Takahashi, 2013) or thalamus–prefrontal cortex connections (Bueno-Junior, Lopes-Aguiar, Ruggiero, Romcy-Pereira, & Leite, 2012), play role in synaptic plasticity and memory formation. Among these structures, NPFF receptors are expressed in the amygdala, hippocampus, thalamus and nucleus of the diagonal band (Gouarderes et al., 2004). In our study, the non-selective muscarinic cholinergic antagonist atropine completely reversed the NPAF-induced memory improvement suggesting that intact muscarinic neurotransmission is mandatory in the action of NPAF on memory.

The serotoninergic system has 7 types of serotonin (5HT) receptors and originates from the dorsal and medial raphe nuclei, which provide afferents to numerous cortical and limbic structures involved in memory processes, including the cingulate gyrus, prefrontal cortex, amygdaloid complex, hippocampus and mammillary bodies, (Grove, Coplan, & Hollander, 1997). NPFF receptors are expressed in the latter three structures and in the dorsal raphe nucleus (Gouarderes et al., 2004). Our study showed that both the non-selective 5-HT2 serotonergic receptor antagonist, cyproheptadine and the mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide completely blocked the action of NPAF on memory suggesting that intact 5-HT1 and 5-HT2 serotoninergic transmissions are also mandatory for the NPAF-induced memory consolidation.

The primary source of forebrain norepinephrine (NE) is the locus ceruleus (LC), which provides adrenergic innervation to the memory-related brain regions, including the amygdala and the hippocampus (Berridge & Waterhouse, 2003). These regions are rich in α- and β-adrenergic receptors (Happe et al., 2004; Pieribone, Nicholas, Dagerlind, & Hokfelt, 1994; Rainbow, Parsons, & Wolfe, 1984) and express NPFF receptors as well (Gouarderes et al., 2004). In our study, both the selective α_1-adrenergic receptor antagonist, prazosin and the nonselective β-adrenergic receptor antagonist, propranolol fully reversed the NPAF-induced memory consolidation. Similarly to the conclusions drawn from the experiments with the cholinergic and the serotoninergic antagonists, our results suggest that intact α- and β-adrenergic transmissions are also required for the NPAF-induced memory improvement.

$D_{1\text{-}5}$ dopaminergic receptors and NPFF receptors have been identified in several structures of the meso-cortico-limbic dopaminergic system, including the ventral tegmental area, ventral striatum, amygdala, hippocampus and frontal cortex (Allard, Zajac, & Simonnet, 1992; Beaulieu & Gainetdinov, 2011; Bonini et al., 2000). Our previous study showed that NPAF increases the dopamine release in striatal and amygdalar slices, in vitro (Jaszberenyi et al., 2009). Our current study demonstrates that the non-selective D2, D3, D4 dopamine receptor antagonist haloperidol blocked completely the effect of NPAF on memory suggesting that – in addition to the above mentioned neurotransmissions – the intact D2, D3, D4 dopaminergic transmission is also obligatory for the stimulatory action of NPAF on memory.

Nitric oxide (NO) is another type of neurotransmitter, which is associated with synaptic plasticity, learning and memory (Moroz & Kohn, 2011). Prior studies revealed the involvement of NO in the NPFF receptor-mediated actions. On one hand, it has been shown that the non-selective NPFF receptor agonist, 1DMe inhibits the anti-nociceptive activity of the NO synthase inhibitor, nitro-L-arginine. On the other hand, nitro-L-arginine potentiated the 1DMe-induced hypothermia (Zajac, Latapie, & Frances, 2000). Our current study demonstrates that in the absence of NO, NPAF is unable to improve the consolidation of memory, since nitro-L-arginine completely reversed the action of NPAF on memory.

It has been shown that the VTA and its dopaminergic target areas, the amygdala and the mPFC encode and retrieve the opiate reward and withdrawal aversion-related memories (Frenois, Stinus, Di Blasi, Cador, & Le Moine, 2005; Sun et al., 2011). μ opioid receptor was found in the VTA and prefrontal cortex, whereas δ and κ receptors were identified in the amygdala (Bodnar, 2011). Recently published place conditioning studies using opiates revealed that NPFF attenuates acquisition of endomorphin 2-induced conditioned place aversion (CPA) (Han et al., 2013) and morphine and cocaine-induced conditioned place preference (CPP) (Kotlinska, Pachuta, Dylag, & Silberring, 2007; Kotlinska, Pachuta, & Silberring, 2008). Accordingly, antagonism of NPFF receptors increases the morphine-induced place conditioning (Elhabazi et al., 2012). Although it has been shown that NPFF and NPFF receptors are involved in opiate-induced place conditioning, our results suggest that opioid transmission is not necessary for NPAF to improve consolidation of passive avoidance learning, since the non-selective opioid receptor antagonist, naloxone did not reverse the action of NPAF.

The doses of the receptor blockers were selected on the basis of our earlier experience as being effective when administered with other neuropeptides, but not affecting the behavioral paradigm per se (Telegdy & Adamik, 2002, 2013). According to our findings and literature data, 5 out of the 6 investigated transmitter systems – namely the cholinergic, the serotoninergic, the adrenergic, the dopaminergic and the nitrergic systems – which were targeted by antagonists play a key role in memory processes in different CNS locations. Therefore, only does this part of our results confirm the target regions of the action of NPAF and reinforce that these specific neurotransmitter groups dominate in memory processes.

Our results suggest that the co-activation of the cholinergic, the serotoninergic, the adrenergic, the dopaminergic and the nitrergic systems is mandatory in the stimulatory effect of NPAF on memory consolidation. A possible mechanism could be that the simultaneous activation of these 5 neurotransmitter systems activates an Extracellular signal-Regulated Kinase (ERK)-dependent mechanism that acts as a coincidence detector (Giovannini, Lana, & Pepeu, 2015). If all the five transmitter systems are concurrently activated, the ERKs may initiate a cascade of intracellular processes that result in synaptic plasticity and learning. This "final common" mechanism has already been demonstrated in case of the combined activation of the muscarinic cholinergic and the β-adrenergic transmissions and ERKs were found in the hippocampus, the striatum, the neocortex and the cerebellum (Giovannini et al., 2015).

Our results demonstrate for the first time that the β-amyloid 25–35-induced memory impairment is reversed by NPAF. β-amyloid 25–35 was found to impair the consolidation of learning of passive avoidance response. This is in accordance with our previous study using the same passive avoidance paradigm (Telegdy et al., 2009) and with the results of studies using similar or different learning tasks and time schedules (Chen et al., 1996; Roesler et al., 2006; Stepanichev, Moiseeva, Lazareva, Onufriev, & Gulyaeva, 2003; Yamaguchi & Kawashima, 2001). Alzheimer's disease (AD) is the most common neurodegenerative disorder, which affects approximately 30 million people. Age is the most important risk factor, therefore the raise in life span of the population predicts a prominent increase in the incidence of AD in the forthcoming years. Neuropathologically, AD is characterized by extracellular deposits (composed of β-amyloid fibrils) and intracellular tangles (composed of hyperphosphorylated protein tau). There is only a

symptomatic treatment available. The aim of developing drugs for the therapy of Alzheimer's disease is to lower the β-amyloid level by either reducing the production of β-amyloid or increasing the clearance of β-amyloid tangles. Post-translational modification may also serve as a target in treatment of AD (Schedin-Weiss, Winblad, & Tjernberg, 2014; Tayeb, Murray, Price, & Tarazi, 2013).

Synaptic dysfunction and cholinergic deficiency are characteristics to AD. In vitro studies showed that β-amyloid impairs synaptic plasticity by suppressing basal neurotransmission, inducing impairment in long-term potentiation and suppressing neurotrophic factors. Experimental application of β-amyloid revealed also that β-amyloid suppresses nicotinic and muscarinic acetylcholine receptor signaling and acetylcholine release from the synaptic terminals (Jiang et al., 2014; Querfurth & LaFerla, 2010). Furthermore, in vivo rodent studies demonstrated decreased choline acetyltransferase activity in several brain regions, such as the hippocampus, the cortex, the medial septum and the striatum as well as impaired learning and memory, following a single or 3-day i.c.v. administration of β-amyloid (Noshita, Murayama, & Nakamura, 2015; Yamaguchi & Kawashima, 2001). Taking into account these findings and our results, one can presume that NPAF may reverse the β-amyloid-induced memory impairment by exerting stimulatory effect on the cholinergic neurotransmission. The clarification of the proper underlying mechanisms require further validation and exploration.

Our study has limitations. The number of animals used in the series of experiments with cyproheptadine, nitro-L-arginine and prazosin was low ($n = 5$). In addition, we used non-selective receptor blockers, therefore we could not investigate the involvement of specific receptor subtypes in the action of NPAF. In the series of experiments with haloperidol, NPAF facilitated considerably the memory consolidation, but this effect did not reach statistical significance. Furthermore, the current exploratory study could not investigate the biochemical mechanisms underlying the action of NPAF on the β-amyloid-induced memory impairment. In spite of these limitations, our study has pinpointed that NPAF has a stimulatory effect on memory consolidation, which requires further validation using larger sample size, selective receptor antagonists and in vitro techniques.

5. Conclusion

NPAF is a member of the RFamide neuropeptide family, which improves the consolidation of passive avoidance learning in mice. This action is mediated through α- and β-adrenergic, muscarinic cholinergic, D2-, D3-, D4-dopaminergic, nitrergic, 5HT1- and 5HT2-serotoninergic neurotransmissions, whereas μ-, δ-, κ-opioid neurotransmissions may not be implicated. Furthermore, NPAF reverses the β-amyloid 25–35-induced memory impairment.

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