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Estrogen receptor beta activation prevents glucocorticoid receptor-dependent effects of the central nucleus of the amygdala on behavior and neuroendocrine function

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Abstract

Neuropsychiatric disorders such as anxiety and depression have formidable economic and societal impacts. A dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis leading to elevated endogenous glucocorticoid levels is often associated with such disorders. Chronically high glucocorticoid levels may act upon the central nucleus of the amygdala (CeA) to alter normally adaptive responses into those that are maladaptive and detrimental. In addition to glucocorticoids, other steroid hormones such as estradiol and androgens can also modify hormonal and behavioral responses to threatening stimuli. In particular, estrogen receptor beta (ERβ) agonists have been shown to be anxiolytic. Consequently, these experiments addressed the hypothesis that the selective stimulation of glucocorticoid receptor (GR) in the CeA would increase anxiety-like behaviors and HPA axis reactivity to stress, and further, that an ERβ agonist could modulate these effects. Young adult female Sprague-Dawley rats were ovariectomized and bilaterally implanted via stereotaxic surgery with a wax pellet containing the selective GR agonist RU28362 or a blank pellet, to a region just dorsal to the CeA. Four days later, animals were administered the ERβ agonist *S*-DPN or vehicle (with four daily sc injections). Anxiety-type behaviors were measured using the elevated plus maze (EPM). Central RU28362 implants caused significantly higher anxiety-type behaviors in the EPM and greater plasma CORT levels than controls given a blank central implant. Moreover, *S*-DPN treated animals, regardless of type of central implant, displayed significantly lower anxiety-type behaviors and post-EPM plasma CORT levels than vehicle treated controls or vehicle treated animals implanted with RU28362. These results indicate that selective activation of GR within the CeA is anxiogenic, and peripheral administration of an ERβ agonist can overcome this effect. These data suggest that estradiol signaling via ERβ prevents glucocorticoid-dependent effects of the CeA on behavior and neuroendocrine function.

Keywords

Glucocorticoids; Amygdala; Estrogen receptor; Stereotaxic; Anxiety

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1. Introduction

Anxiety is one of the most prevalent mental disorders and has an immense impact on society. Approximately 25% of adults will suffer from one or more forms of anxiety disorder in their lifetime, and the yearly economic cost is estimated to be at least $40 billion per year in the United States and Europe (Balak and Elmaci, 2007; Gordon and Hen, 2004; Kessler et al., 2009). In addition to emotional symptoms, patients with anxiety disorder exhibit impaired hypothalamic-pituitary-adrenal (HPA) axis function (Ehlert et al., 2001; McEwen, 2005) as evidenced by the inability to respond appropriately to stress. For example, patients with late-life generalized anxiety disorder (GAD) exhibit higher salivary cortisol levels as compared to normal healthy individuals, and the level of cortisol positively correlates with severity of GAD symptoms (Mantella et al., 2008). Furthermore, anxiety disorders are more prevalent in females than males (Bekker and van Mens-Verhulst, 2007), suggesting a potential role for estradiol in the etiology of anxiety. Interestingly, estradiol has been shown to be both anxiolytic and anxiogenic in rodents (Koss et al., 2004; Leret et al., 1994; Mora et al., 1996; Palermo-Neto and Dorce, 1990). However, selective activation of estrogen receptor alpha (ERα) has been shown to increase anxiety-like behaviors, whereas selective activation of estrogen receptor beta (ERβ) has been shown to decrease these behaviors (Lund et al., 2005; Walf and Frye, 2005; Walf et al., 2008). Furthermore, different stressors can activate distinct neural circuits, each of which may have a different inherent sensitivity to either ERα- or ERβ-dependent signaling (Dayas et al., 2001). This suggests that estrogens can have vastly different effects on hormonal and behavioral responses to stress depending on the predominant ER involved in signaling.

One brain region underlying anxiety behaviors in humans and anxiety-like behaviors in rodents is the amygdala (Chapman et al., 1954; Davis, 1997; Kopchia et al., 1992). This brain region also potentiates HPA axis reactivity (Bhatnagar and Dallman, 1998; Prewitt and Herman, 1994), and neurons within this region contain high amounts of androgen and estrogen receptors (Gray et al., 1993; Laflamme et al., 1998; Shughrue and Merchenthaler, 2001). Consequently, it is possible that the amygdala may be a brain site that is important for the sex-specific or hormone dependent modulation of neurobiological changes underlying anxiety disorders. The amygdala coordinates behavioral, autonomic, and endocrine responses to perceived threats or danger to an animal (Davis, 1992; Davis, 1997; LeDoux, 1998). The central nucleus of the amygdala (CeA) in particular, controls major efferent pathways from the amygdalar complex (for review see (Sah et al., 2003)). The CeA has descending projections to regions involved in autonomic, endocrine, and behavioral responses to emotional stimuli (Gray et al., 1989; Marcilhac and Siaud, 1997; Veening et al., 1984). Accordingly, CeA lesions lead to decreased anxiety and decreased neuroendocrine responses to a spectrum of stressors (Beaulieu et al., 1986; Feldman et al., 1994; Kopchia et al., 1992).

Anxiety disorders are often accompanied by HPA axis dysfunction that results in chronically elevated glucocorticoids. Glucocorticoids typically act through high affinity mineralocorticoid receptors (MR) or low affinity glucocorticoid receptors (GR) (Reul and de Kloet, 1985). During the basal state, circulating levels of glucocorticoids are sufficient to occupy primarily MR, whereas following a stressor, elevated glucocortiocoid levels can occupy both MR and GR. The CeA has a robust expression of GR, and accordingly can respond to acute or chronically elevated CORT (Morimoto et al., 1996; Reul and de Kloet, 1985). In contrast to the paraventricular nucleus of the hypothalamus (PVN), where elevated CORT inhibits CRH expression, similar levels of CORT increase CRH mRNA in the CeA. Correspondingly, adrenalectomy increases CRH expression in the PVN whereas it decreases CRH mRNA within the CeA (Makino et al., 1994; Palkovits et al., 1998; Swanson and Simmons, 1989; Viau et al., 2001; Watts and Sanchez-Watts, 1995) and direct administration of a corticosterone pellet just dorsal to the CeA increases CRH mRNA in the CeA (Shepard et al., 2000). Recent studies also

show that CORT delivery to the CeA causes increased anxiety-type behaviors, and plasma CORT following exposure to the elevated plus maze (Myers et al., 2005; Shepard et al., 2000; Shepard et al., 2003). Such results, when taken together indicate that the CeA likely plays an important role in integrating the response to acutely or chronically elevated glucocorticoids. Alterations in this response by glucocorticoid hormones could precipitate the maladaptive behaviors associated with anxiety.

The gonadal steroid hormone, estradiol, has also been shown to augment HPA axis activity and alter anxiety-type behaviors (Burgess and Handa, 1992; Handa et al., 1994; Lund et al., 2004; Lund et al., 2005). Estradiol can act via two receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Green et al., 1986; Kuiper et al., 1996). Treatment of ovariectomized female rats with the ERβ agonist diarylpropionitrile (DPN) results in decreased anxiety-type behaviors and plasma CORT following exposure to the elevated plus maze, whereas ERα activation appears to have the opposite effect (Lund et al., 2005). Such data suggest that it is the ERα activating component of estradiol that may augment hormonal and behavioral responses to stress, an effect that is opposite of any ERβ activating components.

The CeA is integral in the behavioral responses of fear and anxiety. It also plays a major role in regulating the stress responsiveness of the HPA axis, and expresses low levels of ERβ (Laflamme et al., 1998; Osterlund et al., 1998; Shughrue and Merchenthaler, 2001), and ERα (Laflamme et al., 1998; Shughrue et al., 1997). Therefore, in these studies we sought to determine if local administration of a GR agonist to the CeA can induce anxiety-type behaviors which can be counteracted by peripheral administration of an ERβ agonist.

2. Results

Bilateral implantation of the GR agonist RU28362 results in nuclear localization of GR within the central nucleus of the amygdala

We utilized the translocation of GR immunoreactivity (GR-IR) to the cell nucleus to verify the spread of RU28362 from the wax pellet. GR-IR was examined seven days following bilateral implantation of a beeswax pellet containing either RU28362 or nothing (blank) to a region dorsal to the CeA (Figure 1). RU28362 implants resulted in nuclear GR-IR within the CeA (dark brown staining within cell body) (Figure 2B and 2D). Blank implants resulted in largely cytoplasmic GR-IR within the CeA (light brown staining in cytoplasm) (Figure 2A and 2C). Nuclear GR-IR was primarily contained within the area of the CeA, and observed no further than 1.0mm from the implanted pellet (including basolateral amygdala, paraventricular nucleus of the hypothalamus, and hippocampus). Based on these observations we excluded animals from analysis in which both implants were greater than 1.0mm from the CeA (4/32 or 12.5% of animals, represented by grey dots in Figure 1). There were three animals in which one of the two implants was beyond 1.0mm of the CeA, however the second implant was within 1.0mm of the CeA. The dependent measures obtained from these animals were similar to those that received two correct bilateral implants, and therefore were included in the statistical analysis.

The GR agonist RU28362 administered to the central nucleus of the amygdala decreases anxiolytic behavior and ERβ activation blocks this effect

We next examined whether GR activation in the CeA alters anxiety-like behaviors on the elevated plus maze (EPM), and the possibility that systemic treatment with an ERβ agonist (*S*-DPN) could alter these effects. We measured the open arm entries and open arm time as a percentage of total entries and total time, respectively, to normalize for non-specific treatment effects on and/or individual variations in locomotor activity. Two-way ANOVA analysis showed a trend for systemic treatment-by-central treatment interaction effect for percentage

of open arm entries ($F_{(1,26)}$ = 2.52; P = 0.126) and percentage of open arm time ($F_{(1,26)}$ = 3.07; P = 0.093), but did reveal a significant systemic treatment-by-central treatment interaction effect in total head dips ($F_{(1,26)}$ = 5.86; P = 0.024). *Post hoc* pairwise comparison revealed that RU28362 caused a significant decrease in the percentage of open arm entries (Figure 3A) ($F_{(3,26)}$ = 30.85; P < 0.05), percentage open arm time (Figure 3B) ($F_{(3,26)}$ = 14.44; P < 0.05), and number of head dips (Figure 3C) ($F_{(3,26)}$ = 14.90; P < 0.05) as compared to animals with blank (beeswax) implants. There was no significant effect of GR activation on total entries, rearing, grooming, or number of fecal boli (data not shown).

Two-way ANOVA indicated an overall systemic treatment effect, whereby *S*-DPN treatment significantly increased the percentage of open arm entries (Figure 3A) ($F_{(1,26)}$ = 74.49; P < 0.0001), percentage open arm time (Figure 3B) ($F_{(1,26)}$ = 33.63; P < 0.0001), and head dips (Figure 3C) ($F_{(1,26)}$ = 37.78; P < 0.0001) as compared to animals with vehicle treatment regardless of implant type. There was no effect of *S*-DPN on total entries ($F_{(1,26)}$ = 0.32; P = 0.7025). Therefore, peripheral administration of *S*-DPN effectively blocked the anxiety-like behaviors seen in RU28362 implanted animals, and further increased exploratory behavior on the EPM.

The GR agonist RU28362 administered to the central nucleus of the amygdala increases plasma corticosterone, and peripheral administration of the ERβ agonist S-DPN prevents this effect

To examine the corresponding hormonal response caused by exposure to the EPM, corticosterone (CORT) levels were measured in plasma obtained from trunk blood collected 20 minutes after initial exposure to the elevated plus maze. Two-way ANOVA revealed an overall systemic treatment effect where peripheral *S*-DPN treatment significantly decreased plasma CORT following the EPM regardless of implant type ($F_{(1,23)}$ = 34.73; P < 0.0001). There was no systemic treatment-by-central treatment interaction effect for plasma corticosterone ($F_{(1,23)}$ = 1.25; P = 0.276). *Post hoc* pairwise comparisons indicate that animals with RU28362 implants had significantly higher plasma CORT following the EPM than animals with blank implants (Figure 4; $F_{(3,23)}$ = 13.77; P < 0.05). Therefore, peripheral administration of *S*-DPN effectively blocked the increase in stress-induced plasma CORT seen with RU28362 implanted animals given vehicle injections, and further reduced CORT levels as compared to controls.

3. Discussion

In these studies we examined the ability of selective glucocorticoid receptor activation in the CeA to augment anxiety-like behaviors and determined whether the concurrent activation of estrogen receptor beta could counteract glucocorticoid effects. Our result show that chronic exposure of a GR agonist, RU28362, directly to the CeA results in elevated anxiety-type behaviors and plasma CORT in response to a psychological stressor (elevated plus maze, EPM). Interestingly, concomitant systemic treatment with a selective ERβ agonist, *S*-DPN, completely abolished the behavioral and hormonal effect of local application of RU28362 to the CeA.

The CeA is well poised to integrate glucocorticoid and gonadal steroid hormone signaling during time of perceived or actual danger. The presence of steroid hormone receptors within the CeA allow this brain region to respond to fearful situations in a manner dependent upon the internal hormone milieu (Bingaman et al., 1994; Laflamme et al., 1998; Morimoto et al., 1996; Osterlund et al., 1998; Reul and de Kloet, 1985; Shughrue and Merchenthaler, 2001). The CeA contains the highest density of GR expression in the amygdala, and is responsive to elevated levels of glucocortiocoids (Makino et al., 1994; Morimoto et al., 1996; Swanson and Simmons, 1989; Watts and Sanchez-Watts, 1995). Therefore, in these studies, we delivered

the GR agonist RU28362 bilaterally to an area dorsal to the CeA via stereotaxic implantation. This results in chronic activation of GR within the CeA independent of exogenous CORT levels. The placement of the pellet insures sustained GR occupancy without mechanical damage to the CeA.

In order to test the spread of ligand from the implanted pellet, we examined nuclear localization of GR using immunocytochemistry. The rationale for this approach is based upon the ability of ligand binding to translocate GR to the cell nucleus whereas it is largely cytoplasmic in the absence of ligand (Madan and DeFranco, 1993; Munck and Holbrook, 1984; Raaka and Samuels, 1983; Sackey et al., 1996). Therefore nuclear GR immunoreactivity (GR-IR) provides a good marker to determine the extent of agonist spread. We observed nuclear GR-IR within a radius of approximately 1.0mm from the RU28362 pellet, which included the CeA. This is consistent with what was shown previously for other hormones using the same procedure for estradiol (Lund et al., 2006) and for androgens (Bingham et al., 2009). Predominantly cytoplasmic GR-IR was observed around blank pellets. Previous studies where CORT pellets (30 μg) are placed in this same region resulted in a CORT diffusion radius of 0.5 to 1.0 mm, which included the CeA, but excludes other glucocorticoid sensitive regions such as the BNST, PVN, and hippocampus (Shepard et al., 2003).

Our results demonstrate that administration of a selective GR agonist to the CeA results in increased anxiety-type behaviors in the elevated plus maze (EPM) and increased plasma CORT following maze exposure. Generally, treatments that reduce open arm exploration (time spent on open arm and open arm entries) and increase head dipping and rearing (explorative behaviors) are considered anxiogenic (Handley and McBlane, 1993; Pellow et al., 1985). These results implicate GR activation in the CeA alone as responsible for the generation of anxiety-type behaviors in the rat. Our data are in accordance with previous studies where CORT was delivered to the CeA. CORT exposure was shown to be anxiogenic and augment CeA CRH mRNA expression and plasma CORT secretion (Myers et al., 2005; Shepard et al., 2000; Shepard et al., 2003). Furthermore, this effect of CORT on anxiogenesis can be blocked via peripheral administration of a CRHR1 antagonist, suggesting that the anxiogenic effect of CORT delivered to the CeA is mediated through CRH signaling (Myers et al., 2005). It is important to note that these studies, including the one presented here, examine the consequences of a relatively high dose and long-term (days) exposure to glucocorticoids within the CeA. While this does not entirely match a physiological phenomenon, it may be representative of what occurs during chronic glucocorticoid hypersecretion as is seen with chronic stress and in certain psychological disorders. However, it is necessary to keep in mind that endogenous glucocorticoids are secreted in a diurnal circadian pattern consisting of frequent pulses that are necessary for normal physiology (Lightman et al., 2008; Stavreva et al., 2009).

Since CORT has affinity for both MR and GR and preferentially binds MR, it could not be determined from the studies of Shephard et al. (2000 Shephard et al. (2003) if the effect observed was truly GR mediated. Indeed, elevated glucocorticoid levels allow binding to GR and this increases CRH levels in the CeA, an effect that is opposite that of glucocorticoids in the PVN. Upregulation of CeA CRH may in turn result in increased CRH neurotransmission to other brain regions integral in the behavioral and endocrine responses to stressors. Furthermore, the CeA does have direct stimulatory projections to the medial parvocellular region of the PVN (Gray et al., 1989; Marcilhac and Siaud, 1997), a brain region that contains the CRH and AVP neurons that comprise the main effector arm of the HPA axis. Alternatively, the CeA could access the PVN through an indirect pathway involving the lateral BNST. The CeA sends CRH-positive axons to the lateral BNST, which in turn has direct stimulatory projections to the medial parvocellular PVN (Gray, 1993; Herman et al., 1994; Herman et al., 2003). Correspondingly, stimulation of the lateral BNST increases CRH mRNA expression in

the medial parvocellular PVN and plasma CORT levels (Dunn, 1987; Herman et al., 1994). Interestingly, the hippocampus also accesses the PVN via an indirect path through the BNST (Herman et al., 1998). In this regard, it is possible that the CeA may counteract the inhibitory influence of the hippocampus on HPA axis output, especially during times of chronically elevated glucocorticoids. Studies examining the effect of selective activation of ERβ within the CeA, BNST, or PVN on CRH gene expression is certainly warranted given the results described in this study.

Gonadal steroids play an integral role in modulating mood and HPA axis function (Fink et al., 1998; Handa et al., 1994; McCormick et al., 2002; Shors and Leuner, 2003; Weiser et al., 2008). Estrogen in particular has been reported to have opposing effects on both mood and HPA axis activity. This could be explained, in part, by the existence of two main receptors for estrogen, ERα and ERβ (Green et al., 1986; Kuiper et al., 1996). Animal studies have implicated estrogen signaling via ERα in generating anxiety-type behaviors and in augmentation of HPA axis activity (Lund et al., 2005). Alternatively, studies have shown an opposite role for estrogen signaling through ERβ (Imwalle et al., 2005; Krezel et al., 2001; Lund et al., 2005; Walf and Frye, 2006). Specifically, treatment of OVX females with the selective ERβ agonist, *S*-DPN, decreases anxiety-type behaviors in the EPM, despair in the forced swim test, and plasma CORT secretion in response to restraint (Weiser et al., 2009). However, the mechanism of ERβ-dependent effects on anxiety-like behavior and HPA axis function remain to be determined. Therefore, in this study, we addressed the hypothesis that systemic treatment of *S*-DPN could alter glucocorticoid receptor specific actions on the CeA. Our results indicate that peripheral administration of *S*-DPN blocks the anxiogenic effect of a locally administered GR agonist to the CeA. In fact, *S*-DPN treatment proved to be anxiolytic to the same extent regardless of GR activation in the CeA. Therefore, glucocorticoid-sensitive modulation of anxiety-type behaviors through the CeA can be inhibited or overcome by activation of ERβ.

In this study *S*-DPN, the bioactive enantiomer of the ERβ agonist, diarypropionitrile (Weiser et al., 2009), was administered peripherally, and consequently several different modes of action are possible. DPN has been shown to pass the blood-brain barrier, and thus likely exerts its effect centrally (Lund et al., 2005). The CeA does express ERβ, albeit at low levels (Laflamme et al., 1998; Osterlund et al., 1998; Shughrue and Merchenthaler, 2001), indicating a potential direct effect of ERβ on the activity of CeA neurons. However, it is not known if ERβ and GR co-exist within the same neurons in the CeA. Aside from a direct action on CeA projecting neurons, an alternative possibility is that ERβ is expressed in GABAergic interneurons of the CeA and upon ligand-activation acts to inhibit nearby CRH neurons. Interestingly, infusion into the CeA of the benzodiazepine midazolam, an allosteric modulator of the $GABA_A$ receptor, produces anxiolytic effects that can be blocked by injection of the benzodiazepine receptor antagonist, flumazenil, into the CeA (Pesold and Treit, 1995). Furthermore, local application of the GABA agonist, muscimol, to the CeA results in decreased anxiety in the elevated plus maze (Moreira et al., 2007). This highlights the importance of GABA signaling for CeA-specific modulation of behaviors and raises the possibility that GABA mediates ERβ's actions. Future studies could examine the extent of ERβ, GR and GABA colocalization in the CeA, and to what extent ERβ activation has an effect on GABAergic signaling and/or CRH expression in the CeA.

Another potential mechanism that might explain the anxiolytic effects of ERβ on CeA function could be through ERβ-containing sites that project to the CeA or impinge on projection areas from the CeA. For example, *S*-DPN may act by activation of ERβ-expressing areas that project to the CeA such as the medial amygdala, BNST, PVN, arcuate nucleus, hippocampus, and dorsal raphe nucleus (Canteras and Swanson, 1992; Dong and Swanson, 2004; Dong and Swanson, 2006; Ottersen, 1980; Ottersen, 1981). Alternatively, there could be activation of ERβ containing neurons in brain regions that have direct and indirect projections to the

parvocellular PVN (e.g. BNST, hippocampus, arcuate nucleus) which may act to override the CeA-mediated augmentation of HPA axis drive. Although, ERβ is expressed in a small percentage of CRH-expressing neurons of the PVN (Laflamme et al., 1998; Suzuki and Handa, 2005), suggesting a potential direct effect on HPA axis output, the majority of ERβ-expressing neurons in the PVN are brainstem- and spinal cord- projecting neurons and not neurosecretory neurons (Bingham et al., 2006). Selective activation of ERβ within these discrete brain regions in combination with or without GR activation in the CeA would provide clues into potential sites of action and neural pathways that may be involved.

In summary, the data presented here indicate that GR activation in the CeA can induce anxiety-type behaviors and an elevated hormonal (CORT) response to a psychological stressor. Additionally, treatment with an ERβ agonist can override the effect of GR in the CeA and further attenuates anxiety-type behaviors and dampens CORT secretion following a stressor. These data suggest a role for estradiol signaling via ERβ in modulating glucocorticoid-dependent effects of the CeA on behavior and neuroendocrine function.

4. Materials and Methods

Animals

Young adult female Sprague Dawley rats (~225g) were obtained from Harlan Laboratories (San Diego, CA), housed individually, and maintained on a 12h:12h light schedule (lights on at 0600h) in temperature and humidity controlled rooms at the Laboratory Animal Research Facility at Colorado State University. We chose female rats due to a few factors: one of their primary gonadal hormones is estrogen, there is a significant sex difference in anxiety-type behaviors in female rats as compared to males, and there is a sex difference in anxiety and depression in humans where women suffer greater from either in comparison to men. Animals had *ad libitum* access to a soy free diet (modified AIN-93G, DYET#101591 from DYETS, Inc., Allentown, PA, with corn oil substituted for soy oil) to minimize activation of ERβ by phytoestrogenic compounds. One week after arrival, animals were ovariectomized under isofluorane anesthesia. Following ovariectomy, animals were handled daily (5 minutes each animal) by the same experimenter. All animal protocols were previously approved by the Animal Care and Use Committee at Colorado State University.

Stereotaxic implantation

One week following ovariectomy animals were fitted bilaterally with two 22 gauge stainless-steel cannulae (Small Parts, Miami Lakes, FL) with the aid of a small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA). The tips of the cannulae were previously packed with the glucocorticoid receptor agonist RU28362 (Roussel-Uclaf, Romainville, France), which was dissolved into warmed beeswax (Sigma-Aldrich, St. Louis, MO) to a final concentration of 0.5 uM, and packed to a height of 0.5 mm within the end of the cannulae. We chose this concentration of RU28362 since a similar concentration of estradiol diffuses approximately 0.5mm from a beeswax pellet when examined sven days after implantation in the brain (Lund et al., 2006). Controls received cannulae packed with beeswax alone. Stereotaxic coordinates to allow placement of the cannula tip to the region just above the central nucleus of the amygdala were: 2.3 mm posterior and 3.8 mm lateral to bregma, and 6.3 mm below the skull surface. A 28 gauge stainless steel wire cut to extend 1.0 mm past the length of the cannulae was inserted into the cannulae to expell the pellet. Following animal sacrifice, location of pellet implants was confirmed in cresyl violet-stained sections. Animals in which both implants were more than 1.0mm from the central nucleus of the amygdala (4 out of 32 animals, 12.5%) were excluded from analysis based on the observed spread of RU28362 from the beeswax pellet via glucocorticoid receptor immunocytochemistry.

Drug treatments

Beginning four days following stereotaxic implant, animals were given a single daily subcutaneous injection of either vehicle (hydroxypropyl betacyclodextran; 27% w/w in saline; CTD Inc., High Springs, FL), or the ERβ agonist, *S*-DPN (1.0 mg/kg) in a total volume of 0.2 ml. This dose of DPN has been previously shown to be anxiolytic in female rats (Lund et al., 2005). Injections occurred at 0800 h for 4 days. DPN was synthesized *de* novo as previously described (Lund et al., 2005) and separated into S- and R- enantiomers by HPLC (Weiser et al., 2009). Because we have previously demonstrated that the *S*-enantiomer contains all of the ERβ activity of DPN (Weiser et al., 2009), only the *S*-enantiomer was tested in these studies. Three hours after the injection on day 4, animals underwent behavioral testing on the elevated plus maze (EPM).

Elevated plus maze

Animals were transported in their home cage to the behavioral testing room and maze performance was evaluated for 5 minutes on the fourth day of treatment (seventh day post-implantation) as previously described (Handley and McBlane, 1993). Behaviors measured included number of entries into open and closed arms (where both forelimbs enter maze arm), total time spent in open and closed arms, rearing (picking up both forelimbs from the surface of the maze in an effort to explore), head dips (placing head over the edge of the maze in an effort explore area below and around the maze), total time spent grooming, and the number of fecal boli. Animals that fell off the maze during testing (two total) were excluded from analysis.

Plasma corticosterone analysis

Animals were returned to their home cage following EPM testing and 15 min later (20 min following the start of EPM), animals were rapidly decapitated and trunk blood collected into ice-chilled tubes containing 0.5M EDTA and aprotinin (4 mg/ml, Sigma, St. Louis, MO). Plasma levels of corticosterone were determined by radioimmunoassay as previously described (Lund et al., 2005). Intra-assay and Inter-assay coefficient of variance (CV) were 3.9% and 5.1% respectively.

Immunocytochemistry

To examine the extent of RU28362 spread from implanted pellets, two animals from each central treatment group (four total, blank or RU28362) were intracardially perfused with saline followed by 4% buffered paraformaldehyde 90 minutes after maze exposure. Brains were postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, sectioned at 35uM on a Leitz cryostat, and stored in cryopreservative at −20 C until processed for immunocytochemistry. After standard washes, the free floating sections were incubated in 1.5% NGS in 0.01 M PBS with 0.03% TX to block nonspecific binding, then incubated for 24 hours at 4 C with the GR antibody (1:400) in 0.01 M PBS with TX in the presence of 1.5% NGS. The rabbit antiserum used (PA-511, Affinity Bioreagents, lot# 019-147) was prepared against a synthetic peptide corresponding to residues 346–367 from human GR. This antibody detects a single band via western blot representing the 97 kDa GR protein, and has been shown to produce cytoplasmic staining in the absence of ligand, and nuclear staining after glucocorticoid administration (Cidlowski et al., 1990; Francis et al., 2006). Following primary antibody incubation, the tissue was washed three times for 10 minutes each in PBS with TX and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:400) in PBS with TX in the presence of 1.5% NGS for 2 hours at room temperature. Sections are subsequently washed and processed according to the avidin-biotin-peroxidase procedure (Vector Laboratories; 1:400). After standard washes, the tissue was rinsed in 0.1 M Tris-buffered saline (TBS, pH 8.0) for 15 minutes and then developed with 3,3′-diaminobenzidine (DAB; 0.5 mg/ml; Sigma, St. Louis, MO) in 0.1 M Tris-buffered saline) and 0.01% hydrogen peroxide in 0.1M TBS for 3–

5 minutes to produce a brown reaction product. The reaction is stopped by several washes in 0.01 M PBS. Control sections, where primary antibody was omitted, were run in parallel. Staining for the corresponding antigen was absent in these sections.

Statistics

For behavioral measures, 27 out of 32 total animals were included in the statistical analysis and exclude four animals in which pellet placement was incorrect and two animals that fell off the maze (including one animal that had incorrect pellet placement and fell off the maze). For corticosterone, 24 out of 32 total animals were included in the statistical analysis and exclude four animals perfused for ICC (one of which fell off the maze) and four animals in which pellet placement was incorrect (including one animal that had incorrect pellet placement and fell off the maze). Two-way analysis of variance was performed on data from each experiment using Prism analysis software (v. 5.0, GraphPad Software Inc., San Diego CA). Newman-Keuls multiple comparison test was used *post hoc* for pairwise comparisons. Differences were considered significant when $p < 0.05$. Data are expressed as group means ± SEM.

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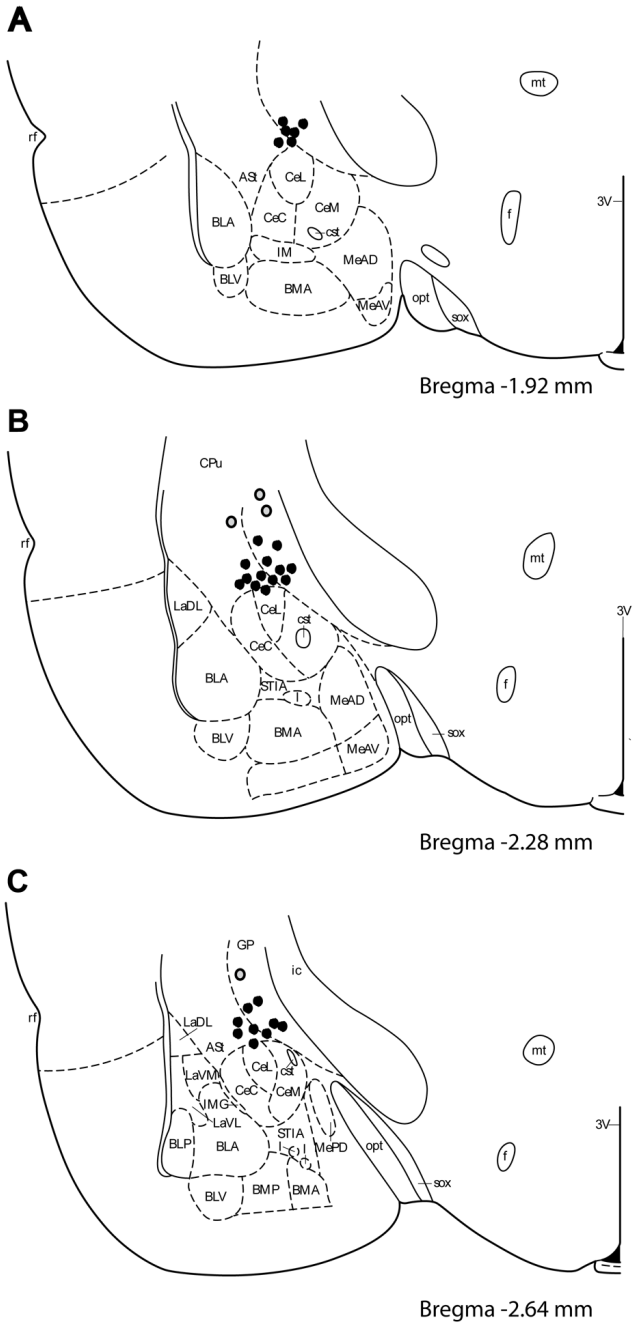

Figure 1.

Location of beeswax pellet implants. One side of the bilateral implant is depicted for clarity. Each dot represents the ventral border of the wax pellet. Grey dots correspond to implants in animals in which placement was beyond 1.0mm of the central nu of the amygdala in both hemispheres (excluded from analysis). Illustration adapted from Paxinos and Watson (Paxinos and Watson, 2007). 3V, third ventricle; ASt, amygdalostriatal transition; BLA, anterior basolateral amygdaloid nu; BLP, posterior basolateral amygdaloid nu; BLV, ventral basolateral amygdaloid nu; BMA, anterior basomedial amygdaloid nu; BMP, posterior basomedial amygdaloid nu; CeC, central amygdaloid nu, capsular part; CeL, central amygdaloid nu, lateral div; CeM, central amygdaloid nu, medial div; CPu, caudate putamen; cst, commissural stria

terminalis; f, fornix; I, intercalated nuclei of the amygdala; ic, internal capsule; IM, intercalated amygdaloid nu, main part; IMG, amygdaloid intramedullary gray; LaDL, lateral amygdaloid nu, dorsolateral part; LaVL, lateral amygdaloid nu, ventrolateral part; LaVM, lateral amygdaloid nu, ventromedial part; MeAD, medial amygdaloid nucleus, ant dorsal; MeAV, medial amygdaloid nucleus, anteroventral part; MePD, medial amygdaloid nucleus, posterodorsal part; MePV, medial amygdaloid nucleus, posteroventral part; mt, mammillotegmental tract; MTu, medial tuberal nu; opt, optic tract; rf, rhinal fissure; sox, supraoptic decussation; STIA, bed nucleus of the stria terminalis, intraamygdaloid division.

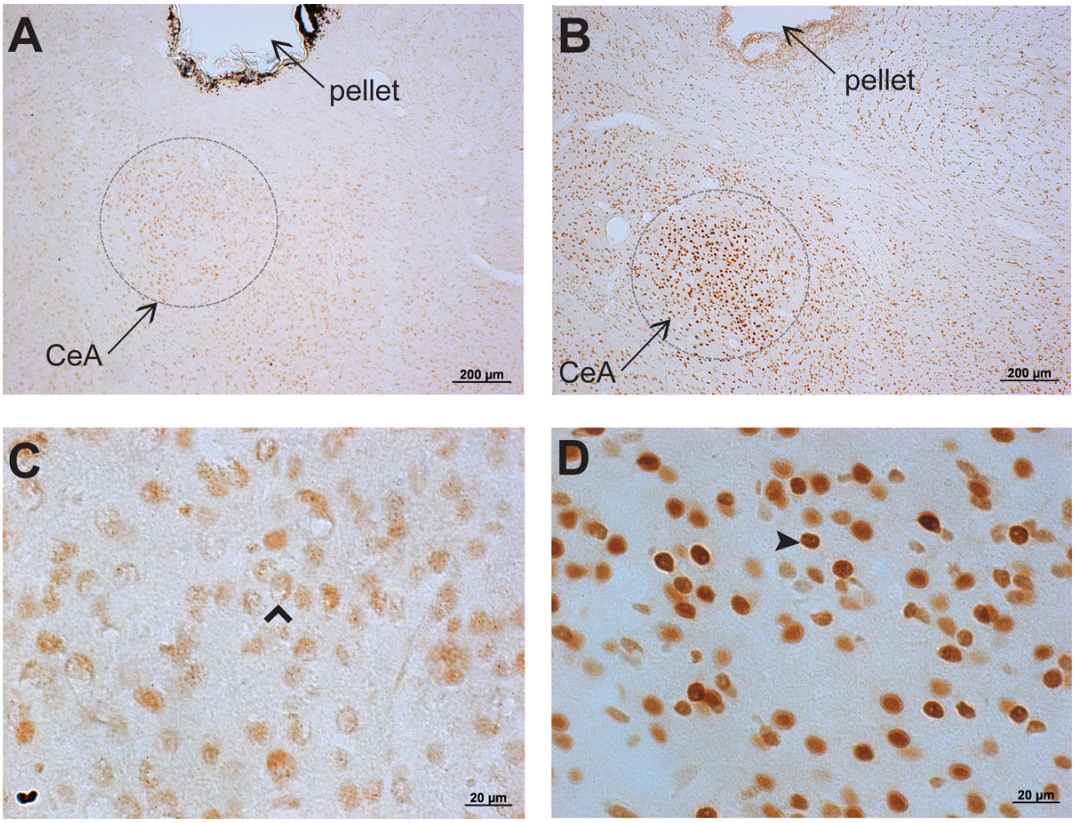

Figure 2.

Glucocorticoid receptor immunoreactivity (GR-IR) in the central nucleus of the amygdala (CeA) following bilateral implantation of RU28362 pellets. Representative photomicrographs (A) and (C) of GR-immunoreactivity (GR-IR) in an animal with bilateral implants of blank pellets to a region dorsal to the CeA show largely cytoplasmic GR-IR within the CeA (open arrowhead in (C), light brown staining in cytoplasm). Representative photomicrographs (B) and (D) of GR-IR in an animal with bilateral implants of the GR agonist RU28362 show nuclear GR-IR within the CeA (closed arrowhead in (D), dark brown staining within cell body). Accumulation of nuclear GR-IR was observed no further than 1.0mm from pellet.

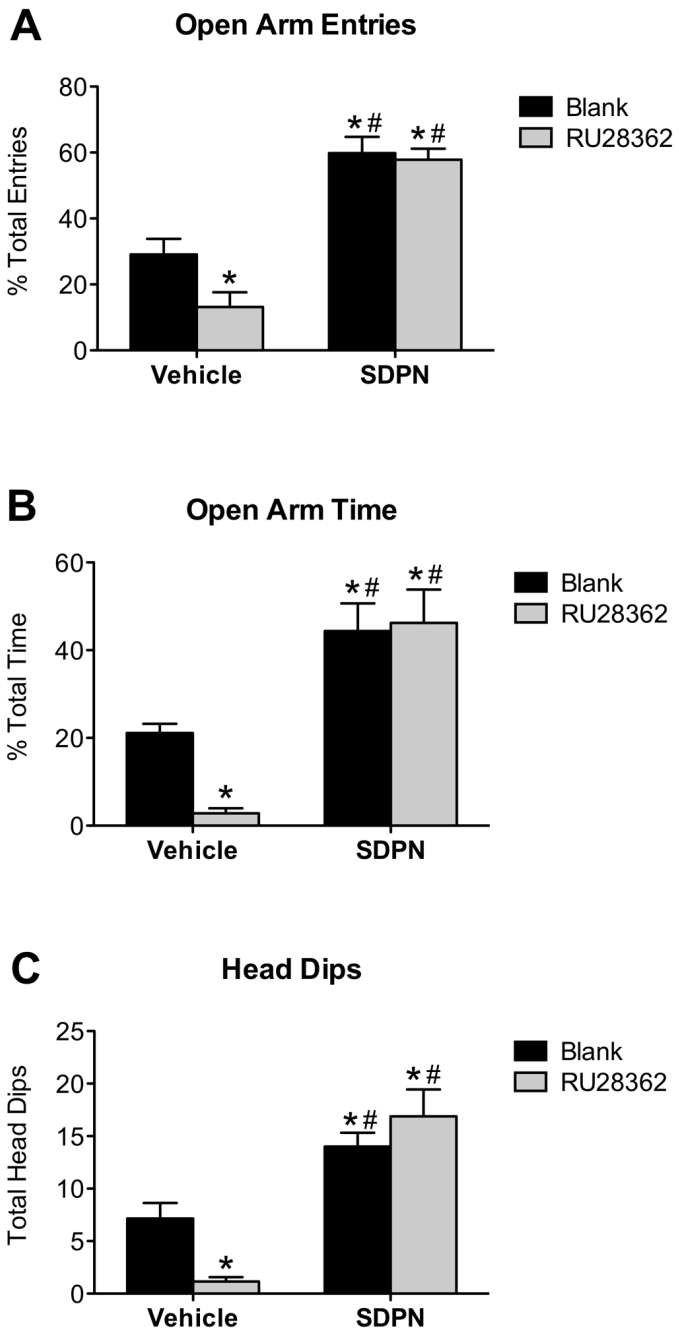

Figure 3.

Behavior on the elevated plus maze. The GR agonist RU28362 delivered bilaterally to the central nucleus of the amygdala (CeA) decreases anxiolytic behaviors on the elevated plus maze (#). Peripheral treatment with the ERβ agonist *S*-DPN increases anxiolytic behaviors and blocks the anxiogenic effect of RU28362 administered to the CeA (*). *Panel A*: Open arm entries expressed as percentage of total arm entries (closed + open). *Panel B*: Open arm time expressed as percentage of total time on maze. *Panel C*: Number of head dips over edge of open arm. Data are represented as mean ± SEM; n = 6–8 animals per treatment group. *, indicates significant difference ($P < 0.05$) compared with animals implanted with blank pellets

and vehicle treated (controls); #, indicates significant effect of peripheral treatment in groups with same central treatment.

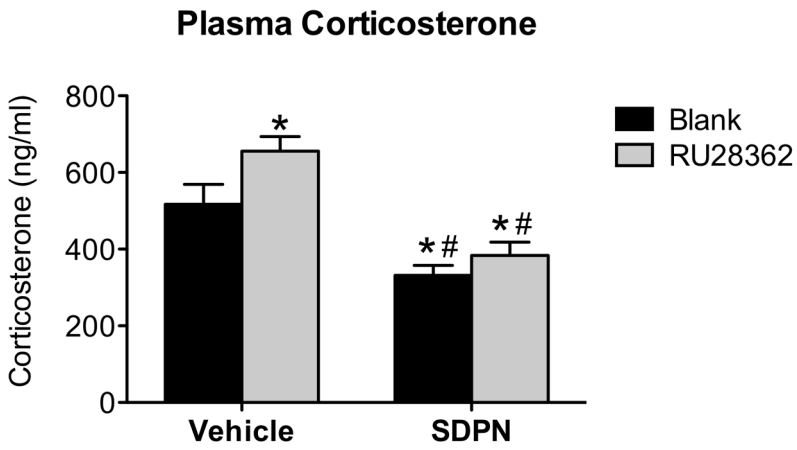

Figure 4.

Plasma corticosterone levels 20 minutes after initial maze exposure. The GR agonist RU28362 delivered bilaterally to the central nucleus of the amygdala (CeA) increases plasma corticosterone 20 minutes following the initiation of elevated plus maze (#). Peripheral treatment with the ERβ agonist *S*-DPN decreases plasma corticosterone following the elevated plus maze and blocks the augmentation of plasma corticosterone seen with RU28362 administration to the CeA (*). Data are represented as mean ± SEM; n = 6–7 animals per treatment group. *, indicates significant difference ($P < 0.05$) compared with animals implanted with blank pellets and vehicle treated (controls); #, indicates significant effect of peripheral treatment in groups with same central treatment.