Full title:

Neuropeptide AF induces anxiety-like and antidepressant-like behavior in mice

Short title:

Anxiogenic and antidepressant action of NPAF

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Abstract

Little is known about the action of neuropeptide AF (NPAF) on anxiety and depression. Only our previous study provides evidence that NPAF induces anxiety-like behavior in rats. Therefore, the aim of the present study was to investigate the action of NPAF on depression-like behavior and the underlying neurotransmissions in mice. In order to determine whether there are species differences between rats and mice, we have investigated the action of NPAF on anxiety-like behavior in mice as well. A modified forced swimming test (mFST) and an elevated plus maze test (EPMT) were used to investigate the depression and anxiety-related behaviors, respectively. Mice were treated with NPAF 30 min prior to the tests. In the mFST, the animals 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 $\alpha_1/\alpha_{2\beta}$-adrenergic receptor antagonist, prazosin or a nonselective β-adrenergic receptor antagonist, propranolol 30 min before the NPAF administration. In the mFST, NPAF decreased the immobility time and increased the climbing and swimming times. This action was reversed completely by methysergide and partially by atropine, whereas cyproheptadine, haloperidol, prazosin and propranolol were ineffective. In the EPMT, NPAF decreased the time spent in the arms (open/open+closed). Our results demonstrate that NPAF induces anti-depressant-like behavior in mice, which is mediated, at least in part, through 5HT2-serotoninergic and muscarinic cholinergic neurotransmissions. In addition, the NPAF-induced anxiety is species-independent, since it develops also in mice.

keywords: neuropeptide AF, forced swimming test, elevated plus maze test, neurotransmitter

1. Introduction

Neuropeptide AF (NPAF, A18F amide, AGEGLSSPFWSLAAPQRFamide) is an amidated octadecapeptide, which was purified together with neuropeptide FF (NPFF, F8F amide, FLFQPQRFamide) from bovine brain extracts on the basis of immunoreactivity using antiserum against the molluscan cardioexcitatory peptide FMRFamide [1]. NPAF and NPFF are RFamide neuropeptides, which share a conserved carboxy-terminal Arg-Phe-NH2 sequence. Subsequently, another RFamide peptide, the neuropeptide SF (NPSF, SLAAPQRFamide), was isolated in rodent spinal cord [2]. These neuropeptides were identified in mouse, rat and human brains. Their related peptides are the prolactin-releasing peptides, LPXRFamide peptides, metastatin and kisspeptin and pyroglutamylated RFamide peptides. These peptides are produced by five different genes (farp-1 to 5), and NPAF and NPFF are alternatively spliced products of the transcription of farp-1 [3]. NPAF and NPFF are present in several medullary nuclei, nucleus of the solitary tract, periaqueductal gray, parabrachial nucleus, amygdala, neurohypophysis, hypothalamus, thalamus and lateral septum [4].

NPAF, NPFF and NPSF signal through two $G_{i/o}$-protein coupled receptors (GPCRs), respectively known as NPFF-2 (GPR74, NPGPR, HLWAR77) and NPFF-1 (GPR147, OT7T022) processed from the product of the same receptor gene rfr-3 [3]. Expression of NPFF-1 and NPFF-2 have been identified in the dorsal division of the septal nucleus, bed nucleus of stria terminalis (BNST), posteromedial cortical amygdaloid nucleus, anterior pretectal nucleus, parafascicular nuclei, medial mammillary nucleus and CA3 region of the ventral hippocampus [5]. NPAF and NPFF play role in several physiological functions, e.g. these peptides lower the nociceptive threshold [1], reduce morphine analgesia [6], inhibit stimulated insulin and somatostatin release from the pancreas [7], exert anorectic action [8], inhibit colonic bead expulsion time [9], regulate body temperature [10], increase mean arterial blood pressure [11], control aldosterone release and adrenal blood supply [12]. Our previous study showed that neuropeptide AF stimulates ACTH and corticosterone release, augments striatal and amygdalar dopamine release and increases exploratory locomotion [13]. NPSF is implicated in control of adipocyte metabolism [14], food and water intake [15] and nociception [16]. We have demonstrated that NPSF increases CRF, ACTH and corticosterone release and stimulates locomotor activity [17].

However, little is known about the action of NPAF on anxiety and depression. Only our previous study provides evidence that NPAF induces anxiety-like behavior in elevated plus maze test in rats [13], whereas the action of NPAF on depression-like behavior has not

been elucidated. Therefore the aim of the present study was to investigate the action of NPAF on depression-like behavior in a modified forced swimming test (FST) in mice. We have also investigated the underlying neurotransmissions. 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 $\alpha_1/\alpha_{2\beta}$-adrenergic receptor antagonist, prazosin or a nonselective β-adrenergic receptor antagonist, propranolol prior to NPAF administration. Furthermore, in order to determine whether there are species differences in the anxiety-like action of NPAF between rats and mice, we have investigated the effect of NPAF in elevated plus maze (EPM) test in mice.

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 stainless steel Luer canulla 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 mice were used after a recovery period of 5 days.

2.3. Materials

NPAF was purchased from Bachem Inc. (Switzerland); atropine sulfate, from EGYS (Budapest, Hungary); cyproheptadine hydrochloride, from Tocris (Bristol, UK); methysergide

hydrogen maleate, from Sandoz (Cologne, Germany); haloperidol, from G. Richter (Budapest, Hungary); phenoxybenzamine hydrochloride was from Smith Kline & French (Herts, UK) and prazosin hydrochloride from Tocris (Köln, Germany).

All the experiments were performed in the morning. NPAF in a quantity of 10 μg per ampoule was lyophilized and stored at -20 ◦C. Immediately before the experiments, NPAF was dissolved in sterile pyrogen-free 0.9% saline and administered i.c.v. in a volume of 2 μl via the cannula.

2.4. Behavioral testing

2.4.1. Forced swimming test

The modified mouse FST was conducted as reported previously [18, 19]. The mice were forced to swim individually in a glass cylinder 12 cm in diameter and 30 cm in height, filled with water to a height of 20 cm. The temperature of the water was adjusted to 25 ± 1 ◦C. The water was changed between the individual mice. A 15 min pretest session was followed 24 h later by a 5 min test session. Atropine (2 mg/kg, i.p.), cyproheptadine (3 mg/kg, i.p.), haloperidol (10 μg/kg, i.p.), methysergide (5 mg/kg, i.p.), phenoxybenzamine (2 mg/kg, i.p.) or prazosin (62.5 μg/kg, i.p.) were administered 1 h before the test session followed 30 min later by NPAF (0.25 μg/2 μl, i.c.v.). 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 [20, 21]. The dose selection of NPAF is based on our previous study, in which 0.25 μg/2 μl was the minimum effective dose [13]. Physiological saline was used as vehicle control. A time-sampling technique was applied to score the climbing, the swimming and the immobility. Every 5 sec, one of the three behaviors was scored. Climbing time was scored when the mouse was participating inactive vertical motion with its forelegs above the water level; swimming time was scored when the mouse was moving in horizontally on the surface of the water; and immobility time was scored when the mouse was in a upright position on the surface with its front paws together and making only those movements necessary to keep itself a float.

2.4.2. Elevated plus maze test

The idea of the elevated plus maze test [22] is that open arms are more fear provoking and that the ratio of the times spent in open versus closed arms or the ratio of the entries into open versus closed arms reflects the relative ‘safety’ of closed arms, as compared with the relative danger of open arms. The wooden maze consisted of two open (25×5 cm) and two

closed (25×5×20 cm) arms, which were connected by a 5x5 cm central square and elevated to a height of 50 cm above the floor. Arms were angled at 90° to each other and the same types of arms were positioned opposite to each other. The animals were placed individually at the center of the maze, facing one of the closed arms. During a 5 min test period, the behavior of the animal was recorded by an observer sitting 1 m from the center of the maze. Recordings were made of the times spent in the open and the closed arms and of the numbers of entries into the open and closed arms. Entry into an arm was defined as the entry of all four feet into that arm. These scores were converted into percentages (open/open + closed). Total number of entries into arms provided a measure of overall activity. Each animal was tested only once in the plus maze apparatus. NPAF (0.5 μg/2 μl; 1.0 μg/ 2 μl; 2.0 μg/ 2 μl, i.c.v.) was administered 30 min prior to the test.

2.5. Statistical analysis

Statistical analysis of the behavioral testing was performed by analysis of variance (ANOVA), which was followed by Tukey's post hoc comparison test. Only the mean percentages were plotted and the standard error of the mean (SEM) is given in the figure captions. The differences between groups were examined by Tukey's post hoc comparison test, and a probability level of 0.05 or less was accepted as indicating a statistically significant difference.

3. Results

NPAF (0.25 μg/2 μl, i.c.v.) decreased significantly the immobility time [$F(3,58) = 12.68$]; $p < 0.01$ and increased significantly the climbing time [$F(3,58) = 7.36$]; $p < 0.01$ and the swimming time [$F(3,58) = 6.51$]; $p < 0.01$ (Figs. 1-6).

Phenoxybenzamine alone (2 mg/kg, i.p.) did not affect the immobility time, the climbing time or the swimming time. In NPAF-treated mice, pretreatment with phenoxybenzamine did not affect considerably the NPAF-induced changes in immobility time, climbing time and swimming time (Fig. 1).

Prazosin per se (62.5 μg/kg, i.p.) did not affect the immobility time, the climbing time or the swimming time. In NPAF-treated mice, pretreatment with prazosin did not affect the NPAF-induced changes in immobility time, climbing time and swimming time (Fig. 2).

Atropine itself (2 mg/kg, i.p.) did not affect the immobility time, the climbing time or the swimming time. In NPAF-treated mice, pretreatment with atropine decreased significantly

the NPAF-induced change in climbing time [$F(3,57) = 15.6$]; $p < 0.01$, but did not affect the NPAF-induced changes in immobility time and swimming time (Fig. 3).

Haloperidol alone (10 μg/kg, i.p.) did not affect the immobility time, the climbing time or the swimming time. In NPAF-treated mice, pretreatment with haloperidol did not affect considerably the NPAF-induced changes in immobility time, climbing time and swimming time (Fig. 4).

Cyproheptadine per se (3 mg/kg, i.p.) did not affect the immobility time, the climbing time or the swimming time. In NPAF-treated mice, pretreatment with cyproheptadine reversed completely the NPAF-induced change in immobility time [$F(3,39) = 15.05$]; $p < 0.01$, climbing time [$F(3,39) = 4.21$]; $p < 0.05$ and swimming time [$F(3,39) = 12.85$]; $p < 0.01$ (Fig. 5).

Methysergide itself (5 mg/kg, i.p.) did not affect the immobility time, the climbing time and the swimming time. In NPAF-treated mice, pretreatment with methysergide did not influence significantly the NPAF-induced changes in immobility time, climbing time and swimming time (Fig. 6).

We present only the duration of entries from the EPM test, because the numbers of entries led to the same conclusion. None of the treatment influenced the overall activity of the animals relative to the controls. Therefore, these results are not shown either. NPAF in a dose of 1 μg/2 μl i.c.v. significantly decreased the time spent in the arms in the plus maze [$F(3,40) = 7.4$]; $p < 0.01$ (Fig. 7).

4. Discussion

The present study demonstrates for the first time that NPAF exerts anti-depressant-like effects in mice. Depression (clinical depression, major depression, major depressive disorder, MDD) is one of the most common psychiatric diseases, which is a major cause of morbidity and affects millions of people worldwide. Depression is characterized by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness and poor concentration (WHO definition). Depression is not only represented by dysregulation of affect and mood but is also associated with neurological disorders (dementia, epilepsy and stroke), metabolic disorders (diabetes and obesity), endocrine, inflammatory and cardiovascular dysfunctions and with the risk to develop cancer. Today's treatment of depression, based on inhibition of serotonin/noradrenaline reuptake or enzyme monoamine oxidase, achieves complete remission in case of only 50% of patients. Therefore, newer medications are needed with higher efficacy and potency [23, 24].

There is a growing evidence base revealing the involvement of RFamide neuropeptides in depression. Human brain imaging studies and autopsies revealed changes in blood flow or related measures and morphological abnormalities respectively in several brain regions, including amygdala, hippocampus, thalamus, striatum and medial prefrontal cortex (mPFC) [24]. Accordingly, autoradiographic and histological studies showed that NPAF and NPFF receptors are present in these brain sites [4, 5]. RFamide related peptides (RFRPs), such as NPSF are encoded by the farp-3 gene and acts through NPFF-1 and NPFF-2 receptors [3]. It has been revealed that the action of citalopram, the most potent serotonin reuptake inhibitor (SSRI) antidepressant, involves modulation of RFRP expression in the dorsomedial hypothalamus [25]. Kisspeptin (KP) is also part of the RFamide peptide family, which is encoded by the farp-4 gene and acts on the G-protein coupled KiSS-1 receptor (GPR54, OT7T175, AXOR12) [3]. KP-13 is an endogenous isoform consisting of 13 amino acids. In our previous study, using an identical forced swimming test to the present experiments, we have demonstrated that KP-13 exerts anti-depressant like effects [26]. Taking into consideration our present results and the previous observations we can conclude that RFamide peptides are implicated in depression. The current results may contribute to the finding of new therapeutic targets and developing of more effective treatments for depression.

Our study demonstrates for the first time that the anti-depressant-like action of NPAF is mediated through 5HT2-serotoninergic neurotransmission and muscarinic cholinergic transmission may also be implicated, since cyproheptadine reversed completely the immobility, the climbing and the swimming times, whereas atropine decreased significantly only the climbing time. Our results also suggest that α- and β-adrenergic and D2-, D3-, D4-dopaminergic neurotransmissions are not involved in this action, since prazosin, phenoxybenzamine and haloperidol were ineffective. Most neuropeptides are co-expressed with at least one classic neurotransmitter in the central nervous system (CNS). Generally, neuropeptides behave as neuromodulators exerting multiple actions on physiological brain functions and, consequently, on behavior. Their effects involve changes in membrane excitability, trophic actions, gene transcription, changes in affinity of receptors and modulation of neurotransmitter release [27]. Modulatory effect of RFamide peptides on neurotransmitter releases has been established. On one hand, RFRPs are associated with serotoninergic transmission [25]. On the other hand, our previous study showed that the anti-depressant-like action of KP-13 is mediated through 5HT2-serotoninergic, α_2- and β-adrenergic neurotransmissions, whereas α_1-adrenergic, muscarinic cholinergic, D2-, D3-, D4-dopaminergic and GABA-A-ergic transmissions are not involved. Comparing the

neuromodulatory profiles of RFRPs, KP-13 and NPAF, we can draw the conclusion that these RFamide neuropeptides are all associated with serotoninergic neurotransmissions. It is interesting that the non-selective 5HT2-serotoninergic antagonist, cyproheptadine reversed completely both the KP-13 and the NPAF-induced changes in FST, whereas the mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide did not influence significantly the KP-13 or the NPAF-induced changes in immobility, climbing or swimming times. This observation coincides with our expectation since 5HT1 inhibits cAMP signaling, while 5HT2 stimulates calcium mobilization, through IP3/DAG. In this paradigm, inhibition of PKA and PKC activity seems to exert opposite activity on behavior.

The current study demonstrates for the first time that NPAF exerts anxiogenic-like action in mice. Anxiety disorders are common psychiatric diseases, which are characterized by emotion of constriction, fear, and inner restlessness that appears physiologically in unfamiliar or threatening situations and is always accompanied by a physical stress reaction [28]. Millions of people are affected, therefore the investigation of newer and more effective medications is essential. There in an increasing base of evidence demonstrating the role of RFamide neuropeptides in anxiety. Autoradiographic and histological studies showed that NPAF and NPFF receptors are expressed in anxiety-related brain regions, such as amygdala, hypothalamus, thalamus and mPFC [4, 5]. Although the involvement of RFamide peptides and NPFF receptors in anxiety has been particularly revealed in rats, this action has not been elucidated in mice. Our previous studies showed that NPAF and KP-13 increase the plasma corticosterone levels and induce anxiety-like behavior in elevated plus maze test in rats [13, 29]. It has also been demonstrated that antagonism of NPFF receptors attenuates the ethanol and the amphetamine withdrawal-induced anxiety-like behaviors in rats [30, 31]. Taking into consideration the present and the previous studies we can conclude that RFamide neuropeptides are involved in anxiety and there are no species differences between rats and mice in terms of NPAF-induced anxiety-like behavior. Based on these observations we believe that NPAF signaling may serve as a target for treatment of anxiety disorders as well.

5. Conclusion

NPAF is a member of the RFamide neuropeptide family, which induces antidepressant-like behavior in forced swimming test and anxiety-like behavior in elevated plus maze test in mice. The NPAF-induced antidepressant-like action is mediated through 5HT2-serotoninergic neurotransmission. Muscarinic cholinergic transmission may also be implicated, whereas α- and β-adrenergic and D2-, D3-, D4-dopaminergic transmissions are

not involved. 5HT1-serotoninergic receptors may have contrary action to the 5HT2-serotoninergic receptors. The NPAF-induced anxiogenic-like action is not species dependent, since it has been observed in both mice and rats. We believe that the present results may contribute to the finding of new therapeutic targets and developing of more effective treatments for depression and anxiety disorders as well.

Acknowledgments

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Figure legends

Fig. 1. The effect of the non-selective β-adrenergic receptor antagonist, phenoxybenzamine on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 15), NPAF 0.25 μg/2 μl, i.c.v. (n = 14), phenoxybenzamine 2 mg/kg, i.p. (n = 15), phenoxybenzamine 2 mg/kg, i.p. + NPAF 0.25 μg/2 μl, i.c.v. (n = 15). *: $p < 0.05$ vs. control. (n: the number of animals, p: probability).

Fig. 2. The effect of the $\alpha_1/\alpha_{2\beta}$-adrenergic receptor antagonist, prazosin on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 5), NPAF 0.25 μg/2 μl, i.c.v. (n = 5), prazosin 62.5 μg/kg, i.p. (n = 5), prazosin 62.5 μg/kg, i.p. + NPAF 0.25 μg/2 μl, i.c.v. (n = 5). *: $p < 0.05$ vs. control. (n: the number of animals, p: probability).

Fig. 3. The effect of the non-selective muscarinic cholinergic receptor antagonist, atropine on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 15), NPAF 0.25 μg/2 μl, i.c.v. (n = 14), atropine 2 mg/kg, i.p. (n = 15), atropine 2 mg/kg, i.p. + NPAF 0.25 μg/2 μl, i.c.v. (n = 14). *: $p < 0.05$ vs. control. **: $p < 0.05$ vs. NPAF (n: the number of animals, p: probability).

Fig. 4. The effect of the D2, D3, D4 dopamine receptor antagonist, haloperidol on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 15), NPAF 0.25 μg/2 μl, i.c.v. (n = 15), haloperidol 10 μg/kg, i.p. (n = 14), haloperidol 10 μg/kg, i.p. + NPAF 0.25 μg/2 μl, i.c.v. (n = 15). *: $p < 0.05$ vs. control. (n: the number of animals, p: probability).

Fig. 5. The effect of the non-selective 5-HT2 serotonergic receptor antagonist, cyproheptadine on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 10), NPAF 0.25 μg/2 μl, i.c.v. (n = 10), cyproheptadine 3 mg/kg, i.p. (n = 10), cyproheptadine 3 mg/kg, i.p. + NPAF 0.25 μg/2 μl, i.c.v. (n = 10). *: $p < 0.05$ vs. control. **: $p < 0.05$ vs. NPAF (n: the number of animals, p: probability).

Fig. 6. The effect of the mixed 5-HT1/5-HT2 serotonergic receptor antagonist, methysergide on NPAF-induced antidepressant-like action in modified mouse forced swimming test. Control (n = 5), NPAF 0.25 μg/2 μl, i.c.v. (n = 5), methysergide 3 mg/kg, i.p. (n = 5),

methysergide 3 mg/kg, i.p. + NPAF 0.25 µg/2 µl, i.c.v. (n = 5). *: $p < 0.05$ vs. control. **: $p < 0.05$ vs. NPAF (n: the number of animals, p: probability).

Fig. 7. The anxiogenic action of NPAF in elevated plus maze test in mice. Control (n = 8), NPAF 0.5 µg/2 µl, i.c.v. (n = 9), NPAF 1.0 µg/2 µl, i.c.v. (n = 14), NPAF 2.0 µg/2 µl, i.c.v. (n = 10). *: $p < 0.05$ vs. control. (n: the number of animals, p: probability).