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Alpha-Melanocyte-Stimulating Hormone Attenuates Behavioral Effects of Corticotropin-Releasing Factor in Isolated Guinea Pig Pups

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Abstract

During a 3-hr period of social isolation in a novel environment, guinea pig pups exhibit an initial active phase of behavioral responsiveness, characterized primarily by vocalizing, which is then followed by a stage of passive responsiveness in which pups display a distinctive crouch, eye-closing, and extensive piloerection. Prior treatment of pups with alpha-melanocyte-stimulating hormone (α -MSH) reduces each of the passive behaviors. The onset of passive responding during separation can be accelerated with peripheral injection of corticotropin-releasing factor (CRF). To examine whether CRF produces its effects through a mechanism similar to that of prolonged separation, we examined the effect of administering α -MSH to pups injected with CRF. As expected, CRF markedly enhanced passive responding during a 60-min period of separation. α -MSH delivered by either intracerebroventricular infusion or intraperitoneal injection significantly reduced each of the passive behavioral responses without significantly affecting active behavior. These findings, together with previous results indicating that it is the anti-inflammatory property of α -MSH that is responsible for its behavioral effects during prolonged separation, suggest that peripheral CRF speeds the induction of passive responding through a mechanism involving enhanced proinflammatory activity.

Keywords

maternal separation; corticotropin-releasing hormone; sickness behavior; alpha-melanocyte-stimulating hormone; proinflammatory cytokines; guinea pig

INTRODUCTION

Corticotropin-releasing factor (CRF) is a principal mediator of the body's responses to stressors. CRF and its receptors are widely distributed throughout the CNS (Korosi & Baram, 2008; Swanson, Sawchenko, Rivier, & Vale, 1983). In its role mediating hypothalamic–pituitary–adrenal (HPA) activity, CRF synthesized by cell bodies of the

paraventricular nucleus of the hypothalamus travels via the hypophyseal portal system to the pituitary to effect release of ACTH and β -endorphin (Owens & Nemeroff, 1991; Rivier, Brownstein, Spiess, Rivier, & Vale, 1982). Glucocorticoid elevations, the end-product of stress-induced increases in HPA activity, mediate adaptive metabolic processes that promote coping and have anti-inflammatory effects (Sapolsky, Romero, & Munck, 2000). In addition, central CRF acts as a neuropeptide to stimulate sympathetic activation, central norepinephrine release, and gastrointestinal effects associated with stress (Tsigos & Chrousos, 2002). Central CRF also mediates a wide range of behavioral changes typical of a stressed state (Britton, Koob, Rivier, & Vale, 1982; Eaves, Thatcher-Britton, Rivier, Vale, & Koob, 1985; Kalin, Shelton, Kraemer, & McKinney, 1983a; Koob et al., 1993).

CRF and CRF receptors also are present in various peripheral organs (Mastorakos et al., 1994, 1996). Although circulating levels of CRF are very low and do not demonstrate measurable increases during stress, increased release of peripheral CRF into local pools near the location of CRF receptors has been observed during stressful conditions (Aguilera et al., 1990; Bruhn, Engeland, Anthony, Gann, & Jackson, 1987; Karalis et al., 1991) and following immune system activation (Zytek & Slominski, 2007). Peripherally CRF affects a variety of physiological functions including testosterone release (Dufau, Tinajero, & Fabbri, 1993), birth (Wadhwa et al., 2004), and colonic motility (Saunders, Maillot, Million, & Tache, 2002). In contrast to the broad anti-inflammatory effects of cortisol, peripheral CRF induces *proinflammatory* activity (Karalis et al., 1991).

Further, although CRF does not readily cross the blood–brain barrier (Martins, Kastin, & Banks, 1996), peripherally administered CRF has been found to affect behavior. For instance, subcutaneous (SC) CRF alters aspects of aversive learning in rats through a mechanism that appears at least partially independent of the HPA system (Veldhuis & DeWied, 1984). Responses on analgesic tests are reduced by peripheral CRF (Ayesta & Nikolarakis, 1989; Hargreaves, Mueller, Dubner, Goldstein, & Dionne, 1987). In rhesus monkeys, effects of intravenous CRF include vocalizing, lying down, and reductions in grooming and exploratory activity (Kalin, Shelton, Kraemer, & McKinney, 1983b).

In guinea pig pups, we have found SC injection of CRF to have robust effects on behavior during social separation. During 30–60 min of isolation in a novel environment, active responses characteristic of such brief periods of separation (vocalizing and locomotor activity) are suppressed by CRF administration, and passive responses that normally become frequent only following several hours of isolation (a characteristic crouched stance, eye-closure, and extensive piloerection) are greatly increased (Hennessy, Becker, & O’Neil, 1991; Hennessy, Long, Nigh, Williams, & Nolan, 1995). That is, peripheral injection of CRF appears to accelerate the onset of the second, passive behavioral stage of separation in guinea pig pups. These behavioral consequences of CRF injection are not secondary to hypotension, impaired motor ability, or emission of behaviors incompatible with active behavior (Becker & Hennessy, 1993; Hennessy et al., 1995). Because effects of peripherally injected CRF could not be duplicated with injection of ACTH, nor reversed with administration of the opiate-receptor antagonist naloxone (Hennessy et al., 1991), pituitary CRF receptors of the HPA axis do not appear to be a primary mediator of these outcomes. The action of CRF on both the active and passive behaviors of pups is abolished by prior

peripheral injection of a CRF-receptor antagonist, indicating that effects are receptor mediated (Hennessy et al., 1995). Moreover, peripheral injection of just a CRF-receptor antagonist produced effects opposite those of peripheral CRF administration, that is, increased vocalization and locomotion rates and a reduction in the proportion of pups exhibiting passive behavior during 60 min of isolation (McInturf & Hennessy, 1996). This finding is as would be expected if endogenous CRF in some way normally modulates behavior during isolation to facilitate the onset of the second, passive stage of behavioral responsiveness.

How and where peripheral CRF acts to affect behavior in guinea pig pups remains unclear. In two experiments, direct intracerebroventricular (ICV) administration of CRF in doses approaching those effective peripherally had little or no effect on vocalizing and locomotor activity (Hennessy et al., 1992; passive behaviors were not examined in this early study). These results, together with the resistance of the blood–brain barrier to CRF, casts serious doubt on the possibility of a direct action of peripheral CRF on central receptors. At present, it would seem that peripheral CRF most likely affects the behavior of guinea pig pups through one of the two general routes. First, CRF might bind to receptors in afferent fibers to transmit neural signals to the nucleus of the solitary tract and brain circuits controlling behavior. It has been observed, for instance, that peripheral CRF mediates colonic motility via vagal afferents to the CNS (Tsukamoto et al., 2006). Second, proinflammatory activity induced by CRF in the periphery may stimulate central proinflammatory activity to produce the behavioral outcomes. In support of this second possibility, it is known that activation of proinflammatory cytokines can stimulate passive behaviors like those shown by guinea pig pups (Hart, 1988), and that peripheral proinflammatory activity can induce central proinflammatory activity through, for instance, stimulation of vagal afferents or the binding of cytokine receptors in CNS vasculature (Blalock & Smith, 2007; Maier & Watkins, 1998).

Recently, we examined the effect of alpha-melanocyte-stimulating hormone (α -MSH), a peptide with broad anti-inflammatory effects (Catania & Lipton, 1993; Lipton & Catania, 1997), on the behavior of guinea pig pups during prolonged separation. We found that central, but not peripheral, administration of α -MSH significantly reduced each of the three passive behavioral responses of guinea pig pups during a 3-hr period of isolation in a novel cage (Schiml-Webb, Deak, Greenlee, Maken, & Hennessy, 2006). In light of those findings, we reasoned that if peripherally injected CRF increases passive behavior through a mechanism similar to that of prolonged separation in untreated pups, then α -MSH might also be expected to reduce the passive behavior of pups injected with CRF. The present study tested this possibility. We examined the ability of centrally (Experiment 1) and peripherally (Experiment 2) administered α -MSH to reduce the passive behavior of pups injected with CRF.

GENERAL METHOD

Subjects

Albino guinea pigs (*Cavia porcellus*) were bred in our laboratory. Each mother and her litter were housed in clear plastic cages (73 cm $\times$ 54 cm $\times$ 24 cm) with sawdust bedding. Water and guinea pig chow were available ad libitum. Lights were maintained on a 12:12 light/dark

cycle, with lights on at 07:00 hr. Cages were changed twice per week. All procedures were approved by the Wright State University Laboratory Animal Care and Use Committee. Pups were maintained with their mothers and littermates for the duration of the experiments, being removed only for surgery for placement of ICV cannulae, behavioral testing, and brief routine colony management procedures (such as weighing of pups). For each experiment, pups were assigned to either CRF or Saline conditions [$n = 10$ (5 males, 5 females) per condition per experiment]. No more than one pup per litter was assigned to either condition in either experiment.

Cannulation Procedures

Between Days 16 and 19 (with day of birth considered Day 0), each pup in Experiment 1 underwent surgery for placement of an indwelling cannula aimed at the right lateral ventricle; pups in Experiment 2 were not cannulated. Pups spent no more than 4 hr out of the home cage for surgical procedures. Pups were pretreated with atropine [.05 mg/kg, intraperitoneal (IP)] and anesthetized with isoflurane before being placed in a stereotaxic apparatus. Surgery was performed with the skull oriented flat in the apparatus. The stereotaxic was equipped with a modified incisor bar/nose cone that delivered a constant dose of isoflurane throughout the surgery. A local anesthetic was administered to the scalp (.25 mg/.1 ml of .25% bupivacaine, SC) prior to the scalp incision being made.

Guide cannulae (26 gauge) were placed relative to bregma with coordinates of -3.0 mm anterior–posterior, -3.0 mm lateral, and -4.0 mm dorsal–ventral (from the skull; Luparello, 1967). A stainless steel screw was placed across the skull's sagittal suture adjacent to the guide cannulae to help secure the cranioplastic cement that held the guide in place. All cannula supplies were sterile at the time of surgery and were purchased from Plastics One (Roanoke, VA). Following surgery, and again 12 hr later, pups were treated with buprenorphine (.015 mg/.05 ml, SC) to control for postoperative pain. Each day postsurgery, pups were weighed, and dummy cannulae (caps) were removed so that the indwelling cannulae could be checked for patency; these procedures required that pups be removed from the home cage for no more than 5 min. A recovery time of at least 4 days was allowed before behavioral testing. All animals were killed after the second behavioral test via carbon dioxide inhalation. Cannulae placement was then verified via infusion of dye (~ 50 μ l India ink) through the cannula; only data from animals in which dye was present in at least one lateral ventricle were included for analysis.

Drugs

A 10 μ g dose of CRF (Sigma, St. Louis, MO) was administered SC. Previously, we found SC doses between 7 and 14 μ g to induce passive responding in guinea pig pups (Hennessy et al., 1991). α -MSH (Sigma) was administered at a dose of 25 μ g, which previously was found to reduce passive responding of guinea pig pups during 3 hr of separation when administered ICV, but not peripherally (Schiml-Webb et al., 2006). On a body weight basis, this dose is in the upper range of ICV doses found to reduce proinflammatory responses in rodents (Catania & Lipton, 1993; Kandasamy & Williams, 1984). ICV infusion volumes were 5 μ l and were administered over 2.5 min. α -MSH was administered by IP injection in Experiment 2. All peripheral injection volumes were .2 ml. Saline served as vehicle for

peripheral injections and artificial CSF (aCSF; Alzet Preparation, Cupertino, CA; www.alzet.com) was used as vehicle for ICV infusions. Pups were first injected with CRF and then immediately were administered α -MSH or its vehicle.

Behavioral Testing

In each experiment, pups of both conditions received α -MSH once and its vehicle once (counterbalanced within condition) with a minimum of 3 days lapsing between tests. Thus, pups were tested twice, and these tests occurred at Days 21–23 and 24–26, that is, pups were tested at approximately the last week of the preweaning period. Following drug administration, pups were immediately returned to their home cages for 60 min, after which they were separated from their mothers and littermates and placed in a clean, empty cage in a novel testing room, adjacent to the animal housing room for 60 min, during which time behavioral data were collected. All behavior tests took place between 10:00 and 14:00 hr under full room lighting. A trained observer scored behavioral events while sitting behind a one-way glass. A single observer was used for both tests for each pup. Passive behaviors of crouch (body hunched down with feet tucked beneath the body), eye-close (one or both eyes completely or nearly completely closed for more than 1 s), and piloerection (occurring over most of the visible body surface) were scored. Because single instances of crouch, eye-close, and piloerection typically occur over an extended period of time, these behaviors were scored with one-zero sampling as in previous studies (Schiml-Webb et al., 2006). Specifically, we noted whether the behavior occurred during any portion of each consecutive 1-min interval (for a total of 60 intervals in each 1-hr observation session). All occurrences of the active behaviors of whistle vocalizations (Berryman, 1976) and line-crossings (the number of times pups crossed lines on the cage floor that divided it into four equal sections) were also recorded. Vocalizations were scored with a hand-held counter; other behaviors were scored with pencil and paper. Interobserver reliability was 85% or above within each behavioral category.

Analyses

Behavioral data were not normally distributed even when summed across observation intervals. Because this was due largely to frequent scores of “zero,” nonparametric tests (rather than parametric tests of transformed scores) were used for analysis. Mann–Whitney *U*-tests were used for between-groups comparisons and Wilcoxon matched-pairs tests were used for within-group comparisons. Preliminary tests revealed that males and females did not differ in their patterns of response, so data were pooled across sex. A two-tailed level of significance of $p < .05$ was used throughout.

RESULTS

Experiment 1: Can ICV α -MSH Reduce Behaviors Induced by CRF?

Passive Behavior—CRF greatly enhanced passive responding. During tests in which pups were administered aCSF vehicle ICV, crouch ($p < .001$), eye-close ($p < .001$), and piloerection ($p < .001$) were all observed much more frequently in the CRF, than in the Saline condition (Fig. 1). α -MSH had no effect on the minimal passive responding shown by Saline pups. However, α -MSH significantly reduced the crouching ($p < .05$), eye-closing (p

<.05), and piloerection ($p < .05$) of pups injected with CRF. Yet, α -MSH did not completely reverse the effect of CRF. That is, when infused with α -MSH, crouch ($p < .01$), eye-close ($p < .01$), and piloerection ($p < .01$) were all observed more frequently in CRF, than in Saline pups. In sum, ICV α -MSH significantly attenuated the passive behaviors induced by CRF.

Active Behavior—CRF suppressed active behavior. When pups were infused with aCSF vehicle ICV, those in the CRF condition exhibited fewer vocalizations ($p < .01$) and line-crossings ($p < .01$) than did those in the Saline condition. There was no significant effect of α -MSH on the active behaviors in either the CRF or Saline conditions, though active behaviors of saline-injected pups tended to be lower following α -MSH. As a result, vocalizations of CRF and Saline pups did not differ when given α -MSH, though line-crossings were still reduced by CRF ($p < .05$). Overall, α -MSH did not significantly affect active behavior in either condition, though there was a tendency for this behavior to be reduced in saline-injected pups.

Experiment 2: Can IP α -MSH Reduce Behaviors Induced by CRF?

Passive Behavior—Pups injected with CRF exhibited high levels of passive behavior, replicating the findings of Experiment 1. As illustrated in Figure 2, CRF increased crouching, eye-closing, and piloerection (p 's $< .05$) regardless of the whether pups were injected with α -MSH or its vehicle. In the CRF condition, crouching ($p < .05$), eye-closing ($p < .05$), and piloerection ($p < .05$) were all observed less frequently when injected with α -MSH than when injected with vehicle. α -MSH had no effect on the low level of passive responding of pups in the Saline condition. Thus, peripherally administered α -MSH, like ICV α -MSH, attenuated CRF's effect on passive responding during separation.

Active Behavior—CRF again suppressed active behavior. When injected with the vehicle for α -MSH, these effects were clear for both vocalizing ($p < .01$) and line-crossing ($p < .01$). There was no significant effect of α -MSH on the active behaviors in either group, though these behaviors again tended to be reduced in the Saline condition: Line-crossings of CRF and Saline pups did not differ, though vocalizing was lower in the CRF pups ($p < .05$). In sum, IP α -MSH, like ICV α -MSH, did not significantly affect active behavior in either condition, though there was a tendency for this behavior to be reduced by α -MSH in Saline pups.

DISCUSSION

Under normal (i.e., nondrug) conditions, the crouching, eye-closure, and extensive piloerection of guinea pig pups become prominent after several hours of isolation in a novel environment (Hennessy et al., 1995). As expected, therefore, the saline-injected pups in the present study exhibited few passive behaviors during 1 hr of isolation. In contrast, SC CRF administration potently inhibited the active behaviors of vocalizing and locomotor activity, and markedly increased the passive responses of crouch, eye-close, and piloerection throughout the separation period, replicating previous results (Becker & Hennessy, 1993; Hennessy et al., 1995). However, if CRF-treated pups were also administered α -MSH, levels of each of the passive behaviors were clearly reduced; active behaviors were not

significantly altered. These results together with our earlier findings that central administration of α -MSH reduced the passive behaviors, but did not affect the active responding, of otherwise untreated pups during a more-prolonged period of isolation (Schiml-Webb et al., 2006) suggests some commonality in the mechanism producing the passive behaviors in the two situations.

The choice of α -MSH for the earlier study was based on this peptide's potent and particularly broad range of anti-inflammatory actions including, but not limited to, inhibition of proinflammatory cytokine and prostaglandin production, enhancement of anti-inflammatory cytokine effects, and inhibition of neutrophil migration (Catania & Lipton, 1993; Lipton & Catania, 1997). Nonetheless, α -MSH is also known to affect behavior through other means, most notably as an anorectic agent (McMinn, Wilkinson, Havel, Woods, & Schwartz, 2000) and, in other species at least, by producing anxiogenic effects (Gonzalez, Vaziri, & Wilson, 1996; Kokare, Chopde, & Subhedar, 2006; Panksepp & Abbott, 1990; Rao et al., 2003). Such actions could be proposed to have influenced behavior in the present study. For instance, the passive behaviors might be a response to the removal of a source of food (the mother), perhaps exacerbated by CRF, so that α -MSH reduced passive behaviors by reducing appetite. However, pups of the age tested here will readily ingest solid food (Harper, 1976) and still display high levels of crouch, eye-close, and piloerection when both chow and water are provided during a 3-hr isolation period (Hennessy et al., 1995). Moreover, even a nonlactating female will partially reduce the level of passive behavior of pups during isolation, though she is not as effective as is the pup's own mother (Hennessy & Morris, 2005). Similarly, an anxiogenic action appears unable to account for the ability of α -MSH to reduce passive behaviors. We have seen no indication that α -MSH increases anxiety in guinea pig pups. α -MSH does not cause pups to "freeze" or emit behaviors incompatible with the scored behaviors. Rather, pups administered α -MSH still engaged in a variety of behaviors typical of pups in this situation, including sitting quietly, shifting position, orienting toward different aspects of the environment, sniffing the cage, consuming feces, grooming, rearing against the sides of the cage, and scratching. Further, α -MSH does not affect the plasma cortisol response to separation (Perkeybile, Schiml-Webb, O'Brien, Deak, & Hennessy, in press).

Though other modes of action cannot conclusively be ruled out, we would contend that it is the anti-inflammatory effect of α -MSH that most likely accounts for its observed outcomes in guinea pig pups. In the time since the present set of experiments were initiated, we have obtained other evidence that passive responses—at least when emitted spontaneously during prolonged isolation—are mediated by proinflammatory factors. Two other anti-inflammatory agents, the COX-2 inhibitor indomethacin, and the anti-inflammatory cytokine interleukin-10, both were observed to reduce passive behaviors of pups during a 3-hr isolation (Hennessy, Deak, Schiml-Webb, & Barnum, 2007; Perkeybile et al., in press). Direct activation of cytokine release with injection of lipopolysacchride (LPS) elevates crouching, eye-closing, and piloerection in a dose-dependent manner (Hennessy et al., 2004), and central administration of 25 μ g of α -MSH reduces LPS-induced passive responses (Hennessy, Deak, et al., 2007). Three hours of isolation also was found to increase expression of a proinflammatory cytokine, tumor-necrosis factor- α , in the spleen (Hennessy, Schiml-Webb, et al., 2007). Based on this evidence, we would argue that CRF administration

induces the passive responses, at least in part, by increasing proinflammatory activity, which in turn is suppressed by administration of α -MSH.

Proinflammatory actions of peripheral CRF seem to be wide spread and are well documented. Local pools of CRF in the vicinity of immune organs and circulating immune cells (thymus and spleen: Chowdrey, Lightman, Harbuz, Larsen, & Jessop, 1984; T cells, B cells, and macrophages: Baker, Richards, Dayan, & Jessop, 2003) appear to act in a paracrine fashion to stimulate proinflammatory responses (Karalis et al., 1991; Webster et al., 1996). Moreover, CRF-deficient mice exhibit a blunted response to LPS challenge, indicating the involvement of endogenous, peripheral CRF in inflammation (Venihaki, Zhao, & Karalis, 2003). The induction of peripheral inflammatory responses is associated with increased peripheral immunoreactivity for CRF and increased transcription of CRF mRNA (Crofford et al., 1992). Proinflammatory effects of CRF have been demonstrated in skin (Singh, Pang, Alexacos, Letourneau, & Theoharides, 1999), which may be particularly relevant for effects of CRF administered subcutaneously. In vitro, CRF was found to release cytokines from immune cells (Leu & Singh, 1992; Singh & Leu, 1990) and to induce synthesis of inflammatory-related acute phase proteins from hepatocytes (Hagen, Poole, & Bristow, 1993). During infection, proinflammatory cytokines released from peripheral cells (e.g., macrophages) appear to induce central cytokine activity underlying passive behavior associated with sickness (Maier & Watkins, 1998). In the present study, then, both peripherally and centrally administered α -MSH may have been effective because these procedures inhibited proinflammatory activity at two separate points along the pathway by which peripheral CRF injection induced behavioral change. Yet, it should also be emphasized that not all of CRF's behavioral effects appeared to be mediated by proinflammatory processes. That is, not only was there no significant effect of α -MSH on the active behaviors of vocalizing and locomotor activity, but the passive responses of CRF-injected pups were only partially reversed by α -MSH.

In summary, the results of the present study demonstrate that the induction of passive behavioral responses following peripheral CRF injection—like the induction of passive responding following prolonged isolation—can be attenuated by α -MSH. The results suggest that these two means of inducing passive behavior share a common underlying mechanism, one which may involve induction of proinflammatory activity. In addition, the findings raise the possibility that endogenous peripheral CRF activity might contribute to proinflammatory-induced passive behavior during prolonged separation in untreated pups, though this possibility remains speculative pending additional research.

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FIGURE 1.

Effects of central (ICV) administration of α -MSH on CRF-induced active and passive behaviors. Medians and semi-interquartile ranges (numbers in parentheses) are depicted. Levels of significance were calculated with Wilcoxon matched-pairs tests for within-subject comparisons and Mann-Whitney tests for between-subject comparisons (* $p < .05$; ** $p < .01$; *** $p < .001$).

FIGURE 2.

Effects of peripheral (SC) administration of α -MSH on CRF-induced changes in active and passive behaviors. Medians and semi-interquartile ranges (number in parentheses) are depicted. Levels of significance were calculated with Wilcoxon matched-pairs tests for within-subject comparisons and Mann-Whitney tests for between-subject comparisons (* $p < .05$; ** $p < .01$; *** $p < .001$).