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BETA-ENDORPHIN LEVELS IN LONGTAILED AND PIGTAILED MACAQUES VARY BY ABNORMAL BEHAVIOR RATING AND SEX

Carolyn M. Crockett^a, Gene P. Sackett^a, Curt A. Sandman^b, Aleksandra Chicz-DeMet^b, and Kathleen L. Bentson^a

^aNational Primate Research Center, Box 357330, University of Washington, Seattle, WA 98195-7330

^bDepartment of Psychiatry & Human Behavior, University of California, Irvine, CA 92697

Abstract

Frequent or severe abnormal behavior may be associated with the release of endorphins that positively reinforce the behavior with an opiate euphoria or analgesia. One line of research exploring this association involves the superhormone, proopiomelanocortin (POMC). The products of POMC appear to be dysregulated in some human subjects who exhibit self-injurious behavior (SIB). Macaque monkeys have POMC very similar to humans, and some laboratory macaques display SIB or frequent stereotypies. We investigated associations between plasma levels of three immunoreactive POMC fragments with possible opioid action and abnormal behavior ratings in macaques. In 58 adult male and female macaques (24 *Macaca fascicularis* and 34 *M. nemestrina*), plasma levels of intact beta-endorphin (β E) and the N-terminal fragment (BEN) were significantly higher in animals with higher levels of abnormal behavior. The C-terminal fragment (BEC) was significantly higher in males but unrelated to ratings of abnormal behavior. Levels of ACTH, cortisol, and (β E-ACTH)/ β E dysregulation index were unrelated to abnormal behavior. None of the POMC products differed significantly by subjects' species, age, or weight. The finding that intact beta-endorphin is positively related to abnormal behavior in two species of macaque is consistent with some previous research on human subjects and nonprimates. The positive relation of the N-terminal fragment of β E to abnormal behavior is a new finding.

Keywords

Self-injurious behavior (SIB); stereotypy; abnormal behavior; opioids; beta-endorphin; proopiomelanocortin (POMC); adrenocorticotrophic hormone (ACTH); cortisol; *Macaca fascicularis*; *Macaca nemestrina*

Introduction

Severe behavioral disorders, including self-injurious behavior (SIB) and frequent stereotypies, can compromise the well-being of humans and of captive animals [22,46,49]. Once established,

Corresponding author: Carolyn M. Crockett, Ph. D. National Primate Research Center Box 357330 University of Washington Seattle, WA 98195-7330 crockett@u.washington.edu Ofc: 206-543-2211 FAX: 206-685-0305

Co-authors' emails:

Gene P. Sackett: jsackett@bart.rprc.washington.edu

Curt A. Sandman: casandma@uci.edu

Aleksandra Chicz-DeMet: ademet@uci.edu

Kathleen L. Bentson: kbentson@u.washington.edu

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abnormal behaviors can become intractable to treatment. The persistence of behaviors that interfere with normal life, in some cases causing pain and suffering, has inspired various hypotheses. The self-stimulating properties of stereotypies may develop to increase arousal in unstimulating environments; or stereotyped behavior may occur during states of increased stress, serving as a coping mechanism by dissipating tension, frustration or anxiety; or both [13,47]. The long-term maintenance of abnormal behavior may result if the individual displaying it is somehow rewarded through its exhibition [34].

One hypothesis for the manifestation of frequent or severe abnormal behavior is that it is associated with the release of endorphins that positively reinforce the behavior with an opiate euphoria [44]. A related hypothesis – the pain hypothesis – is based on the idea that behaviorally released or atypically high levels of endogenous opioids increase the pain threshold through analgesia [44,53]. The role of endogenous opioids in behavior has been investigated using opioid antagonists, or blockers, such as naloxone and naltrexone, which eliminate the potentially rewarding properties of euphoria and pain attenuation [3,30,35]. For example, analgesic effects were reversed (i.e., pain thresholds were lowered) by naloxone in most animal studies [3]. Experiments with rats have shown that the exogenous opioid morphine can activate a part of the brain that mediates the hedonic (reward) experience while making the animal less sensitive in another area of the brain that subserves pain [20].

A study of pigs found an association between stereotypies and the release of endorphins: injections of naloxone temporarily reduced stereotypies in tethered sows but exerted no effect on movements associated with normal exploratory behavior of unrestrained sows [11]. Inhibition of stereotypies by opioid antagonists was also reported for poultry, voles, and horses [21]. Opiate blockers reduce SIB in humans, being most effective in patients with elevated levels of endorphin [42,44]. Naltrexone reduced the frequency and severity of SIB in male rhesus macaques (*Macaca mulatta*) with a history of severe self-wounding [36]. Rhesus macaques with a history of SIB directed self-biting preferentially toward body areas associated with acupuncture analgesia [26]. This association also has been reported in human subjects [50]. Collectively, these data are consistent with the hypotheses that SIB and stereotypies might be positively reinforced and maintained by the pleasurable sensations or analgesic properties associated with the release of endogenous opioids.

One line of research exploring the role of opioids and abnormal behavior involves the cleaving of the superhormone, proopiomelanocortin (POMC). POMC and its numerous hormonal products and receptors influence a wide range of physiological functions [16]. POMC produces several behaviorally active products including the opioid beta-endorphin (β E) and adrenocorticotrophic hormone (ACTH), which stimulates the synthesis of cortisol. These products of POMC appear to be dysregulated in some humans who exhibit SIB, including self-inflicted tissue damage and potential injury. This “uncoupling,” expressed as a “dysregulation index,” results in different ratios of ACTH and opioids in the bloodstream, especially under conditions of stress [45]. Humans with “contagious” SIB, where bouts of SIB follow one another in rapid succession, have especially high levels of intact β E relative to ACTH [45]. Rhesus macaques with high rates of SIB have blunted cortisol responses to mild stress [31, 52]. Autistic individuals, with and without SIB, have higher ACTH and lower cortisol basal levels compared to controls [12]. HPA dysregulation has been observed among adults with a history of post-traumatic stress disorder [17,57].

Previous research has identified three POMC products with possible opioid activity, the highly specific “intact” β E component (β E₁₋₃₁), a smaller sequence (β E₁₋₂₃), and another fragment that is highly cross-reactive with beta-lipotropin (β -LP₁₋₉₁) [44]. These latter fragments are sometimes referred to as N-terminal and C-terminal, respectively, because of their proximity to the H₂N terminus and COOH terminus peptides surrounding the supermolecule, POMC

[48]. In research on humans, two of the immunoreactive fragments (C-terminally directed and intact β E) have been reported to be dysregulated at rest among autistic individuals, with and without SIB [44]. The third fragment (N-terminal-directed) has not been reported to differ among SIB patients. Some autistic subjects with SIB were reported to have normal ACTH levels but elevated levels of the C-terminally directed β E-immunoreactivity relative to the N-terminally directed immunoreactivity [23,24]. In another study, autistic subjects were found to have markedly elevated C-terminal β E concentrations compared to normal individuals, but did not differ significantly in N-terminal β E concentrations; six of the ten subjects had a history of mild SIB [4]. The possible opioid activity of the third fragment was suggested by an analysis of beta-endorphin fragment activity on human monocyte chemotaxis indicating that the N-terminal, but not the C-terminal, has opiate effects [39].

Some laboratory primates display SIB or frequent stereotypies. As a consequence, they may experience diminished psychological well-being, and the research projects in which they participate may be compromised [1,14,31,32]. Recent data [53] contribute to our understanding of the potential role of endogenous opioids in the development and maintenance of severe abnormal behavior in nonhuman primates but do not address the contribution of different β E fragments and are restricted to rhesus macaques (*Macaca mulatta*). High sequence homologies in POMC across a variety of mammalian species and vertebrates in general [19,48] support comparisons between human and nonhuman primates. Of particular relevance to the current research is the strong similarity in β E: The sequence of the last 31 amino acids coded by the POMC gene, corresponding to β E₁₋₃₁, is identical for *M. mulatta* and *M. nemestrina*, and the first 30 of these are identical to *Homo sapiens*; the only difference is the last amino acid, in the C-terminal region [29,33] [NCBI Entrez Protein Database Accession Numbers **P01201**, **XP_001082999**, **NP_000930**].

In this study, we investigated associations between POMC fragments and abnormal behavior ratings in two laboratory monkey species, longtailed macaques (*M. fascicularis*) and pigtailed macaques (*M. nemestrina*). Significant associations with abnormal behavior were used to assess opioid activity of the products investigated. We investigated in these nonhuman primates plasma levels of three different immunoreactive POMC fragments with possible opioid action: intact β E (BET-IR, further abbreviated to BET), N-terminally directed β E-immunoreactivity (BEN-IR, or BEN), and C-terminally directed β E/ β LP (beta-lipotropin) immunoreactivity (BEC-IR, or BEC). Our goals were to determine if ratings of severity of abnormal behavior correlate with intact β E and other POMC products or with dysregulation between β E and ACTH, and whether levels of the POMC products differ by species or sex.

1. Methods

1.1. Subjects and sample collection

Venous blood samples were drawn from 58 adult macaques (24 *Macaca fascicularis*, and Mf, 34 *Macaca nemestrina*, Mn) under ketamine sedation between 8:10 AM and 12:30 PM (most between 9:30 and 11:30 AM). Blood was collected into EDTA tubes containing aprotinin, a serine protease inhibitor. The plasma was stored at -70 degrees C until assayed in the lab of A. Chicz-DeMet.

We requested research support staff to collect samples from similar numbers of adult males and females and at least 20 per species. Subjects were scheduled for routine blood draw for other research projects over a three-month period and were not selected for this study on the basis of behavioral or other criteria. The number of subjects drawn per day varied, as did the interval between sedation and venipuncture. Eleven male and 11 female Mf were subjects in a long-term study of prenatal exposure to mercury (10 controls, 12 dosed) [15]. The mercury subjects did not differ significantly from their undosed control subjects in any analysis in this

study. All subjects were housed in individual cages at the National Primate Research Center (NPRC) at the University of Washington, Seattle, an Institutional Animal Care and Use Committee (IACUC)-approved and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility with a fully implemented Environmental Enhancement Plan, as required by law [55].

1.2. POMC Peptide Assays

β -Endorphin Assay (intact β E₁₋₃₁ immunoreactivity, BET-IR, or BET)—Plasma levels of BET-IR were determined by a commercially available direct solid phase two-site immunoradiometric assay (IRMA) using antibodies against synthetic human β E (Nichols Institute Diagnostics; San Juan Capistrano, CA). Cross-reactivity of pertinent analytes is summarized in Table 1. The BET IRMA method measures intact β E₁₋₃₁ in a radiolabeled soluble sandwich complex bound to two antibodies with high affinity and specificity for β E coupled to a solid bead matrix. Briefly, samples (200 μ l) were incubated with an antibody-coated bead and labeled antiserum (100 μ l) for 20 \pm 1 hours at room temperature. The labeled antibody complex bound to the solid phase was measured using an ICN Biomedical (formerly Micromedex) Isoplex Gamma Counter. All plasma β E values were calculated using a four-parameter logistics program [37] obtained from a modified standard curve enriched with two additional low-level standards. The calculated assay sensitivity was approximately 5.7 pg/ml. Intra- and inter-assay coefficients of variation (CVs) were 4.2% and 8.3%, respectively.

β -Endorphin N-terminally directed immunoreactivity (BEN-IR, or BEN) and β -Endorphin C-terminally directed immunoreactivity (BEC-IR, or BEC)—Three ml of plasma were extracted with acetone activated Sep-pak C18 cartridge (Waters, Milford, MA) and eluted with acidified acetone (1.5 ml of 0.2 M HCl/acetone (25:75)). The extracts were dried under vacuum and reconstituted in assay buffer. BEN-IR and BEC-IR were measured by competitive radioimmunoassays (RIAs) employing antisera directed at human BEN and BEC (Euro-Diagnostica AB, Malmo, Sweden). These assays specifically detect human BEN (<1% cross-reactivity with β LP) and human BEC (102% cross-reactivity with β LP), with non-significant cross-reactivity with ACTH and related opiate analogs as listed in Table 1. Reconstituted samples were incubated (100 μ l/assay tube) with antiserum (200 μ l) for 24 hr at 4°C, followed by a second overnight incubation with ¹²⁵I- β E (200 μ l) at 4°C. Labeled and unlabeled β E was collected by immunoprecipitation and the aspirated pellets were quantified using a gamma counter (ICN Biomedical, formerly Micromedex, Isoplex Gamma Counter). BEN-IR and BEC-IR levels were calculated using a standard curve computed by a four-parameter logistics program [37] corrected for dilution. The detection limits of the BEN-IR and BEC-IR RIAs are 10 pg/ml. For BEN-IR and BEC-IR, respectively, the intra-assay CVs were 5.2% and 9.1% and the inter-assay CVs were 17.6% and 21.6%.

Adrenocorticotropin Hormone (ACTH) Assay—Plasma levels of ACTH were measured directly in plasma by an IRMA method using human ACTH antibodies with non-significant cross-reactivity with β E and ACTH fragments, and with reported detection limits of 1.0 pg/ml (Nichols Institute Diagnostics; San Juan Capistrano, CA). Briefly, samples (200 μ l) combined with ACTH labeled antibody (100 μ l) and a coated bead were incubated at room temperature for 20 \pm 1 hours. The bound radiolabeled antibody complex was quantified using an ICN Biomedical (formerly Micromedex) Isoplex Gamma Counter. Concentrations of ACTH were determined directly from the standard curve computed by a four-parameter logistics program [37]. The intra and inter-assay CVs were 4.4% and 10.8%, respectively.

Plasma Cortisol Assay—Plasma cortisol levels were determined by a competitive antibody coated tube RIA with reported sensitivity of 0.21 μ g/dL (GammaCoat™, DiaSorin, Stillwater, MN). Plasma samples (10 μ l) were incubated with ¹²⁵I-labeled cortisol (1.0 ml) in antibody-

coated tubes for 45 min in a 37°C water bath. The aspirated antibody-bound labeled tubes were counted on an ICN Biomedical (formerly Micromedex) Isoflex Gamma Counter. Cortisol concentrations were interpolated from a standard curve computed by a four-parameter logistics program [37]. Cross-reactivities of the cortisol assay were 6.3% with 11-deoxycortisol, <1.5% with cortisone, prednisone, corticosterone, and <0.1% with other naturally occurring steroids. The intra- and inter-assay CVs were 6.6% and 10.6%, respectively.

1.3. Abnormal behavior ratings

Abnormal behavior ratings were determined by staff in the Psychological Well-being (PWB) Program at the Washington National Primate Research Center (WaNPRC). Raters were blind to assay results. Behavioral records were summarized in a rating scale (Table 2). The rating scale ranged from 1 = no abnormal behavior to 15 = self-inflicted injury sufficient to break the skin that was the closest in time to the date of blood draw. The subjects were assigned the rating that corresponded to the most serious abnormal behavior(s) recorded for the animal.

As part of its role in overseeing behavioral well-being of laboratory primates, the PWB Program compiles data on abnormal behavior of monkeys. The list of subjects was provided to PWB staff in the absence of assay results. Existing behavioral data were searched, and at least four observations were made on remaining subjects with no relevant behavioral information. For subjects that had been referred to the PWB Program for assessment of abnormal behavior, the occurrence or absence of abnormal behaviors from a defined list was scored since referral during weekly assessment visits. Some of these referred animals also had been scored repeatedly during systematic 10-minute behavioral assessment observations [1]. The clinical animal records of all 58 subjects were searched for evidence of self-wounding. Some monkeys were housed individually but had grooming contact with a partner through widely spaced bars [9] and were observed systematically once a week to assess ongoing compatibility irrespective of having been referred for abnormal behaviors. Although the focus was on social behaviors, any abnormal behaviors were also noted. Most of the remaining subjects were observed systematically at least four times by PWB staff to record the occurrence or absence of abnormal behaviors. Fourteen subjects, deceased before the observations could be completed, were assigned rating 2 (Table 2). Subjects with behavioral information and no reports of abnormal behavior were assigned rating 1 (Table 2).

Eight of the 58 subjects had a history of self-inflicted injury sufficient to break the skin (PWB definition of SIB); some injuries were serious enough to be recorded in the animal's clinical record. SIB is the most concerning abnormal behavior to PWB staff. These 8 subjects were given unique ratings of 8 through 15 based on the temporal proximity of SIB to the date of blood draw. PWB behavioral definitions require breaking of the skin for self-directed behavior to be classified as self-injurious behavior (SIB); behavior such as self-biting or self-hitting that does not break the skin is classified by PWB as potential SIB. In the human literature, SIB definitions generally include behaviors that can potentially cause tissue damage, but may not necessarily break the skin [4, p. 193 includes examples]. In our rating scale, infrequent abnormal behavior including low levels of potential SIB was rated 5, below frequent stereotypy without potential SIB (rating 6) and rating 7: frequent abnormal behavior including any level of noninjurious self-biting or other potentially self-injurious behavior, but no history of actual SIB (Table 2).

We chose this rating method because it combines measures of severity and frequency across all types of abnormal behaviors into a continuous scale. For statistical analyses, each abnormal behavior rating was converted to an Abnormality Rank (AbnRank) score (minimum rank = 7.5, the average rank of 15 animals with tied ratings of "1"; maximum rank = 58, the rank of the subject rated 15).

1.4. Statistical tests

The peptide measures analyzed include plasma levels of: BET (BET-IR pg/ml), BEC (BEC-IR pg/ml), BEN (BEN-IR pg/ml), ACTH (pg/ml), and Cortisol ($\mu\text{g/dl}$). A dysregulation index, $\text{DYSBET} = ((\text{BET}-\text{ACTH})/\text{BET}) \times 100$, was computed to characterize the degree of uncoupling between BET and ACTH and to eliminate sources of variation inherent in measures of absolute concentration [42]. When BET and ACTH are equal, the index equals zero. A positive index means that BET is elevated above ACTH, and a negative index means that ACTH is elevated above BET.

Statistical analyses were performed using SPSS (Spearman correlations; Univariate Analysis of Variance), and Data Desk 6.2 (General Linear Model ANOVA-MANCOVA, Type 3, Partial; Fisher exact tests) [56]. Two-tailed probabilities of $\leq .05$ are reported as statistically significant.

2. Results

Table 3 summarizes the means and SDs for all dependent variables and the independent variable AbnRank by species-sex category and across all samples. Univariate ANOVAs found no significant differences between mercury-dosed monkeys and their controls on any dependent variable or AbnRank, so their data were combined with the remaining subjects. Across all 58 subjects, AbnRank was strongly positively correlated with plasma levels of intact beta-endorphin (BET), moderately correlated with BEC, BEN, and DYSBET, and weakly correlated with ACTH and (negatively) with cortisol (Table 4). A concordant pattern of significance for both species and both sexes held only for the positive correlation between plasma levels of BET and AbnRank (Figure 1). Correlations between AbnRank and ACTH or Cortisol were significant only when all 58 subjects were included. Other correlations only were significant for one sex and/or species.

To further investigate factors contributing to the significant correlations, we ran GLM-MANCOVAs with AbnRank as a continuous independent variable, where higher ranks reflected more severe behavior disorders, and with sex (M, F) and species (Mf, Mn) as discrete factors. Because the species and sexes varied in weight, and the mercury project subjects were older than most others, weight and age at time of blood draw were also included as continuous covariates. The first MANCOVA included the beta-endorphin variables (Table 5a: BET, BEC, BEN). The second MANCOVA included the dysregulation, ACTH, and Cortisol variables (Table 5b: DYSBET, ACTH, Cortisol). AbnRank significantly predicted plasma levels of BET and BEN (positive relation), but BEC was unrelated to abnormal behavior rank (Table 5a). Sex was significantly related to BEC (levels higher in males) and approached significance for BET (Table 5a). No independent variables (Abnormal Rank, Sex, Species, Age, Weight) significantly predicted levels of the dependent variables DYSBET, ACTH, or Cortisol (Table 5b). Species, weight, and age were not significantly associated with any of the dependent variables (DV).

Abnormal behavior rank was used as the DV in GLM-ANCOVA. AbnRank differed significantly by sex (males more abnormal) ($F_{1,53} = 6.40, p < .02$), but not by species, weight or age (all $p > .36$). Nonparametric statistics also found males to be significantly more likely to have an abnormality rank above the median (abnormal behavior ratings 4-15, Table 2) (Fisher's exact test, $p < .0001$, 2-tailed). Comparing species, Mf and Mn did not differ significantly in abnormality rank above the median (Fisher's exact test, $p = .17$, 2-tailed).

In summary, while simultaneously controlling for the effects of abnormal behavior rank, sex, species, weight, and age, GLM-MANCOVA found that plasma levels of intact βE (BET) and the N-terminally directed immunoreactive fragment (BEN) were both significantly higher in

animals with higher levels of abnormal behavior. Plasma levels of the C-terminally directed immunoreactive fragment (BEC) was significantly higher in males and unrelated to abnormal behavior rank in these macaques. Males had higher levels of abnormal behavior rankings than females, but the GLM controls for sex differences in abnormal behavior. The dysregulation index DYSBET, ACTH, and cortisol did not vary significantly by sex or AbnRank. Plasma levels of none of the POMC products varied significantly by species, age, or weight of subjects.

3. Discussion

3.1 The relation between abnormal behavior and different beta-endorphin fragments

This study characterizes in nonhuman primates three different immunoreactive POMC fragments with possible opioid action. Multivariate analyses identified significant factors while controlling for the effects of others. The finding that intact β E (BET) is positively related to abnormal behavior rank scores in two species of macaque is consistent with previous research on nonprimates associating stereotypy and endorphins [11,21]. However, the significant positive relation of BEN, but not BEC, to abnormal behavior is a new finding. In the monkeys studied here, the N-terminal fragment (BEN) is significantly related to the overall abnormal behavior rank score, but not to sex. The C-terminal fragment (BEC) is significantly related to a monkey's sex (higher levels in males) and unrelated to their abnormal behavior. This suggests that, in these monkeys, intact β E and the N-terminal, but not the C-terminal, have similar opioid activity.

The macaque results differ from those reported for some autistic humans with SIB, whose plasma levels of the C-terminal fragment are elevated relative to the N-terminal fragment [23,24]. The Leboyer group found that naltrexone responses in autistic humans suggested opioid activity in the C-terminal fragment but not the N-terminal. In subjects whose autistic symptoms were markedly reduced under low-dose naltrexone treatment, their normally elevated plasma levels of C-terminal β E were also reduced to normal levels under naltrexone [4]. The N-terminal assay found no significant differences in plasma levels between autistic subjects and controls, nor any changes under naltrexone versus baseline conditions.

These contrasting results may be due in part to differences in assays. Many assays have been reported in the literature for measuring POMC products, and they are not necessarily comparable. The profile of cross-reactivity for the three antisera used in the present study (Table 1) indicates that all three antibodies we used to detect these fragments are highly sensitive to the 31 amino acid β E peptide, but that patterns are very different for other peptides. Furthermore, the patterns of immunoreactivity of the antisera used in this study (Table 1) differ considerably from those used by Leboyer, et al. [23, Table 1, p. 1798]. The cross-reactivity profile and the very high level of β -LP peptide measured by antiserum for the C-terminal fragment suggests that perhaps their assay is primarily detecting the intact (or nearly intact) β -LP fragment, whereas our BEC-IR also detects more β E₁₋₂₇ (75% cross-reactivity). The antibody for the fragment they labeled "N-terminal" is not highly cross-reactive with β E₁₋₁₆ (11%) whereas ours cross-reacts 69%. Possibly the failure of the Leboyer group to detect elevated BEN in autistic patients is related to their assay being less immunoreactive to the N-terminal than our BEN-IR assay. However, it is not clear that our BEC-IR assay is sufficiently different from theirs to explain the differing C-terminal results. Their research has found markedly elevated BEC in 80% of autistic patients, many of which had SIB symptoms [4, 23].

We used human antisera to assess macaque POMC fragments since there are notable genetic similarities between macaques and humans. The μ -opioid receptor coding region in rhesus is approximately 98% homologous to the corresponding human coding region [28]. Naltrexone antagonizes particularly μ -type opioid receptors [4]. The amino acid sequence for *M*.

nemestrina βE_{1-31} is identical to that of *H. sapiens* except for one substitution in the last position, at the end of the C-terminal [29] that may possibly contribute to the contrasting results we found for BEC, but not for BEN.

In the two macaque species studied using the assays described in Table 1, there is clear evidence of an association between abnormal behavior and the N-terminal, but not the C-terminal, βE fragment. This is the first demonstration of a link between abnormal behavior and the N-terminal fragment (BEN) in a nonhuman primate, supporting the role of opioid action in that region of the POMC molecule. Previous human research has identified the N-terminal as a region of opioid activity. A study of human monocyte chemotactic activity of beta-endorphin and related peptides found that N-terminal-mediated effects were naloxone reversible, whereas the C-terminal effects were not; the authors concluded that the intact N-terminal is necessary for opioid-like effects [39].

In this study, C-terminal immunoreactivity (BEC) is related to a monkey's sex, but not its abnormal behavior. The trend toward significance of the sex factor in BET (Table 5) appears to be related to sex differences in the C-terminal, because the MANCOVA controlled for sex differences in abnormal behavior. It is possible that some human studies indicating opioid action in the C-terminal are confounded by uncontrolled sex differences, although this was not the case for Bouvard [4] in which five subjects were male and five were female. In our study, male macaques were more likely to have higher abnormal behavior rank scores than females. In general, male macaques are significantly more likely than females to exhibit SIB and other serious abnormal behaviors [31,32]. Among developmentally delayed (DD) humans, SIB is slightly more prevalent in males [38].

The species did not differ significantly in levels of abnormal behavior. This study revealed no statistically significant species differences in any of the peptides. Mf males had the highest levels of BET and Mn females, the lowest, consistent with Mf males having the highest average AbnRank scores and Mn females, the lowest (Table 3). The beta-endorphin (BET) levels of ~12 pg/ml reported previously for female Mf [18,27] are intermediate between the 17.7 pg/ml BET we found for female Mf and the 8.5 pg/ml for female Mn (Table 3). McCubbin, et al. [27] measured βE -like immunoreactivity with a commercially available radioimmunoassay kit with <5% cross-reactivity with β -LP.

3.2 ACTH, cortisol, and dysregulation index

The GLM-MANCOVA found no significant relation between abnormal behavior and the ACTH and cortisol variables. These factors were weakly significant in Spearman correlations (Table 4), but the significance evaporated when MANCOVA took into account multiple factors simultaneously (Table 5). The uncontrolled interval between sedation and venipuncture, and variations in time of blood draw, may have contributed variability in ACTH and cortisol levels, which appear to be somewhat elevated. However, opioid levels can also be influenced by these factors, yet significant BET, BEC, and BEN effects were found. In macaques, both ACTH and cortisol vary with time of day, being higher in the morning [7]. The plasma cortisol values in the present study averaged 38 $\mu\text{g}/\text{dl}$, compared with baseline morning values of 27 $\mu\text{g}/\text{dl}$ from *M. mulatta* [2,5] and ~23 $\mu\text{g}/\text{dl}$ from *M. fascicularis* [10]. In response to a stressor of mock capture, morning cortisol values for *M. fascicularis* rose from 30 $\mu\text{g}/\text{dl}$ to 46 $\mu\text{g}/\text{dl}$ [27]. ACTH values in the present study averaged 63 pg/mg, compared with morning values of 47-56 pg/ml for male *M. mulatta* [7] and afternoon values of 15-32 pg/ml for male *M. mulatta* [6]. In female macaques (*M. mulatta*, *M. fascicularis*, *M. radiata*), plasma ACTH and cortisol levels rose significantly after exposure to acute stressors (awake venipuncture, hand-capture, transport, telemetry jacket); values (average of morning and afternoon) were ~40 pg/ml baseline and ~67 pg/ml stressed for ACTH, and ~27 $\mu\text{g}/\text{dl}$ baseline and 50 $\mu\text{g}/\text{dl}$ stressed for cortisol [8]. Cortisol levels can rise within minutes of a stressor such as sedation for blood draw. Further

sampling (Bentson, in progress), in which the time of day and rapidity of blood draw are more tightly controlled, may clarify the significance of the dysregulation index, ACTH, and cortisol with respect to abnormal behavior in these macaque species.

3.3 Positive versus negative associations between beta-endorphin and behavior disorders

Our longtailed and pigtailed macaque subjects showed a positive relation between abnormal behavior and BET and BEN. Recent research on rhesus macaques (*M. mulatta*) found significantly reduced basal levels of β E-like immunoreactivity in blood plasma of monkeys with a veterinary record of SIB wounding [53]; the specific assay was not described. Their results for *M. mulatta* appear to be inconsistent with the present results for *M. nemestrina* and *M. fascicularis*, where β E-immunoreactivity (BET and BEN) are positively associated with overall rating of abnormal behavior. In our study, SIB subjects received the top 8 rankings because they were regarded as the most severe abnormal behavior cases. Because our sample included only eight SIB subjects and because the human literature defines SIB more broadly, we did not directly compare SIB versus non-SIB subjects.

These contrasting results may reflect species or genetic differences, or differences in assays, in severity or frequency of SIB, methods for quantifying abnormal behavior, or other factors. For example, Tiefenbacher, et al. [53] cite several studies supporting their findings in which autistic humans were reported to have significantly lower levels of beta-endorphin, yet the Leboyer group found elevated BEC-IR in most autistic patients [4,23]. SIB and other forms of abnormal behavior may represent a variety of etiologies [1,14,51,53], as might their relation to endogenous opioids. Tiefenbacher et al. [53] suggested that rhesus macaques with lower basal levels of β E may be more prone to engage in self-biting to increase low levels of endogenous opioids. SIB in their monkeys might have some parallels with alcoholism. The mu-opioid receptor plays a role in regulating the effects of alcohol, and alcohol consumption increases levels of β E, probably through increased release of POMC [58]. Compared to a low-risk group, humans at high risk for developing alcoholism, have lower levels of β E and increased plasma β E in response to small doses of ethanol [58]. Some macaques have an allele of the mu-opioid receptor gene that produces more than three times the affinity for β E, i.e., for a given level of β E, greater β E binding produces increased opioid effects in the body [28]. Rhesus macaques with this allele have lower plasma cortisol and higher rates of aggressive threat [28]. Possibly macaques with this allele are also more likely to self-bite. However, another study found SIB and externally directed aggression in male rhesus macaques to be unrelated [25].

The negative relation between SIB and β E reported for rhesus macaques [53] is in the opposite direction suggested by the present study of longtailed and pigtailed macaques, and that reported for humans. Opioid blockers generally are associated with reductions in abnormal behavior, although the type of behavior affected varies across studies. Although some studies have specifically linked stereotypy to endorphins [11], in other studies opioid antagonists have reduced both stereotypic and normal activities [21]. One speculation is that opioid antagonists reduce the persistence of behavior but not necessarily all stereotypic behavior [21]. Human patients with SIB and stereotypy showed decreased SIB but no systematic effects on stereotypy when administered naltrexone [41]. Subsequent research found SIB to be more effectively treated by naltrexone in patients with higher levels of β E [43,44]. Among developmentally delayed (DD) humans, those with SIB plus stereotypy had significantly higher morning plasma β E levels than DD patients without these behaviors. Those patients with SIB or stereotypy, but not both, had slightly higher levels of β E than those without either [40]. However, the combined patient groups had lower levels of β E than non-DD controls [40].

Some answers may lie in individual differences in opioid responses to stress. Stress is known to increase the level of POMC products, including β E [54]. A study of female *M.*

fascicularis exposed to a stressor (person wearing monkey capture glove) found naloxone to produce significant increases in plasma cortisol and β E-like immunoreactivity compared with saline [27]. The monkeys with high heart-rate reactivity to stress had a very different response to naloxone than those with lower heart-rate reactivity, suggesting that opioids were less effective in inhibiting adrenocortical and circulatory responses in the former [27]. The less reactive naloxone-treated monkeys mounted a much greater increase in β E in response to stress than did the high heart-rate reactors. This suggested that “normal” monkey opioid systems effectively modulate responses to stress.

It may be that individuals with abnormally low levels of β E that are exposed to stressors -or are more reactive to stressors - need to engage in higher levels of stereotypy or SIB to mount an effective stress response. If performing SIB, noninjurious self-biting, or stereotypies increase β E, then β E should be positively correlated with abnormal behavior rank if other factors are equal. Because other factors usually are not equal, reported patterns may vary. As this study and some others have shown, the pattern of beta-endorphin response varies depending upon the fragments measured. Clearly, more research is warranted.

4. Conclusions

The significant positive correlation between beta-endorphin and abnormal behavior ratings in longtailed and pigtailed macaques is consistent with the hypothesis that abnormal behavior might be maintained by the sensations produced by endogenous opioid release. This could explain why such behaviors, once established, are difficult to treat. However, these results do not address hypotheses about origins of the abnormal behavior, nor whether the sensations proposed to be reinforcing are pleasurable (euphoric), pain-reducing (analgesic), or both.

The finding in the present study that intact beta-endorphin is positively correlated with AbnRank may reflect heightened stress responsiveness to sample collection and/or higher baseline levels of POMC products in monkeys that exhibit behavioral abnormalities. The positive correlation is consistent with two hypotheses regarding endorphins and abnormal behavior. Endorphins provide analgesia and euphoria. Monkeys with high endorphin levels may be less sensitive to physical discomfort that may result from self-biting. Alternatively, monkeys may engage in abnormal behaviors if doing so increases endorphin levels and produces euphoria.

The present results contrast with the negative correlation between beta-endorphin and SIB reported for rhesus macaques [53]. However, both studies confirm that an association exists between abnormal behavior and endogenous opioids in macaques. The present study, by examining three beta-endorphin fragments using sensitive assays, is the first to demonstrate a positive relation of the N-terminal fragment to abnormal behavior in a nonhuman primate. Another new finding is that, in these macaques, C-terminal variation is associated with a macaque's sex but not its level of abnormal behavior.

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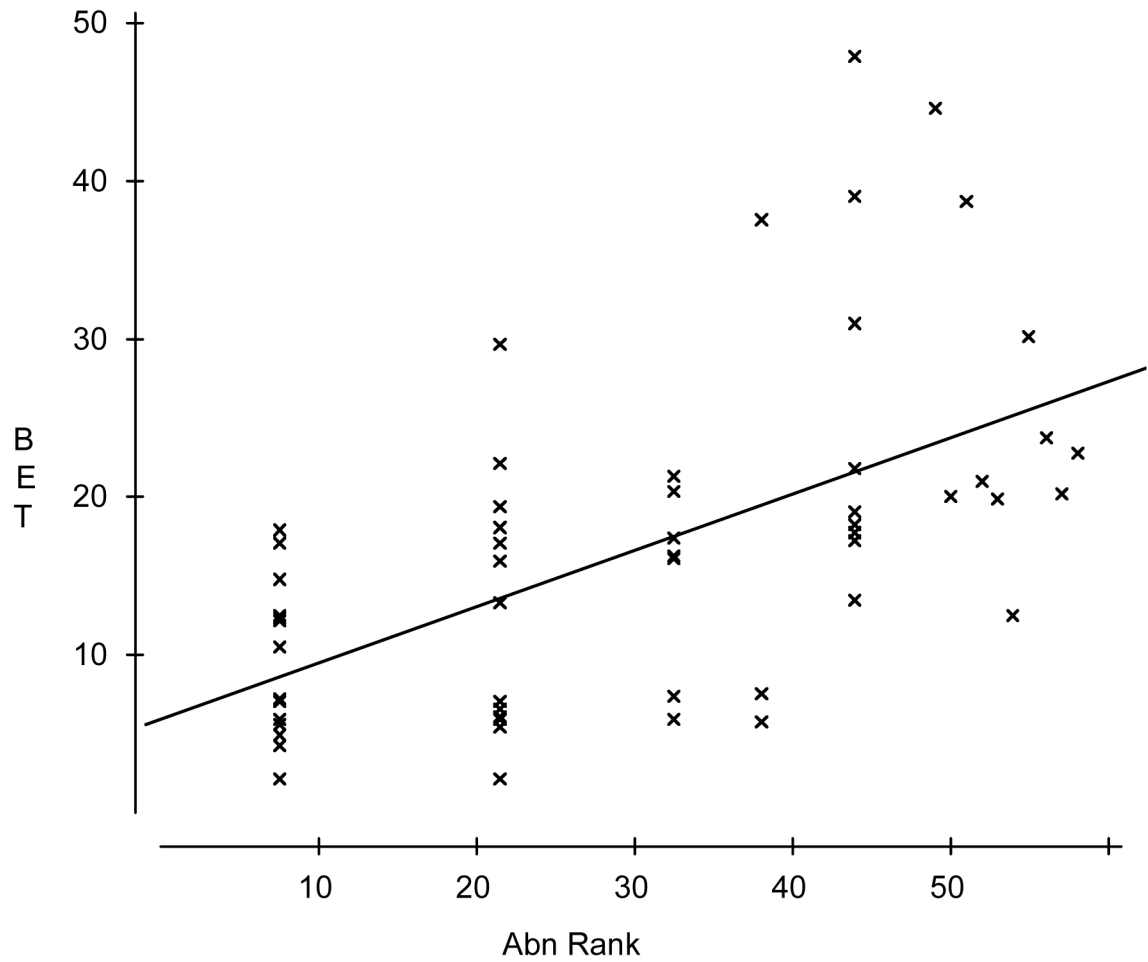


Figure 1. Plasma levels of intact beta-endorphin (BET, pg/ml) increased significantly with abnormal behavior rank (Abn Rank). Spearman correlation = $+0.63$ ($p < .001$, $n=58$).

Table 1
Crossreactivity (%) with POMC Fragments for Beta-Endorphin Directed Antisera for POMC-Peptide Assays

| Peptide | C-terminal / β LP assay ^a BEC-IR | N-terminal assay ^a BEN-IR | Specific β E ₁₋₃₁ assay ^b BET-IR |
|-------------------------------------|---|--|--|
| β E ₁₋₃₁ | 100 | 100 | 100 |
| β -Lipotropin ₁₋₉₁ | 102 | <1.0 | 16 @ 500 pg/ml |
| β E ₆₋₃₁ | 84 | 2 | N/A |
| β E ₁₋₂₇ | 75 | 43 | 0.007 @ 5mg/ml |
| β E ₁₋₁₇ | N/A | N/A | 0.007 @ 5mg/ml |
| β E ₁₋₁₆ | <0.01 | 69 | 0.005 @ 5mg/ml |
| Leu-enkephalin | <0.01 | <0.04 | <0.001 @ 5mg/ml |
| Met-enkephalin | <0.01 | 9 | <0.001 @ 5mg/ml |

N/A not available

^aDirectional insert; Euro-Diagnostica AB, Malmo, Sweden

^bDirectional insert; Nichols Institute Diagnostics, San Juan Capistrano, CA

Table 2

Abnormal behavior ratings

| Rating | Abnormal behavior category |
|--------|--|
| 1 | No abnormal behavior detected. Never referred to Psychological Well-being Program staff for abnormal behavior. Behavioral observations by trained staff support absence of abnormal behavior. |
| 2 | Never referred; no other information; no SIB (defined as self-injury sufficient to break the skin) in animal's clinical record. |
| 3 | Low levels of abnormal behavior (excluding self-injurious behavior – SIB - or potentially self-injurious behavior); e.g., infrequent locomotor stereotypy, overgrooming, feces smearing, self-clasping. Low levels defined as >0 to <15% of time budget spent engaging in abnormal behavior or observed in ≤25% of behavioral assessment visits. Half of the 58 subjects were rated 3 or below (median split). |
| 4 | Minor levels of abnormal behavior (without any self-injurious or potentially self-injurious behavior). Minor levels defined as 15% to <20% of time budget spent engaging in abnormal behavior or observed in >25% to ≤50% of behavioral assessment visits. |
| 5 | Minor or low levels of abnormal behavior that include potentially self-injurious behavior (e.g., self-biting or self-hitting that does not break the skin); no SIB in animal's clinical record. |
| 6 | Frequent abnormal behavior (primarily frequent locomotor stereotypy such as pacing) but no SIB or potentially self-injurious behavior. Frequent defined as ≥20% of time budget spent engaging in abnormal behavior or observed in >50% of behavioral assessment visits. |
| 7 | Frequent abnormal behavior including any level of potentially self-injurious behavior; no SIB in animal's clinical record. Example: animals that self-bite without injury and also engage in frequent locomotor stereotypy. |
| 8-15 | Animals with history of injury-producing SIB, ratings ordered by temporal proximity to POMC blood collection: 8, the animal with its last SIB episode most distant in time, to 15, the animal with SIB episodes around the time of POMC blood collection. |

Table 3

Summary statistics for dependent and independent variables

| Variable | Spec-Sex | N | Mean | StdDev | Min | Max |
|------------------|----------|----|---------|--------|----------|---------|
| AbnRank | all | 58 | 29.5 | 16.59 | 7.5 | 58 |
| AbnRank | Mf-F | 10 | 28.8 | 15.05 | 7.5 | 56 |
| AbnRank | Mf-M | 14 | 39.93 | 15.62 | 7.5 | 57 |
| AbnRank | Mn-F | 19 | 17.11 | 11.81 | 7.5 | 53 |
| AbnRank | Mn-M | 15 | 35.93 | 14.41 | 7.5 | 58 |
| BET (pg/ml) | all | 58 | 16.40 | 10.59 | 2.07 | 47.86 |
| BET (pg/ml) | Mf-F | 10 | 17.70 | 6.94 | 7.01 | 29.64 |
| BET (pg/ml) | Mf-M | 14 | 25.85 | 11.36 | 12.33 | 47.86 |
| BET (pg/ml) | Mn-F | 19 | 8.51 | 5.29 | 2.07 | 19.78 |
| BET (pg/ml) | Mn-M | 15 | 16.69 | 9.71 | 5.73 | 44.65 |
| BEN (pg/ml) | all | 58 | 51.43 | 15.16 | 32.79 | 114.24 |
| BEN (pg/ml) | Mf-F | 10 | 55.94 | 10.16 | 43.87 | 81.34 |
| BEN (pg/ml) | Mf-M | 14 | 55.73 | 12.81 | 33.27 | 83.12 |
| BEN (pg/ml) | Mn-F | 19 | 42.41 | 5.95 | 32.79 | 53.12 |
| BEN (pg/ml) | Mn-M | 15 | 55.84 | 22.51 | 34.99 | 114.24 |
| BEC (pg/ml) | all | 58 | 66.64 | 45.66 | 22.83 | 233.70 |
| BEC (pg/ml) | Mf-F | 10 | 56.54 | 25.55 | 25.49 | 120.24 |
| BEC (pg/ml) | Mf-M | 14 | 116.30 | 64.15 | 27.16 | 233.70 |
| BEC (pg/ml) | Mn-F | 19 | 39.30 | 10.91 | 22.83 | 64.05 |
| BEC (pg/ml) | Mn-M | 15 | 61.67 | 22.68 | 39.41 | 117.41 |
| DYSBET | all | 58 | -386.12 | 311.37 | -1625.76 | 26.10 |
| DYSBET | Mf-F | 10 | -341.17 | 298.20 | -1138.03 | -128.21 |
| DYSBET | Mf-M | 14 | -292.09 | 171.96 | -722.11 | -35.59 |
| DYSBET | Mn-F | 19 | -577.47 | 403.66 | -1625.76 | -91.80 |
| DYSBET | Mn-M | 15 | -261.45 | 154.87 | -545.79 | 26.10 |
| ACTH (pg/ml) | all | 58 | 62.90 | 44.57 | 18.85 | 317.52 |
| ACTH (pg/ml) | Mf-F | 10 | 66.03 | 21.03 | 32.59 | 95.44 |
| ACTH (pg/ml) | Mf-M | 14 | 100.38 | 73.00 | 30.50 | 317.52 |
| ACTH (pg/ml) | Mn-F | 19 | 42.58 | 10.44 | 27.54 | 75.49 |
| ACTH (pg/ml) | Mn-M | 15 | 51.56 | 24.60 | 18.85 | 123.90 |
| Cortisol (ug/dl) | all | 58 | 37.70 | 10.09 | 14.49 | 61.17 |
| Cortisol (ug/dl) | Mf-F | 10 | 34.96 | 10.77 | 14.49 | 55.41 |
| Cortisol (ug/dl) | Mf-M | 14 | 34.12 | 11.01 | 18.16 | 61.17 |
| Cortisol (ug/dl) | Mn-F | 19 | 41.07 | 8.38 | 23.64 | 56.98 |
| Cortisol (ug/dl) | Mn-M | 15 | 38.59 | 10.11 | 22.31 | 59.63 |
| AGE (yr) | all | 58 | 13.55 | 4.48 | 5 | 20 |
| AGE (yr) | Mf-F | 10 | 18.40 | 0.97 | 17 | 19 |
| AGE (yr) | Mf-M | 14 | 16.00 | 3.55 | 7 | 19 |
| AGE (yr) | Mn-F | 19 | 12.74 | 3.83 | 7 | 20 |
| AGE (yr) | Mn-M | 15 | 9.07 | 2.34 | 5 | 13 |
| Weight (kg) | all | 58 | 8.85 | 4.43 | 3 | 19.4 |
| Weight (kg) | Mf-F | 10 | 3.69 | 0.38 | 3 | 4.1 |
| Weight (kg) | Mf-M | 14 | 6.31 | 0.92 | 5.2 | 8.8 |
| Weight (kg) | Mn-F | 19 | 8.76 | 2.05 | 5.9 | 14.5 |
| Weight (kg) | Mn-M | 15 | 14.79 | 3.20 | 6 | 19.4 |

| Variable | Spec-Sex | N | Mean | StdDev | Min | Max |
|----------|----------|---|------|--------|-----|-----|
|----------|----------|---|------|--------|-----|-----|

Table 4
Spearman Rank Correlations between AbnRank, POMC fragments, and cortisol

| AbnRank vs. | All | p ^a | Mf | p | Mn | p | Males | p | Females | p |
|-----------------------------|---------------|----------------|--------------|----|--------------|-----|--------------|----|--------------|----|
| BET ^{****b} | 0.632 | *** | 0.611 | ** | 0.49 | ** | 0.522 | ** | 0.507 | ** |
| BEC | 0.49 | *** | 0.268 | NS | 0.54 | ** | 0.334 | NS | 0.187 | NS |
| BEN | 0.496 | *** | 0.244 | NS | 0.398 | * | 0.507 | ** | 0.201 | NS |
| DYSBET ^c | 0.45 | *** | 0.091 | NS | 0.553 | *** | 0.166 | NS | 0.471 | * |
| ACTH | 0.314 | * | 0.341 | NS | 0.087 | NS | 0.265 | NS | 0.092 | NS |
| CORTISOL | -0.285 | * | -0.229 | NS | -0.251 | NS | -0.136 | NS | -0.229 | NS |
| n of subjects | 58 | | 24 | | 34 | | 29 | | 29 | |

^a * p < .05, ** p < .01, *** p < .001, two-tailed (**bold**)

^b **** Concordant pattern across species and sexes (**bold**)

^c DYSBET = ((BET - ACTH) / BET) × 100

Table 5

GLM-MANCOVAs^a overview of p-values with percentage of unique variance accounted for by independent variable (factor) given in parentheses (%UniqV).

| a. Dependent variables Intact Beta-endorphin (BET), N-Terminal (BEN), and C-Terminal (BEC) | | | | |
|--|--------------|-------------------|-------------------|-------------------|
| Source (factor) | Wilks | BET (%UniqV) | BEN (%UniqV) | BEC (%UniqV) |
| AbnRank | 0.007 | 0.01 (6.7) | 0.03 (6.1) | 0.55 (0) |
| Sex | 0.053 | 0.06 (2.8) | 0.82 (0) | 0.01 (8.5) |
| Species | 0.49 | 0.42 (0) | 0.46 (0.7) | 0.56 (0) |
| Weight | 0.66 | 0.84 (0) | 0.55 (0) | 0.62 (0) |
| Age | 0.64 | 0.19 (0.8) | 0.73 (0) | 0.18 (1.1) |
| Regression adjusted R-squared | | 43.3% | 14.0% | 35.3% |
| b. Dependent variables DYSBET, ACTH or Cortisol | | | | |
| Source (factor) | Wilks | DYSBET (%UniqV) | ACTH (%UniqV) | Cortisol (%UniqV) |
| AbnRank | 0.11 | 0.10 (3.0) | 0.64 (0) | 0.15 (2.0) |
| Sex | 0.07 | 0.61 (0) | 0.15 (1.7) | 0.51 (0) |
| Species | 0.11 | 0.40 (0) | 0.46 (0) | 0.15 (2.1) |
| Weight | 0.38 | 0.29 (0.3) | 0.69 (0) | 0.70 (0) |
| Age | 0.06 | 0.6 (0) | 0.37 (0) | 0.30 (0.2) |
| Regression adjusted R-squared | | 15.2% | 18.7% | 5.0% |

^a df = 1, 52 for each factor. **Bold indicates p ≤ .05.** Wilks's Lambda probability indicates significance of factor across all DVs.