Published in final edited form as:

Neurosci Lett. 2019 January 18; 690: 214–218. doi:10.1016/j.neulet.2018.09.049.

Neuropeptide Y impairs the acquisition of conditioned defeat in Syrian hamsters

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Abstract

Recent evidence indicates that Neuropeptide Y (NPY) may function as a potent anxiolytic as well as a resilience factor that can insulate the brain from the effects of stress. However, most of these studies have utilized physical stressors such as shock or restraint. In the present study, we use an ethologically-based model in Syrian hamsters (*Mesocricetus auratus*) called Conditioned Defeat (CD) to investigate whether NPY can ameliorate the effect of social defeat stress. In the CD model, a male Syrian hamster is socially defeated by a larger, more aggressive conspecific. Subsequently, when paired with a smaller, non-aggressive intruder (NAI) in its own home cage, changes in its behavioral repertoire occur, including a reduction in aggression and chemosensory (social) investigation, and a concomitant increase in submissive behaviors. In Experiment 1, hamsters were infused intracerebroventricularly (icv) with NPY prior to social defeat, and 24-hours later, hamsters were exposed to a NAI. Results indicate that NPY significantly reduced submissive/defensive behaviors in socially defeated hamsters compared to control animals. In Experiment 2, we examined whether this effect was mediated by the NPY Y1 receptor. Subjects were first pre-treated with the Y1 receptor antagonist BIBP 3226 or vehicle, followed by NPY and then socially defeated. Upon testing with a NAI 24-hours later, pretreatment with BIBP 3226 failed to block the NPY effect compared to controls. These results demonstrate that NPY may function as an important resilience factor in socially defeated hamsters, but that these effects are not mediated by the Y1 receptor.

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Disclosures. The authors declare no conflict of interest, financial or otherwise to declare.

Keywords

Social defeat; voluntary exercise; stress; anxiety; defensive behaviors

1. Introduction

Traumatic life events are a significant contributor to psychiatric illness such as mood and anxiety disorders. Individuals exposed to psychological [1] and physical abuse [2] as well as combat duty [3, 4] are at a significantly higher risk for developing PTSD. Indeed, most studies have investigated the link between maladaptive behaviors associated with traumatic and/or stressful events and its susceptibility to these disorders, surprisingly few have examined factors that may make an individual more resilient to these psychopathologies. While these factors are important in elucidating the neural mechanisms of trauma and stress, the fact is that most individuals that experience a traumatic life event do not develop mental illness [5, 6]. We believe that one way to examine these protective resilience factors is to utilize an animal model of stress that employs an ethologically relevant, social conflict stressor that has high face validity.

Social conflict in most species can lead to a host of long-lasting behavioral and physiological changes, especially in the defeated individual. In Syrian hamsters, social defeat will result in various physiological changes such as increased levels of plasma adrenocorticotrophin (ACTH) and glucocorticoids [7]. In addition, hamsters that experience social defeat will show increased levels of submissive and defensive behaviors even when it is subsequently paired with a smaller, non-aggressive conspecific. This phenomenon, known as Conditioned Defeat (CD) is a direct consequence of the social defeat experience [8]. Germane to the current study, however, is that we have found that some socially defeated hamsters exhibit none of these behavioral changes, and instead show increased social behavior and, occasionally, even a re-establishment of territorial aggression when exposed to the NAI. A recent analysis of past CD experiments conducted in our lab shows that up to 40% of subjects that experience social defeat exhibit little to no submissive/defensive behaviors when subsequently paired with a NAI (unpublished findings). Importantly, this effect seems to be independent of the level of social defeat incurred by the hamster, indicating that this finding may reflect the inherent individual variations among our subjects. Similar findings of a wide range of individual differences following social defeat stress in other species have been reported. For example, a recent study showed that in a model of social defeat in mice, up to 50% of defeated subjects exhibited a high level of social interaction, similar to that of non-defeated controls, when tested against a caged conspecific [9]. Even considering species and methodological differences (a chronic defeat protocol was used in the mouse study) these results are remarkably comparable with our own findings.

Several studies have shown that NPY may play a role in reducing anxiety-like behaviors and inducing resilience in animals exposed to stress. NPY, a 36-amino acid peptide that is among the most widely distributed peptides in the central as well as the peripheral nervous system, has been shown to play a role in reducing anxiety-like behaviors and inducing resilience in animals exposed to various stressors. Brain areas expressing NPY include the lateral septum,

cerebral cortex, hippocampus and the amygdala [10], areas that have all been implicated in mediating the acquisition and/or expression of CD. Additionally, given its wide distribution, it is not surprising that NPY appears to be involved in a multitude of functions, including regulation of food intake [11], sleep regulation [12] and mediation of fear and anxiety responses. With regard to fear and anxiety, intracranial infusion of NPY has been shown to have anxiolytic-like effects in a variety of animal models and that these effects may be mediated by the NPY Y1 receptor [13, 14, 15]. In addition, recent studies have begun to suggest that NPY is also involved, at least in part, in counteracting the effects of environmental stressors and in inducing a resilience-like state in rodents [16,17]. Furthermore, in clinical studies low levels of NPY were detected in the postmortem brain tissue of suicide victims with a history of depression [18], while in study of combat veterans, Sah and colleagues [19] found that trauma-exposed veterans with PTSD had a lower concentration of cerebrospinal fluid NPY compared to healthy controls.

Together, these findings suggest that NPY may play a key role in reducing anxiety responses as well as inducing a resilience-like state, and that these effects are mediated by the Y1 receptor. Because no studies to date have been conducted examining the effect of NPY on social defeat in hamsters, the purpose of the current experiment was to first test the hypothesis that icv infusion of NPY will reduce submissive/defensive behaviors in socially defeated hamsters and to determine whether this effect is mediated by the Y1 receptor. Our hypothesis was that infusion of NPY prior to social defeat will reduce submissive behaviors while pretreatment with the Y1 receptor antagonist will block this effect.

2. Materials and Methods

2.1 Animals and Housing Conditions

Experimental subjects were adult male Syrian hamsters (*Mesocricetus auratus*; Charles River Laboratories, Wilmington, MA) approximately 10 weeks old and weighing between 110–125 g at the time of testing. All subjects were housed in groups of 5–6 per cage for one week prior to surgery in a temperature ($22^{\circ} \pm 2.5^{\circ}\text{C}$) and humidity-controlled room with free access to food and water and kept on a 14:10 h light:dark cycle (lights out at 10:00 h). Animals were single housed after surgery. Resident aggressors (RA) used for defeat training were older (>6 mo), singly housed males weighing between 175 and 205 g. NAI's were younger (8 weeks), group-housed (5–6 per cage) males weighing between 100–110g. All testing occurred during the first three hours of the dark phase of the light/dark cycle in order to minimize circadian variation of the behaviors. Behavioral testing was conducted in one of two dedicated rooms located adjacent to the vivarium. All procedures and protocols were approved by the Morehouse College School of Medicine Institutional Animal Care and Use Committee (IACUC) and are in accordance with the standards outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2 Surgical Procedures

Subjects were anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and placed into a stereotaxic frame. A single stainless steel guide cannula (26-gauge) was implanted into the lateral ventricles using the following coordinates: 0.3 mm caudal and ± 1.8 mm lateral

relative to bregma [20]. The guide cannula was lowered to 2.7 mm below dura to minimize damage to the cortex. On the day of injection, a 33-gauge injection needle projecting 1.2 mm below the guide cannula was used to reach a final depth of 3.9 mm below dura. Following surgery, dummy stylets were placed in the guide cannula to help maintain patency. Post-operative care consisted of subcutaneous injections of 0.1ml of 10mg/ml Ketofen and 0.9ml of physiological saline to aid in recovery. Hamsters were allowed 7–10 days to recover from surgery were handled each day by gently restraining them and removing and replacing the dummy stylet in order to maintain patency. Cages were changed once during the post-operative recovery period.

2.3 Social Defeat and Behavioral Testing

On day of social defeat, animals were transported from the vivarium to the testing room and allowed to acclimate for 30 minutes. The defeat session consisted of a single 10-min exposure to the RA in the aggressor's homecage. The RA reliably attacked and defeated the experimental subject within 60 s (Table 1). It should also be noted that subjects displayed submissive and defensive behaviors during the defeat and that the defeats occurred in "bouts" and do not last the entire 10-min defeat session. In addition, analysis of the defeat sessions revealed no significant differences in the duration of the attack behavior between the NPY and control group, indicating that NPY itself did not alter the behavior of the RA or the experimental animal. Twenty-four following defeat, subjects were again transported to the vivarium and allowed to acclimate for 30 min. Next, a NAI was placed into its cage for 5 min. The total duration of four classes of behaviors were scored during the test session: (a) social behaviors (approach, sniff, nose touching, grooming of the NAI and flank marking), (b) non-social behaviors (locomotion, exploration, rearing, grooming, nestling, feeding and sleeping), (c) submissive/defensive behaviors (flight, avoidance, tail up, upright and side defense, submissive posture, stretch attend, head flag and jump escape), (d) aggressive behaviors (upright and side offense, chase, attack and bite). Both defeat and testing sessions were recorded on a DVD disc via a CCD camera and were scored by an observer blind to the experimental conditions using the behavioral analysis software program Hindsight (developed by Scott M. Weiss, Ph.D., University of Hawaii).

2.4 Experiment 1: Effect of Intracerebroventricular infusion of NPY on Acquisition of CD

The goal of Experiment 1 was to determine whether icv infusion of NPY will reduce the acquisition of submissive behaviors in socially defeated hamsters. Animals ($n=30$) were weight matched and randomly assigned to one of three conditions. Hamsters received one of two doses of NPY (Tocris Bioscience, Minneapolis, USA; 200 or 400 pmol in 1 μ l saline) or vehicle control (1 μ l saline) thirty minutes prior to being placed into the home cage of a resident aggressor for 10 min. Twenty-four hours after social defeat, subjects were exposed to a NAI in their own homecage for 5 min to examine CD. Doses and timing of NPY injections were determined based on previously published research in hamsters [21] as well as pilot studies.

2.5 Experiment 2: Effect of pretreatment with the NPY Y1 antagonist BIBP 3226 on the Acquisition of Conditioned Defeat

The goal of Experiment 2 was to determine whether pretreatment with the NPY Y1 antagonist BIBP 3226 (Tocris Bioscience, Minneapolis, USA) will reverse the NPY-induced amelioration of submissive behaviors observed in Experiment 1. Animals ($n=28$) were weight matched and randomly assigned to one of four conditions. Hamsters were first pretreated with BIBP 3226 (1.5 μg or 3.0 μg in 1 μl saline) or vehicle control (1 μl saline), followed by the 400 pmol dose of NPY (the most effective dose from Experiment 1) 15 min later. Behavioral testing was conducted 30 min after the second infusion by placing subjects into a cage with a RA for 10 min. A behavioral control group was also run consisting of saline only infusions ("saline-saline"). As in Experiment 1, subjects were exposed to a NAI in their own home cage for 5 min 24-hours later. BIBP 3226 was dissolved in distilled water. Dose and timing of the injections were based on pilot studies and previously published research using rats [22], since no studies using BIBP 3226 administered centrally or in specific brain regions have been conducted using hamsters.

2.5 Site Verification

At the end of the experiments, hamsters were euthanized with Pentobarbital and infused with India ink to histologically verify the placement of the cannula. All subjects had correct cannula placements.

2.6 Statistical Analysis

The total duration (in seconds) of each behavior displayed (submissive/defensive, social, nonsocial) was determined and the mean duration of these behaviors were then analyzed using one-way analysis of variance (ANOVA) for Experiment 1 with dose as the grouping variable and ANOVA with Newman-Keuls post-hoc test to compare pairwise differences, where appropriate. Significant differences for all analysis was set at $p < 0.05$.

3 Results

3.1 Experiment 1: Effect of icv infusion of NPY on Acquisition of CD

As shown in Figure 1, infusion of NPY prior to social defeat significantly reduced submissive and defensive behaviors during CD testing. Specifically, ANOVA revealed a main effect of dose on submissive behaviors ($F_{(2,25)}=10.47$; $P < 0.0005$). Post-hoc analysis showed that animals in both the high (400pmol) and low (200pmol) groups exhibited a lower mean duration of submissive behaviors compared to the control groups ($P < 0.005$ and 0.0001, respectively). No other group differences were observed in the other behaviors measured (aggression, social and non-social), although non-social behaviors approached significance ($F_{(2,25)}=3.096$; $P < 0.059$; Figure 2).

3.2 Experiment 2: Effect of pretreatment with the NPY Y1 antagonist BIBP 3226 on the Acquisition of Conditioned Defeat

As shown in Figure 3, one-way ANOVA revealed a significant main effect of dose on submissive behaviors during CD testing ($F_{(3, 24)}=3.64$; $P < 0.05$). Post-hoc testing showed

that while the Saline/NPY groups exhibited significantly lower levels of submission compared to the Saline/Saline control group, pretreatment with either dose of BIBP 3226 did not significantly increase submission compared to the Saline/NPY group. None of the other behaviors examined (social, non-social, aggressive) were significantly different compared to the controls (data not shown).

4. Discussion

The present experiment demonstrates that icv infusion of NPY blocks the acquisition of CD in Syrian hamsters, providing further evidence that NPY may impart resilience to stressful experiences. However, we also show that this effect may not be mediated by the NPY Y1 receptor. In Experiment 1, icv infusion of NPY prior to social defeat training significantly reduced submissive/defensive behaviors in hamsters that were subsequently paired with a NAI. In Experiment 2, results show that pretreatment with the NPY Y1 receptor antagonist BIBP 3226 did not reverse the NPY-induced impairment of submissive behaviors following social defeat, although the saline-NPY group showed the expected reduction in submission compared to the behavioral control group (Saline-Saline). While Experiment 1 confirm previous reports that NPY may mediate resilience during the acquisition of fear and stress-related stimuli, our data from Experiment 2 was unexpected, as the majority of studies have indicated that the resilience and anxiolytic-like effects of NPY is mediated, at least in part, via the Y1 receptor system.

As mentioned above, both clinical and preclinical studies have demonstrated that NPY is involved in counteracting the effects of environmental stressors via anxiolytic-like actions and inducing a resilience-like state. Indeed, icv infusion of NPY has been shown to ameliorate the effects of stress in a variety of animal models, including the forced swim test [25], fear potentiated startle [13, 26] and the elevated plus maze [15]. In addition, Thorsell and colleagues showed that overexpression of NPY in transgenic rats can induce stress resilience [23], while NPY knockout mice exhibited increased anxiety and stress responses [17] as well as territorial aggression (24). Although we demonstrate that NPY plays a key role in mediating resilience to social defeat stress, we specifically did not examine its role on agonistic behaviors in non-defeated animals, as it was beyond the scope of the present study. However, future studies should examine whether central or site-specific infusion on NPY will increase territorial defense in non-defeated hamsters.

The present results also provide important information to begin targeting key structures in the neural circuit mediating the acquisition of CD. One possible site is the basolateral nucleus of the amygdala (BLA), which has been shown to play a significant role in the acquisition of CD [27] and an area that also expresses high levels of NPY [10]. Several studies have demonstrated that NPY in the BLA may mediate anti-stress responses. For example, Gutman and colleagues showed that intra-amygdala infusion of NPY impaired both baseline acoustic startle amplitude as well as the expression of fear-potentiated startle [13]. Others have demonstrated that infusion of NPY into the BLA ameliorated the stress inducing effects of restraint stress [22] by inducing an increase in social interaction in previously restrained animals. Another potential target is the hippocampus, which, like the BLA, is not only rich in NPY [10] but also plays a critical role in the acquisition of CD via

its connection to the BLA [28]. In a study examining the role of the hippocampus on social defeat in rats and tree shrews, Zambello and colleagues showed that NPY and NPY receptor mRNA were both downregulated in the hippocampus [29]. Together, these data provide an important foundation to allow us to examine the role of NPY on stress related behaviors using the CD model. We believe that because CD uses an ethologically relevant, social conflict stressor with high face validity it may be more akin to traumatic stressors that are a known antecedent to various psychopathologies including PTSD and will provide additional support for the role of NPY on stress and anxiety.

In Experiment 2, pretreatment with the NPY Y1 receptor antagonist BIBP 3226 did not prevent the inhibition of submissive behaviors induced by NPY, as indicated by the lack of significant difference between the Saline/NPY and the BIBP 3226/NPY groups. However, we do show that, consistent with our finding in Experiment 1, NPY did significantly reduce submissive behavior compared to the behavioral control group (Saline-Saline). These data demonstrate that the Y1 receptor does not play a role in reversing the effect of stress, at least in the CD model. As indicated above, these results were surprising considering several reports showing that the resilience inducing and anxiolytic-like effects of NPY is mediated primarily via the Y1 receptor. Hendriksen and colleagues [30] for example, showed that infusion of [Leu31, Pro34]-NPY, a Y1 agonist, into the BLA significantly reduced the behavioral effects of stress from inescapable foot shock. Furthermore, pretreatment with BIBO 3304 or BIBP 3226, both Y1 antagonists, were shown to block the antidepressant effect of NPY in the forced swim test [24, 31]. One possible explanation for these findings is that the above-mentioned studies specifically assessed stress effects using non-ethologically based models (foot shock and forced swim). Because social defeat, and specifically the CD model, is more complex in that the subject must, by definition, interact with the stressor itself (a conspecific animal), this interaction necessarily induces responses on multiple sensory (visual, olfactory) and behavioral (defensive flight, upright, risk assessment, freezing) levels. Consequently, it is likely that the resulting effect simultaneously engages multiple systems. There are several lines of evidence that supports this hypothesis. First, while some studies have suggested that centrally administered NPY potentiates anxiety and defensive behaviors via the Y2 receptor [32], others have shown the opposite effect [33]. In addition, a recent report by Longo and colleagues suggest that the NPY Y1 receptor, co-expressed with the Y5 receptor, may also play a critical role in mediating anxiety responses in mice [34]. Further investigations using other NPY antagonists on social defeat will be necessary to elucidate this possibility. Another potential explanation for the findings in Experiment 2 is that studies have utilized several different types of Y1 antagonists [24, 31, 35], and, given that we did not observe a reversal of the resilience-inducing effect using BIBP 3226, it is possible that any number of other Y1 antagonists will yield results. Studies are currently underway investigating this possibility, using BIBO 3304. Finally, there are also reports that indicate that while the Y1 receptor may be involved in specifically mediating unconditioned fear and anxiety responses [24, 30, 31] it may not play a critical role in conditioned fear [36]. This finding would be consistent with our data considering we examined the conditioned fear response of socially defeated hamsters.

In summary, the findings from the present experiment add to the growing body of evidence that NPY plays a critical role in ameliorating the effects of social defeat stress by reducing

submissive/defensive behaviors in Syrian hamsters. Importantly, while most studies have utilized physical stressors such as restraint and electric shock, our data shows for the first time that NPY may play a significant role in ameliorating the effect of social stress and may provide critical data for possible insight into the basis of individual differences in the susceptibility to stress-related psychopathologies as well as to suggest a novel treatment strategy for psychiatric diseases related to stress-induced traumatic events.

Acknowledgements.

This work was supported by 5 R25 GM060566–17 awarded to the Spelman College Research Initiative for Scientific Enhancement (RISE) Program.

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Highlights

- Intracerebroventricular infusion of Neuropeptide Y (NPY) reduced submissive behaviors in previously defeated Syrian hamsters.
- Pretreatment with the NPY Y1 receptor antagonist BIBP 3226 did not restore the NPY induced reduction in submission.
- We conclude that while NPY may impart resilience to stressful experiences, these effects are not mediated by the Y1 receptor.

Figure 1.

Total duration (mean $\pm$ SEM) of submissive behavior during the 5-min test with the NAI in Experiment 1. * and # indicates significant difference compared to the saline control group at $p < 0.005$ and 0.0001 , respectively).

Figure 2. Total duration (mean $\pm$ SEM) of other behaviors during the 5-min test with the NAI in Experiment 1.

Figure 3. Total duration (mean $\pm$ SEM) of submissive behaviors during testing with NAI. * Indicates significant difference compared to the Sal-Sal control group

Table 1.Mean Latency to First Attack in Experiments 1 and 2 (means $\pm$ SEM)

<u>Experiment 1</u>	<u>Mean Duration</u>
0 pmol NPY	13.5 $\pm$ 2.56
200 pmol NPY	19.7 $\pm$ 6.22
400 pmol NPY	16.6 $\pm$ 3.90
<u>Experiment 2</u>	<u>Mean Duration</u>
0 μ g BIBP 3226/0 pmol NPY	10.5 $\pm$ 3.33
0 μ g BIBP 3226/400 pmol NPY	27.8 $\pm$ 4.33
1.5 μ g BIBP 3226/400 pmol NPY	4.8 $\pm$ 3.15
3.0 μ g BIBP/3226400 pmol NPY	13.51 $\pm$ 8.85