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Journal
Peptides, 21(6)

ISSN
0196-9781

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Publication Date
2000-06-01

DOI
10.1016/s0196-9781(00)00209-6

ELSEVIER

PEPTIDES

Peptides 21 (2000) 785–791

Uncoupling of proopiomelanocortin (POMC) fragments is related to self-injury☆

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Received 18 February 2000; accepted 3 May 2000

Abstract

Proopiomelanocortin (POMC) contains several interesting, behaviorally active peptides. Release patterns of these fragments have been related to bizarre episodes of self-injurious behavior (SIB) among autistic individuals. Moreover, elevation in β-endorphin (βE) but not ACTH levels was associated with a positive response to an acutely administered, centrally acting opioid blocker among autistic individuals exhibiting SIB. In the present study, POMC fragments were measured in 12 self-injurious patients before and after long term (3 month) treatment with an opiate blocker naltrexone (NTX). POMC fragments were sampled from blood collected at the beginning of the baseline and placebo-controlled treatment phases of the study. Results indicated that the co-release (coupling) of POMC fragments were stable over time and the profile of POMC fragments in plasma predicted the effectiveness of a CNS acting drug in autistic subjects who self-injure.

Keywords: Self-injury; Autism; β-endorphin; Naltrexone; ACTH; POMC; Opioids

1. Introduction

Expression of proopiomelanocortin (POMC) involves a well-understood system with several levels of control [5–7, 22]. POMC is a 31 kDa molecule that contains eight pairs of basic amino acids and one sequence of four basic amino acids that are the cleavage sites for prohormone convertases (PCs). POMC expression is controlled by the cellular response to corticotrophic-releasing factor (CRF). Binding of CRF to specific G-protein-coupled receptors stimulates the accumulation of cAMP and intracellular calcium and promotes POMC gene transcription. POMC, as is true for most neuropeptide precursors, initially is synthesized as an inactive molecule that requires post-translational modifications to generate bioactive products. A family of subtilisin-like PCs have been identified that are responsible for proteolytic processing of POMC. Two of these, PC1 and PC2, convert the POMC molecule into bioactive peptides [28,29]. Among the active peptides processed from POMC by PC1 are ACTH (39 amino acids) and B-LPH (91 amino acids) whereas PC2 cleaves βE (31 amino acids) in addition to several smaller fragments (e.g. a-MSH). Both convertases are expressed by midgestation but the great differences in their distribution that are evident prenatally begin to disappear as organisms reach adulthood [33] and explains that, in normal circumstances, the concentrations of POMC products are perfectly correlated in adults [5,31].

Recent studies from our laboratory [20,22] indicate that uncoupling of the POMC fragments, B-endorphin (βE_{1-31}) and adrenocorticotropic hormone (ACTH) may be an indicator of opioid involvement in self-injurious behavior (SIB) among autistic individuals. Plasma concentrations of βE_{1-31} were uncoupled from ACTH immediately after an episode of SIB. In contrast, morning concentrations of these peptides were tightly coupled (i.e. positively correlated) in these same patients, when levels of βE_{1-31} and ACTH normally are elevated [14,22].

Reports [14,15] of massive resting level differences be-

☆ Supported by a grant award (HD31571) and MRRC grant (HD28202) from the NICHD.

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0196-9781/00/$ – see front matter
PII: S0196-9781(00)00209-6

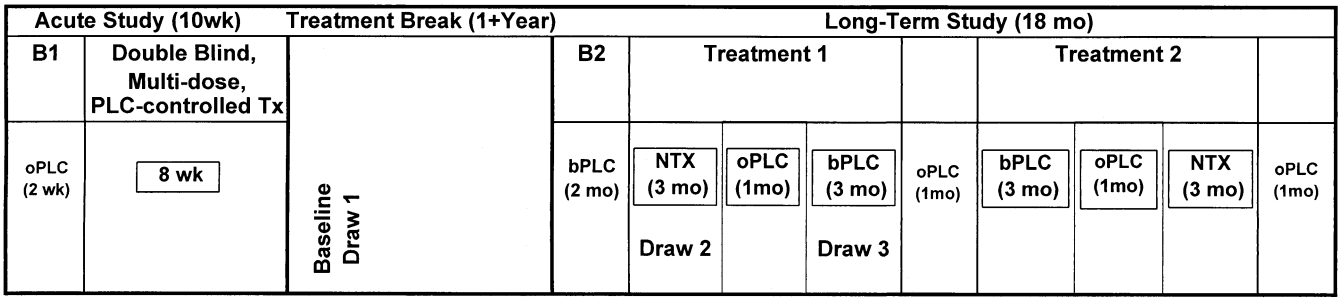

Fig. 1. Study timeline. The baseline blood draw was collected after the acute study. All other samples were drawn during the last week of the treatment phase corresponding with the placebo-controlled, double-blind, multiple baseline design.

tween N-terminal-directed βE antibody $_{(1–23)}$ and C-terminal-directed βE antibody $_{(20–29)}$ in autistic patients indicate another interesting dissociation of POMC fragments. Leboyer et al. [14,15] found that only the C-terminal βE fragment was elevated in plasma resting levels and that the N-terminal βE fragment was depressed or not different compared with controls. In a separate study, this group [7] also reported that the C-terminal βE fragment was decreased (or "normalized") in plasma after treatment with naltrexone (NTX) in patients categorized as good responders. Consistent with this finding, we reported that uncoupling of POMC fragments was related to the effectiveness of an acutely administered, centrally acting opioid blocker, NTX, in reducing SIB among autistic individuals [22].

This collection of findings provides evidence of abnormal expression of the POMC gene in subgroups of autistic individuals [24] and offers a biological explanation for the wide range of variability in response to opioid blockers [8,21,22]. In the present study, POMC fragments were measured in patients exhibiting SIB before and after long term (3 month) treatment with NTX.

2. Methods

2.1. Participants

Informed consent for evaluation of long-term effects of NTX and collection of plasma was obtained from guardians/conservators of twenty-one participants residing in a 900-bed residential treatment center for individuals with neurodevelopmental disabilities. From these subjects, fifteen were selected as positive responders to NTX in an acute study [21] and 12 of these completed the 24-month protocol [23]. Procedures of initial subject selection and characteristics have been described elsewhere [21–23]. Briefly, 129 subjects were observed directly by the research staff resulting in selection of a target sample of 24 subjects with the highest frequency SIB, who met the criteria for inclusion in the acute study. From this group, 15 were selected for the present long term study that met the criterion of at least a 25% reduction in SIB on any of the three active doses of NTX.

Subjects were excluded if there was evidence of debilitating disease or perceived risk from naltrexone as determined by chart review or results of the most recent annual physical examination, including chest radiography, urinalysis, complete blood count, and blood chemistry. Subjects were excluded if they had seizures within 2 years of initiation of the protocol. Although six of the subjects (50%) were administered anticonvulsants during the study, none had recent seizure histories. Four of the six subjects on anticonvulsant medication (plus an additional subject for a total of five or 41.6%) were also administered neuroleptic medication, which remained constant after entry into study protocol. Five subjects were naïve to psychotropic and anticonvulsant medication throughout the study.

2.2. Procedures

The study design was placebo-controlled, double-blind, and multiple baseline with a single (most effective) dose (described in detail by Sandman et al. [23]). The single dose was chosen based upon the previous acute challenge (i.e. 0.5, 1.0, or 2.0 mg/kg) with NTX [21] that produced a positive response in these patients compared to a naïve baseline (B_1). A positive response to NTX was defined as at least a 25% decrease in SIB. For long-term treatment the lowest effective, acute dose of NTX was chosen. The majority of patients (64%) responded most favorably to the 2.0 mg/kg dose. The study time line is shown in Fig. 1.

Behavioral baseline (B_1) observations were conducted at the beginning of the acute study, before any patient received NTX. Patients who responded to treatment in the acute phase of the study [21] were re-examined one year later, before long-term treatment, by collecting two months of open placebo baseline (B_2) SIB data. Subjects then entered the long-term treatment protocol, an 18-month study of the effects of NTX on SIB. In balanced order, subjects received a three-month treatment with NTX (A, called NTX_1) and a 3-month blind PLC condition (B, called bPLC) after the baseline period (see Fig. 1). After each 3-month treatment period (either A or B), an open PLC (oPLC) month was

inserted. For example, a possible order was: blind PLC for 2 months, 3 months of NTX_1 (blind), 1 month PLC (open), and 3 months PLC (blind). The order of treatment was balanced across patients. Blood level information was available corresponding to the baseline (B_1), treatment with naltrexone (NTX_1), and placebo conditions (B_2) of the study but not for the second half of the 18 month study (NTX_2).

2.3. Measurements

Fifteen-minute observational samples/week (3, 5 min/samples) were collected randomly (videotaped) for each patient for at least 28–32 weeks generating between 420 and 480 min (7–8 h) of direct observation for each subject [23]. The videotaped sessions were scored by raters with a computerized observational program [12]. The program permitted computer-assisted measurement of frequency and duration of target behavior (i.e. SIB). Inter-rater reliabilities were $r = 0.81$ for frequency of SIB, $r = 0.89$ for duration of stereotypy, and $r = 0.96$ for duration of self-restraint.

2.4. Procedures for collection of blood

All patients in this study had blood drawn fairly regularly as a matter of clinical care and as a group were not resistive to the procedures. Study protocol required that the procedures were to be terminated, if, in the opinion of hospital staff (not research staff), the patient exhibited signs of distress. In this group of patients, no blood draw was aborted. Venous blood was drawn for each patient and deposited immediately in tubes on ice. Baseline blood samples were drawn in the morning (8 a.m.) and afternoon (4 p.m.) of the same day immediately after the acute study before the 18-month treatment break. This served as the baseline (B_2) for blood samples. In addition, blood was drawn once in the morning (8 a.m.) during the last week of each 3-month treatment phase (drug and blind placebo phase; see Fig. 1).

2.5. Assays for ACTH and βE

Blood samples (10 ml/draw) were withdrawn by antecubital venipuncture into EDTA (purple top) vacutainers and chilled on ice immediately. Samples were centrifuged at $2000 \times g$ (15 min.) and the plasma decanted into polypropylene tubes containing 500 KIU/ml aprotinin (Sigma Chemical Company; St. Louis, MO, USA). The samples were stored at −70°C until assayed. Plasma levels of βE were determined by a commercially available direct solid phase two-site immunoradiometric assay (IRMA; Nichols Institute Diagnostics; San Juan Capistrano, CA, USA). The βE assay incorporates two antibodies, both with high affinity and specificity for both N-terminal and C-terminal defined amino acid regions of the $\beta E_{1–31}$ molecule. Both antibodies bind βE without competition or steric interference and form a sandwich complex between the immobilized βE antibody on the plastic bead and 125I-labeled βE antibody. The antiserum has 16% cross-reactivity with BLH at 500 pg/ml and has <0.01% cross-reactivity with related opiates at 5 μg/ml. Samples were assayed in duplicate (200 μl per assay tube). 125I-anti-βE (rabbit) solution (100 μl) was added to each tube and vortexed. The reaction was initiated by adding one anti-BE (rabbit) coated polystyrene bead to the assay tube followed by a stationary incubation at room temperature for 20 + 4 h. The beads are then washed twice with phosphate-buffered saline (PBS) and aspirated to dryness. The labeled antibody complex bound to the solid phase was measured using an ICN Biomedical (formerly Micromedic) Isoflex Gamma Counter. The amount of radioactivity is directly proportional to the amount of intact $\beta E_{1–31}$ because the formation of the sandwich complex occurs in the presence of an intact βE molecule containing both N-terminal and C-terminal regions. The Allegro Beta–Endorphin Immunoassay system has a minimum detectable dose MDD = 14 pg/ml (95% confidence limit) with a coefficient of variance CV = 4.1% (intra-assay) and CV = 9.0% (inter-assay) at the highest concentrations in the present study.

ACTH ^{125}I-antibody solution (100 μl) was added to the samples, vortexed and incubated at room temperature for 20“2 h after the addition of an avidin coated bead. The solid matrix was washed with buffered surfactant in PBS to remove unbound components and the bound radiolabeled antibody complex quantified using a Micromedic Isoflex Gamma Counter. The ACTH assay has a MDD = 1.0 pg/ml (95% confidence) with CV = 3.0 percent (intra-assay) at 35 pg/ml and CV = 7.8 percent (inter-assay) at 36 pg/ml.

Levels of βE and ACTH were expressed as pg/ml. In addition, a disregulation index (D.I. = ((βE-ACTH)/βE) × 100; [21,32] was computed [25,32] using morning (AM) resting blood levels to characterize the degree of uncoupling between βE and ACTH and to eliminate sources of variation inherent in measures of absolute concentrations (i.e. genetic and or environmental factors). When βE and ACTH are equal, the index equals zero. A positive index means that $\beta E_{1–31}$ is elevated above ACTH, and a negative index means that ACTH is elevated above βE.

3. Results

Baseline levels of the POMC fragments $BE_{1–31}$ and ACTH in the total sample ($N = 21$) at B_1 and B_2 were positively and significantly correlated with each other in the morning but not the evening samples (see Table 1). These findings confirm that POMC fragments are coupled (i.e. co-released) during periods when basal levels normally are highest [22]. Similar relationships among POMC fragments were observed for the subsample of subjects who completed the treatment phase of the protocol ($n = 12$).

Analyses for the twelve subjects who completed the protocol yielded significant correlations between baseline

Table 1
Correlation Matrix for Morning and Evening Blood Peptide Levels (N = 21)

		AM			PM		
		BE	ACTH	Cortisol	BE	ACTH	Cortisol
AM	BE		.67**	.37	.79**	.14	−.06
	ACTH			.57*	.33	.42	.14
	Cortisol				.10	−.07	.16
PM	BE					.31	.13
	ACTH						.63*
	Cortisol						

* Significant at the $p < .05$ level
** Significant at the $p < .001$ level

and placebo levels of βE ($r = 0.86$, $P < 0.0001$) and ACTH ($r = 0.78$, $P < 0.003$). Relationships between basal (B_2) and treatment periods were slightly less significant for both βE ($r = 0.65$, $P < 0.02$) and ACTH ($r = 0.66$, $P < 0.02$). Levels of βE and ACTH were not significantly different across treatment conditions. Measures of coupling (DI) were different, however, between groups and among treatments.

Based on previous analysis [23] it was determined that half of the subjects ($N = 6$) exhibited *persisting improvement* (25–80% decrease in SIB) that lasted over one year (responders) after acute treatment with NTX. The remaining half showed no *persisting effect* of acute exposure to NTX (non-responders) even though their initial response was positive. The DI of these two groups across treatments is illustrated in Fig. 2a. A positive value indicates greater levels of βE relative to ACTH. A significant ($F = 6.30$, $P < 0.01$) interaction between DI and morning levels of βE indicates that subjects with reduced SIB extending over a year after acute treatment also have lower relative levels of βE at baseline (B_2). After treatment with NTX each of these six subjects significantly increased their SIB (i.e. they got worse; Fig. 2b) and their DI ratios increased reflecting higher relative levels of βE (Fig. 2a). Return to placebo resulted in improved behavior and lower relative levels of βE. The pattern for non-responders suggests relatively higher levels of βE at baseline (B_2; when SIB is prevalent) with no discernable effect after long-term treatment but then a precipitous rise in relative βE during subsequent placebo periods. (Fig. 2b extends the behavioral analysis to a second treatment period, however plasma was not collected during this period for analysis of POMC levels).

To investigate further the relationship between DI and the long-term response to the acute dose of NTX, the percent change in disregulation and SIB was correlated [21–23]. Comparison between the change in DI from B_2 to long-term treatment and change in SIB from B_1 to B_2 revealed that a persisting behavioral response to an acute dose of NTX predicts the subsequent ratio of βE and ACTH ($r = 0.77$, $P < 0.01$). Results reveal that a significant portion of the variance was accounted for by a quadratic regression equation ($r = 0.62$, $P < 0.05$) and that group membership (i.e., responders/non-responders) accounts for the non-linear expression of disregulation of the peptides (Fig. 3). Thus, long-term positive response to acute doses of NTX is associated with less disregulation (i.e. greater association) of ACTH and βE. A negative response, in contrast, is associated with an increase in uncoupling (i.e. as βE levels are increased, ACTH levels are decreased).

4. Discussion

The primary findings are that POMC fragments are co-released (coupled) at rest and that these patterns are reliable over time (also see LeBoyer et al. [14]). In addition, the degree of coupling between these co-released peptides was related to response to an opiate blocker, in agreement with previous conclusions [8,22]. Elevated βE relative to ACTH predicted positive response to NTX.

Previous results [23] suggest that there are subgroups of SIB patients with persisting behavioral sensitivity to acute, intermittent exposure to opiate blockers. One group (responders) in our study displayed persisting improvement in SIB and lower relative levels of βE after acute exposure to NTX. Chronic administration of NTX to this group was associated with increased SIB and elevated relative levels of βE. Return to placebo improved their behavior (reduced SIB) and their levels of βE returned to basal levels. The second group (non-responders) was characterized by absence of persisting improvement after acute treatment with NTX and by elevated basal βE levels. Chronic treatment with NTX improved their behavior but did not alter their βE levels. Their behavior continued to improve during the placebo period and their levels of βE were elevated relative to ACTH.

The current findings suggest that there may be fundamental differences in the profile of POMC fragments between sub-groups of individuals who self-injure that account for the sensitivity to opiate blockers. The two groups responded differently and relationships among POMC fragments tracked their response. Relationships between βE and ACTH predicted long term response to acute and chronic exposure to opiate blockers. Persistent responders displayed greater coupling of the POMC fragments than the non-responders. Moreover, patient behavioral response to NTX was reflected in the relationship among the peptides.

The present findings are consistent with basic studies that indicate response to opiate blockers is regulated by level of exposure to opioids and by the chronicity of treatment [16]. Chronic elevation of opioids result in supersensitive responses to the effects of opiate antagonists [9,30]. This effect was illustrated in the "non-responders" who entered the long-term study with high relative levels of βE and high rates of SIB. They responded swiftly and significantly (i.e. supersentively) to long-term NTX, with a decrease in SIB. Different patterns were detected in the subjects who had persisting reduction in SIB to the acute trial. They entered

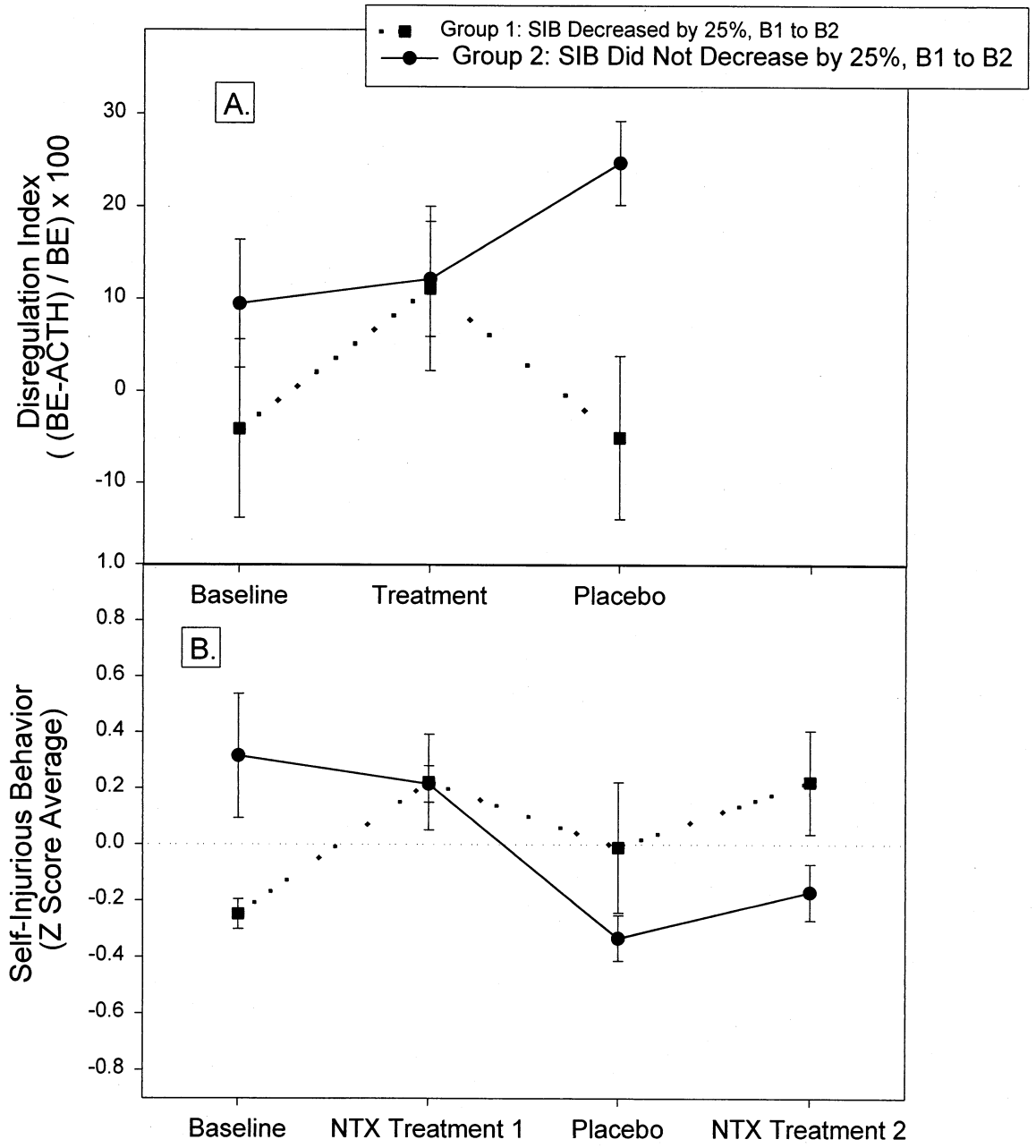

Fig. 2. Disregulation index and self-injurious behavior across long-term treatment with naltrexone. Section A shows the pattern in the disregulation index separately for the group that responded to acute treatment (Group 1) and the group (2) that did not respond to acute treatment. Section B shows the corresponding changes in self-injurious behavior, including the second long-term treatment phase (3-months bPLC and three months NTX).

the chronic phase of the study with low rates of SIB and low levels of βE relative to ACTH, and, when administered NTX, their behavior deteriorated (i.e. SIB increased) and their DI increased (i.e. βE increased relative to ACTH).

Consideration of the relationship among POMC fragments by using the DI, rather than absolute levels, eliminates important sources of between-subject variability [25]. For instance, expression of the POMC gene is controlled by dispositional/genetic factors [23] as well as environmental factors such as circadian rhythms. Measures of absolute levels of POMC fragments can be influenced by these uncontrollable sources of individual variation. Because βE and ACTH are both within the same precursor molecule and both subject to similar sources of variation the specific effects of the opioid system in SIB can be determined by consideration of their ratio.

The importance of the specific elevation of plasma βE levels in SIB patients was illustrated by its ability to predict response to NTX. Consistent with several earlier studies (see [20,21]), 2 mg/kg of NTX in these patients effectively

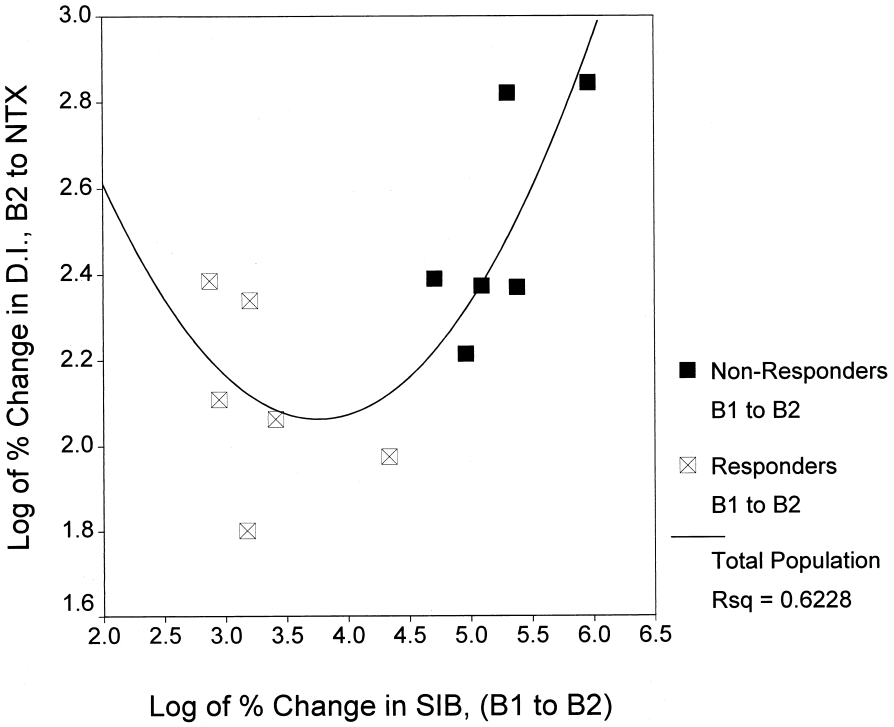

Fig. 3. Relationship between percent change in self-injurious behavior and disregulation index. This illustrates the non-linear relationship between change in behavior and the change in POMC. Subjects who responded to acute treatment had an increase in βE relative to ACTH (higher log D.I.) when administered naltrexone long term. Conversely, subjects who did not respond to acute treatment showed a relative decrease in βE relative to ACTH when treated with naltrexone in a long-term fashion.

reduced SIB in many, but not all patients. In the current study, patients with elevated βE relative to ACTH had the greatest reduction in SIB when administered NTX. These results are closely associated with two other reports. First, Ernst et al. [10] reported that baseline levels of βE was positively related to changes in behavior (Clinical Global Impressions, CGI) after treatment with NTX in autistic children. Second, Bouvard et al. [8] found that C-terminal βE decreased after NTX only in good responders. Our findings and those of Ernst et al. [10] are similar to studies [26] in which naloxone blocked behavior in rats when opiate levels were high (induced by administration of 3.0 to 5.6 mg/kg of morphine) but not when they were low (0.3–1.0 mg/kg of morphine). These findings may suggest that patients who have elevated βE comprise a subgroup whose aberrant behavior is associated with elevated endogenous opioids and whose SIB can be treated with opiate antagonists. Patients who do not have elevated βE may be a different subgroup with different mechanisms controlling their behavior.

There is speculation [20,21] that some patients engage in self-injury to release βE as an opioid "fix." The often compulsive and ritualistic patterns of behavior associated with SIB and its resistance to intervention are consistent with this possibility. Inferential support for the addiction hypothesis derives from similar peptide patterns (specific elevation of βE) reported for SIB patients [20,22] and for heroin addicts [17]. Responses to NTX in SIB patients with elevated βE were similar to the responses of rats to naloxone that had been pre-treated with high doses of morphine [26], supporting the significant relationship between systemic levels of opioids and sensitivity to centrally acting opiate blockers. Moreover, there is evidence that circulating peptides not only participate in the regulation of brain and pituitary release of peptides but also directly influence the central nervous system [27]. There is evidence also that many neuropeptides (in small amounts), including βE, cross the BBB [1]. For instance, the effects of peripherally injected endogenous opioids on the brain are prevented by centrally acting opiate blockers (e.g. NTX) but not by blockers unable to pass the BBB (e.g. methylnaltrexone; [13]). It is well known that although only small quantities of peripherally administered opiates such as morphine cross the BBB, they can have profound effects on the central nervous system [18]. Our findings further suggest that the patterns (uncoupling) of plasma peptides can influence response to a centrally acting drug.

There are several possible mechanisms that can account for uncoupling of the POMC molecule. These mechanisms include: the expression of the CRH gene that controls transcription of the POMC gene, variants of POMC gene expression [6,11,23], and expression of the genes for the converting enzymes PC1 and PC2. Because all elements of this CRH-POMC-PC-peptide system are developed early in

fetal life, uncoupling of POMC products, or abnormal levels of POMC products can provide evidence of genetic disturbance expressed during the prenatal period. The association between early (perinatal) development and SIB have been reported by us previously [2–4,19] and adds further support to the rationale for examining the POMC system among these patients.

Acknowledgments

The assistance of Kimberley Cox is greatly appreciated.

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