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Neuropeptide Y-Y_2 receptors mediate anxiety in the amygdala

Tammy J. Sajdyk[a], Douglas A. Schober[b], David L. Smiley[b], Donald R. Gehlert[b,*]

[a]*Indiana University Medical Center, Indianapolis, IN 46202, USA*

[b]*Neuroscience Division, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, DC 0510, Indianapolis, IN 46285, USA*

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Abstract

The behavioral effects of direct injection of the neuropeptide Y (NPY) Y_2 receptor agonist C2-NPY into the basolateral nucleus of the amygdala (BLA) was assessed in rats utilizing the social interaction test (SI). C2-NPY decreased SI time in a dose-dependent manner with a significant change observed at a dose of 80 pmol/100 nl. The anxiogenic effects produced by intra-amygdalar C2-NPY injections were reversed with intraperitoneal administration of alprazolam (1 mg/kg), a known anxiolytic. These findings support the hypothesis that Y_2 receptors are involved in the regulation of the anxiety response.

Keywords: Amygdala; Anxiety; Benzodiazepines; NPY; Social interaction; Stress

1. Introduction

Neuropeptide Y (NPY) is one of the most abundant peptides in the central nervous system. To date there have been five receptor subtypes cloned (Y_1, Y_2, Y_4, Y_5, and y_6) from different species (Michel et al., 1998). One of the major biological actions attributed to NPY has been its regulatory role in anxiolytic-like responses. Results from behavioral experiments indicate that central administration of NPY will induce anxiolytic-like responses in several animal tests (Heilig et al., 1989, 1992). Several lines of evidence indicate the anxiolytic-like effects seen following central administration of NPY are mediated via the Y_1 receptor. For example, intracerebroventricular injection of an antisense oligodeoxynucleotide targeted at the Y_1 receptor messenger RNA (mRNA) results in a decreased density of Y_1 receptors and a reduction of the anxiolytic effects of intra-amygdalar NPY (Heilig, 1995). Similarly, we have shown that injections of the Y_1 antagonist, BIBO 3304, into the basolateral amygdala (BLA) will block the anxiolytic-like effects of NPY in the social interaction (SI) test (Sajdyk et al., 1999). The role of the other NPY receptor subtypes in anxiety is not as clear. Y_1 and Y_2 receptor mRNAs are abundant in the amygdala (Parker and Herzog, 1999) including the BLA. Recently, the Y_1 agonist [Leu^{31},Pro^{34}] NPY and the Y_2 agonist NPY_{13-36} were administered to mice who were tested using the elevated plus-maze. Consistent with other studies, Y_1 receptor stimulation produced anxiolytic-like behavior, however, Y_2 receptor stimulation resulted in an anxiogenic-like response (Nakajima et al., 1998). These findings are in contrast to those by Heilig et al. (1989) who found that intracerebroventricular administration of NPY_{13-36} produced no measurable effect in rats subjected to the elevated plus-maze or the conflict test. In addition, Kask et al. (1998) administered NPY_{13-36} near the area of the locus coeruleus (LC) of rats and found that it produced an anxiolytic-like response using the elevated plus-maze model. Given the mixed results observed in behavioral studies and since the BLA contains a dense concentration of mRNA for the Y_2 receptor subtype, we investigated the role of the Y_2 receptor in anxiety-like behavior. In the present set of experiments, we injected the selective Y_2 agonist, C2-NPY (Gerald et al., 1996) into the BLA of rats and then assessed their behavior in the SI test, a validated test for anxiety (File, 1980).

2. Methods

All experiments utilized male Wistar rats (250–275 g) from Harlan Laboratories (Indianapolis, IN). Animals were

* Corresponding author. Tel.: +1-317-276-1810; fax: +1-317-276-5546.

E-mail address: gehlert_donald_r@lilly.com (D.R. Gehlert).

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individually housed in a temperature-controlled room (72 °F) and maintained on a 12-h light–dark cycle. Rats were given free access to food and water.

All animals were anesthetized with isoflurane using a Plexiglas chamber. After obtaining the full anesthetic effects, the rats were transferred to a stereotaxic instrument where they continued to receive isoflurane via a nose cone on the incisor bar, which was set at −3.3 mm. The scalp was shaved, cleaned, and cut to expose the skull. Bilateral 26-gauge cannulae (10 mm in length; Plastic Products, Roanoke, VA) were implanted into the BLA (A: −2.1, L: 5.0, V: −8.5 relative to bregma). Coordinates for placement of cannulae were determined using the atlas of Paxinos and Watson (1986). The guide cannulae were secured in place using cranioplastic cement and three 2.4-mm stainless steel screws anchored to the skull. Dummy cannulae were inserted to seal the guide cannulae. Animals were allowed 72 h to recover.

Two 33-gauge microinjection cannulae (11 mm in length; Plastic Products) were used to bilaterally administer C2-NPY (Eli Lilly, Indianapolis, IN and Neosystem, Strasbourg, France). C2-NPY was delivered in 100 nl of 1% bovine serum albumin (BSA). The cannulae were attached to polyethylene tubing (PE-50; Fisher Scientific, Pittsburgh, PA), which were connected to a 10-μl Hamilton syringe. The Hamilton syringes were situated on an infusion pump (Model PHD 2000; Harvard Apparatus, Holliston, MA) and the pump was programmed to automatically deliver 100 nl per site over a 30-s time period. The injection cannulae remained in place for an additional minute following infusion. Precise flow of the solutions was verified before and after each injection to ensure peptide delivery.

Alprazolam was mixed with 1 drop of Tween 80 and 1 drop of DMSO to dissolve the compound, then saline was added to bring the final concentration to 1 mg/ml. The suspension was injected at a dose of 1 mg/kg in a 1-cc syringe with a 25-gauge needle. The intraperitoneal injection was given 30 min prior to C2-NPY intracerebral administration.

Experimental anxiety was measured using the SI test (File, 1980). The 5-min test period was recorded via a video camera mounted on the ceiling above the SI arena ($36 \times 36 \times 12$ in.^{3} $L \times W \times H$) and was conducted under red light (25 W) familiar conditions. The "experimental" rat and the "partner" rat were simultaneously placed in the SI arena and the total time (seconds) was measured that the "experimental" animal initiated contact with the "partner" rat as previously described (Sajdyk and Shekhar, 1997).

The experimental protocol was designed to obtain data for three different groups of animals. The first group of rats was used to determine the dose of C2-NPY into the BLA necessary to induce behavioral effects. Bilateral cannulae were placed into the BLA of six rats. Approximately 72 h later, they were injected with 1% BSA and 30 min later assessed in the SI test. The same procedure was repeated on experimental days 2–4 with all animals receiving all doses (20, 40, and 80 pmol), but only a single dose on any given day. The second set of rats were utilized to measure the anxiety-like behavior of a single acute threshold dose of C2-NPY. Ten rats received bilateral cannulae into the BLA. Approximately 72 h after surgery, all rats were injected with vehicle (1% BSA) and 30 min later placed in the SI test. Forty-eight hours later, all animals were reinjected with C2-NPY (80 pmol) and 30 min later placed in the SI test. The third group of animals were used to determine the behavioral effects of a known anxiolytic on the anxiogenic properties of C2-NPY in the BLA. Six animals received bilateral cannulae into the BLA. Approximately 72 h following surgery, all rats were administered saline intraperitoneally (vehicle), then 30 min later, vehicle (1% BSA) into the BLA. Then 30 min later, the animals were assessed in the SI test for baseline anxiety. Forty-eight hours later, all animals were injected with saline intraperitoneally, then 30 min later, rats were administered C2-NPY (80 pmol) into the BLA. Again, 30 min later, all rats were assessed in the SI test. This same protocol was repeated 48 h later, except that alprazolam (1 mg/kg) was given intraperitoneally 30 min prior to C2-NPY (80 pmol).

Upon completion of the study, all rats were sacrificed with carbon dioxide. Their brains were immediately removed, rinsed with saline, wrapped with parafilm and foil, and stored in a −70 °F freezer. Later, the brains were removed from the freezer and sliced into 40-μm sections, mounted onto slides, stained with Cresyl violet, and verified for cannulae placement. Only data from the animals that had correct bilateral placement of cannulae in the BLA were utilized for data analysis.

A repeated-measures ANOVA with a Newman–Keul's post hoc test was used to analyze data from the dose response and the alprazolam studies. A paired t test was used to analyze data from the acute study with C2-NPY. The significance level for all analysis was set at $\alpha = .05$ and data on graphs are represented with S.E.M.

3. Results

3.1. C2-NPY dose response

In these experiments, baseline behavior was initially established on Day 1, then various doses of C2-NPY were administered, in a counterbalanced design, via bilateral cannulae and the behavior was assessed 30 min after infusion. A significant difference in treatment effect was observed using a repeated-measures ANOVA [$F(3,12) = 7.226$, $P = .0090$], while a significant decrease in SI at the 80-pmol dose from all other doses (20 and 40 pmol) as well as baseline was observed using post hoc analysis (Fig. 1a).

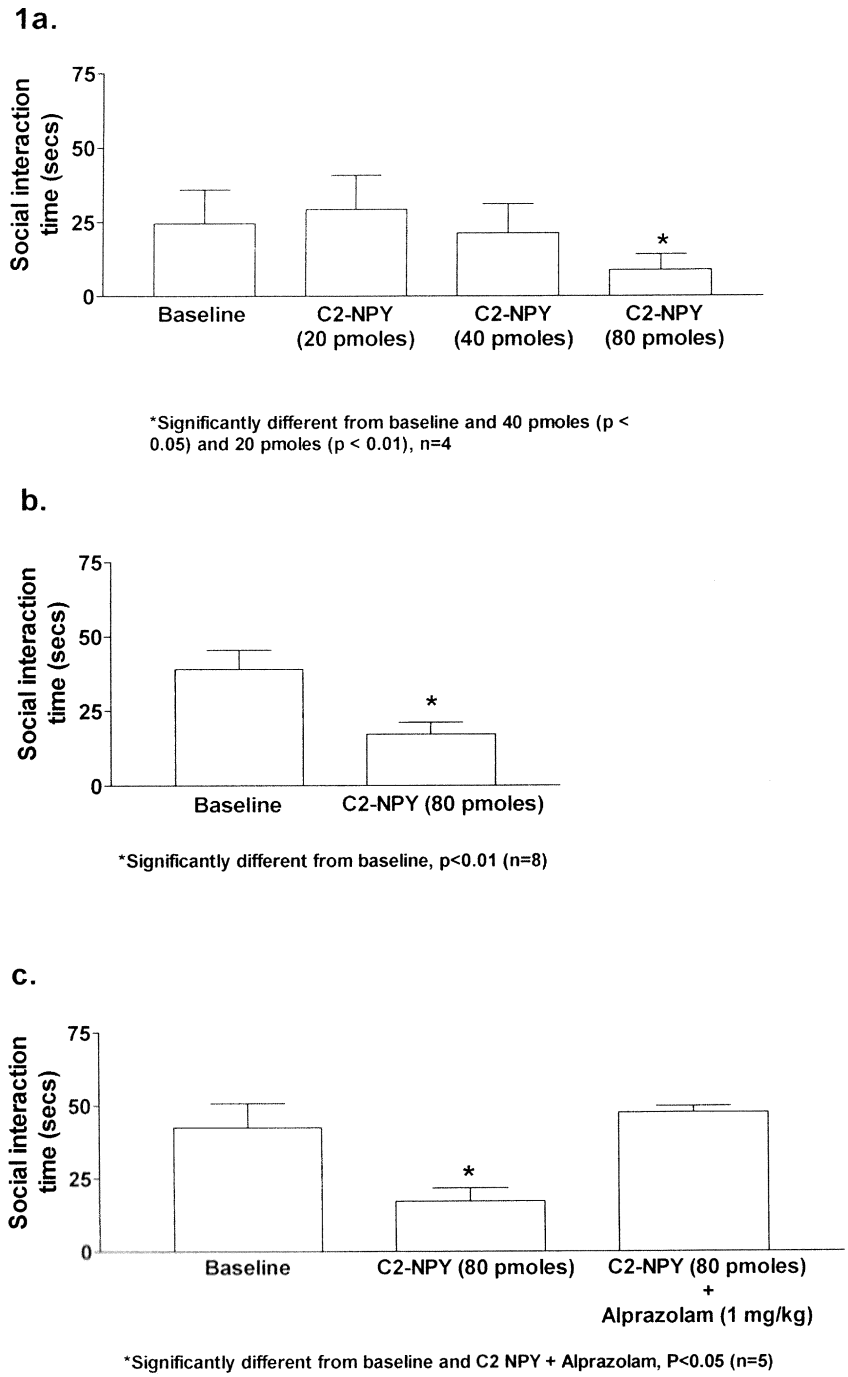

Fig. 1. Changes in SI time of rats 30 min following administration of (a) either vehicle (1% BSA) or C2-NPY (20, 40, or 80 pmol), (b) vehicle or C2-NPY (80 pmol), and (c) vehicle, C2-NPY (80 pmol), or C2-NPY (80 pmol) with a 30-min pretreatment of alprazolam (1 mg/kg). $P < .05$ (S.E.M.).

3.2. C2-NPY (80 pmol) acute response

Due to repeated administration of C2-NPY in the animals included in the dose response experiments, a second experiment was performed to assess the effects of a single effective dose of the agonist. Fig. 1b is a graph indicating the changes in SI time for rats 30 min following administration of 80-pmol C2-NPY into the BLA of rats. Analysis with a paired t test showed a significant difference between treatment and baseline [$t(7,6) = 3.801$, $P = .0067$].

3.3. C2-NPY (80 pmol) and alprazolam (1 mg/kg)

To determine whether the anxiety-like behavior was reversible with an anxiolytic, rats were administered alprazolam prior to C2-NPY infusion. Fig. 1c shows the changes in

the SI time of rats intraperitoneally administered with alprazolam (1 mg/kg) 30 min prior to an injection of C2-NPY into the BLA. A significant difference was noted among treatment groups following data analysis with a repeated-measures ANOVA [$F(4,12) = 1.029$, $P = .0103$], while a significant difference between the animals receiving C2-NPY alone compared to the other two treatment groups was seen following a Newman–Keul's post hoc analysis ($P < .05$).

4. Discussion

Our lab has focused on the role of NPY receptor subtypes in anxiety-like behavior. When administered into the BLA, NPY decreased anxiety-like behavior via the Y_1 receptor subtype (Sajdyk et al., 1999). These findings are consistent with the current literature on Y_1 receptors and anxiety (Greibel, 1999). In contrast, the experiments investigating the role of Y_2 receptors in anxiety have resulted in differential effects depending on the area studied. Therefore, we investigated the role of the Y_2 receptor in anxiety-like behavior by directly infusing the selective Y_2 agonist C2-NPY into the BLA, an area known to be involved in anxiety responses. Activation of the Y_2 receptor with C2-NPY (80 pmol) significantly decreased SI time in rats compared to their baseline (see Fig. 1a and b). In addition, the anxiogenic response produced by C2-NPY could be blocked by pretreatment of the anxiolytic alprazolam (see Fig. 1c).

Several reasons may account for the differential results observed in the studies using selective Y_2 agonists. First, Kask et al. (1998) conducted their studies by infusing peptides into the area of the LC, another brain region associated with behavioral responses to fear. The NPY-containing neurons in the LC also produce the excitatory neurotransmitter noradrenaline (NA; Everitt et al., 1984), while in the BLA, NPY is found in the interneurons that contain the inhibitory neurotransmitter GABA (McDonald and Pearson, 1989). In electrophysiology studies of the LC, NPY enhanced the hyperpolarizing effects of NA via the Y_2 receptors (Illes et al., 1993). This type of neuronal regulation by Y_2 receptor activation probably accounts for the anxiolytic-like actions seen in the studies by Kask et al. (1998). Conversely, in the suprachiasmatic nucleus, where NPY is colocalized with GABA, NPY decreased the inhibitory effects of GABA via a presynaptic Y_2 mechanism (Chen and van den Pol, 1996). This presynaptic mechanism has also been observed in the hypothalamus (King et al., 2000). This group showed that the Y_2 selective antagonist BIIE0246 could prevent NPY_{13-36}-induced reduction in basal and K^+-stimulated NPY release. Thus, it is likely that this mechanism is also occurring within the BLA. Earlier work in our lab showed that disruption of inhibition within the BLA by antagonizing the $GABA_A$ receptor led to an increase in glutamatergic excitation and a subsequent increase in heart rate, blood pressure, and anxiety-like behavior (Sajdyk and Shekhar, 1997).

The current findings may differ from those of Heilig et al. (1989) due to the site of injection of the Y_2 agonist and the specificity of the agonist used. It could be the case that the area of diffusion of the intracerebroventricular injection of the NPY_{13-36} did not directly stimulate the Y_2 receptors in the necessary brain regions involved in the NPY Y_2 mediated behavioral response. NPY_{13-36} has a high affinity ($K_i = 1.8$ nM; Gehlert et al., 1996) for the human Y_2 receptor and human Y_5 receptor ($K_i = 1.9$ nM; Statnick et al., 1998) while having substantial affinity for the human Y_1 receptor ($K_i = 12.5$ nM). Therefore, it is likely that an effective dose of NPY_{13-36} at the Y_2 receptor would also stimulate Y_5 and, perhaps, Y_1 receptors. Therefore, it is possible that nonselective activation of the Y_2 and Y_5 receptors was occurring in rats receiving the intracerebroventricular dose of 400 pmol of NPY_{13-36}, as compared to the 20-pmol dose in the mouse. To determine whether NPY_{13-36} and C2-NPY are exerting their effects selectively via the Y_2 receptor it would be necessary to carry out these same studies with a Y_2 antagonist. Since an antagonist was not available at the time of testing, we reversed the C2-NPY induced anxiogenic behavior using the benzodiazepine alprazolam at a dose of 1 mg/kg ip. These results are consistent with the hypothesis that the response seen in the SI test due to intra-amygdalar Y_2 receptor activation is anxiety-like. It is possible that two independent processes are occurring during the injection of alprazolam, that is, C2-NPY could be exerting an anxiogenic-like effect while alprazolam is exerting an equal but opposite anxiolytic-like effect thus creating an overall net effect of zero behavioral change, it is probably not the case. Experimental studies in our lab have shown that alprazolam given at a dose of 1 mg/kg has no effect on baseline SI behavior (data not published).

In conclusion, Y_2 receptor activation in the BLA of rats resulted in anxiety-like behavior. This provides additional evidence that the BLA is an important region for mediating the behavioral effects of NPY. Further understanding of the role of the Y_2 receptor in behavioral regulation will require the development of specific brain penetrant Y_2 antagonists.

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