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Proopiomelanocortin (POMC) Disregulation and Response to Opiate Blockers

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Autism is a collection of disorders or subtypes with distinctive or prominent phenotypes and genotypes. The study of adults with autism offers a unique opportunity to examine its phenotypic diversity. Recent evidence has identified disturbances in specific neurochemical systems that are associated with primary autistic symptoms. Establishing biological markers, such as specific neurochemical disturbances, not only confers greater precision in phenotyping individuals but also provides the basis for rational intervention. Our initial studies in adult individuals exhibiting self-injurious behavior (SIB) generated evidence that the proopiomelanocortin (POMC) system, specifically the endogenous opioid system, may be disregulated in subgroups of autistic patients. These findings, corroborated in at least 15 other laboratories, indicated that treatment with an opiate blocker, naltrexone (NTX), reduced SIB in 30–70% of individuals observed. However, the effects of NTX on SIB were not simple. We and others have found that concentration of plasma POMC fragments, specifically opioid fragments, contributed to the symptoms of autism and to the response to treatment. Uncoupling of the release of POMC products predicted the efficacy of NTX treatment on the expression of SIB. Uncoupling of POMC fragments among autistic and SIB patients suggested a basic, underlying defect, perhaps in the POMC gene. The findings of a maternal influence on the C-terminal BE fragment among individuals with autism (Leboyer et al. [1999] Soc Biol Psychiatry 45:158–163) and our preliminary findings, reported here, of a mutation in the opioid region of the POMC gene in an autistic individual, were consistent with the prospect that a subgroup of patients will be identified who share a POMC genetic defect.
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Key Words: autism; proopiomelanocortin; opiate blockers; naltrexone

PHENOTYPIC AND GENOTYPIC DIVERSITY IN AUTISM

During the past 25 years, our programs of research [Sandman et al., 1983, 1990/1991, 1997; Spence et al., 1973] have focused on biological and genetic factors contributing to the autism spectrum. One inescapable conclusion from our observations is that autism is a very complex disorder. The complexity becomes especially apparent in the behavior of adults who were diagnosed at an early age with autism. The behavioral phenotypes of adult individuals with autism vary widely even though as young children their presentation may have satisfied a relatively narrow range of symptoms. Adult presentation of autism may include individuals with well-developed expressive verbal language skills or with extremely limited expressive abilities; some may have relatively advanced capacity for social attachment and others may remain withdrawn and uninvolved; some may be relatively high functioning and others may be profoundly mentally retarded; some may engage in overt stereotypic activities and for others these behaviors seem to disappear; for some either the hypo- or hypersensitivity to stimulation evident during childhood has disappeared. Thus, the study of adults with autism offers the opportunity to examine the diverse expression of the autistic spectrum.

Phenotypic diversity is an important advantage because contemporary theories propose that autism is a developmental disability of multiple etiologies [Rapin, 1997; Rutter, 1996]. Moreover, the familial relative risks yield estimates that the number of genetic loci involved are between three and six [Bristol et al., 1996]. The evidence suggests that the multiple genetic mechanisms contributing to this complex disorder include the EN2 region on chromosome 7, the HRAS oncogene region, various alterations on chromosome 15 (partial tetrasomy and duplication) [Flejter et al., 1996; Hotopf and Bolton, 1995; Martinsson et al., 1996], SLC6A4 (serotonin transporter) on chromosome 17, q11-q12 [Cook et al., 1997], and as we will suggest here, the proopiomelanocortin (POMC) region of chromosome 2.

It is most reasonable to consider autism to be a collection of disorders or subtypes with distinctive or prominent phenotypes [Wing, 1989] and genotypes [Cook et al., 1997; Rutter, 1996]. Psychobiological evidence defines some patients as expressing significant disturbances in neurochemical systems that are associated with their primary autistic symptoms [Gillberg, 1992, 1995]. Establishing biological markers in autism, such as specific neurochemical disturbances, not only confers greater precision in phenotyping individuals but also suggests that a candidate gene strategy can be used. Genes encoding proteins and enzymes involved in neurochemical pathways that are

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shown to be disturbed in autism represent candidate genes. Molecular analysis of these genes in individuals with autism may lead to identification of mutations such as insertions and deletions. This approach has generated interesting preliminary findings for serotonin. For instance, the role of serotonin (5-HT) in autism has been studied since the early 1960s, when it was discovered to be increased in whole blood of autistic children [Schrain and Freedman, 1961]. Subsequent studies reported that between 30% and 50% of individuals with autism have increased 5-HT in their blood [Anderson and Hoshino, 1987; Anderson et al., 1987; Leboyer et al., 1999]. Evidence also indicates that serotonin uptake inhibitors are effective in controlling stereotypy and repetitive movement disorders exhibited by some patients with autism [Lewis et al., 1995]. Potent serotonin transporter inhibitors have been partially effective in reducing behaviors related to anxiety and aggression among individuals with autism [Gordon et al., 1993; McDougle et al., 1997]. These findings led to the study of the serotonin transporter gene (HTI) as a primary candidate in autistic disorder. Two polymorphisms for the serotonin transporter (SLC6A4) gene have been reported including a variable number tandem repeat (VNTR) in the second intron and a deletion/insertion polymorphism in the promotor that is central to the ''fine tuning'' of brain serotonergic neurotransmission [Cook et al., 1997]. In a study of 86 autistic triads (probands and both parents) using the transmission/disequilibrium test, Cook et al. [1997] reported evidence of linkage between halotypes of two HTI markers and autism. The short variant of the HTI promoter was preferentially transmitted. However, disturbances of serotonin do not explain all of the variable symptoms associated with autism. Additional studies implicate disturbances in several other neurochemical systems among individuals with autism. Insel [1997], for instance, has argued convincingly that the neuropeptides oxytocin and vasopressin are important in the pathophysiology of social attachment that is prominent in disorders such as autism. Stereotyped behavior, common among individuals with autism, has been associated with hypodopaminergic function [Bodfish et al., 1995; Lewis et al., 1996a, 1996b]. The studies we summarize here have focused on individuals with autism who present with a defined behavioral phenotype (self-injurious behavior, SIB) that is associated with disregulation of a specific neurochemical system (proopiomelanocortin, POMC).

Self-Injurious Behavior (SIB) and Proopiomelanocortin (POMC)

SIB is mysterious, expensive to control, often unmanageable, destructive, sometimes life threatening, and unpredictable. It is reported to occur in between 7 and 30% of neurodevelopmentally delayed individuals, especially those with autism [Green, 1967; MacKay et al.,1974; Sandman and Barron, 1992; Schroeder et al., 1978]. The most influential explanations of SIB have been behavioral (e.g., attention seeking; escape/avoidance), and many interventions were effective without consideration of possible biological mechanisms [Iwata et al., 1982; Matson and Taras, 1989; Matson and Keyes, 1990; Rincover and Devaney, 1982; Rincover and Koegel, 1975; Romanczyk and Goren, 1975]. Understanding putative biological mechanisms provides a basis for rational pharmacological treatment strategies.

The majority of contemporary studies support a biological basis for some forms of SIB. One prominent biological explanation of SIB among individuals with autism involves the hypothalamic–pituitary–adrenal system, specifically the POMC molecule and its various opioid peptide fragments. POMC was the first mammalian endocrine or neuronal precursor to be cloned and the first prohormone shown to be differentially processed in a tissue-specific manner to yield multiple active neuropeptides. Expression of POMC involves a very well understood system with several levels of control [Bertagna, 1994; Bicknell et al., 1996; Boutillier et al., 1995; Sandman et al., 1997]. POMC is a large (31 kDa) proteinlike molecule that contains eight pairs of basic amino acids and one sequence of four basic amino acids that are the cleavage sites for enzymes (prohormone convertases; PC1 and PC2). POMC expression is controlled by the cellular response to a hypothalamic message, corticotrophic-releasing factor (CRF), a 41-amino-acid peptide. Binding of CRF to specific G-protein-coupled receptors stimulates the accumulation of cyclic adenosine monophosphate (cAMP) and intracellular calcium and promotes POMC gene transcription. Usually cAMP and calcium induce transcription factors that bind to the promoter region at DNA-responsive elements. POMC CRF-responsive element (PCRF-REB-1) binding protein, which binds to a specific region [-171/-160]) of the POMC promoter, increases transcription of that gene sevenfold.

POMC, as is true for most neuropeptides precursors, initially is synthesized as an inactive molecule that requires posttranslational modifications to generate bioactive products (Fig. 1). The crucial step is proteolytic (enzymatic) cleavage, usually at the carboxyl side of specific dibasic residues, followed by exoproteolytic removal of the terminal basic residues for the peptides. The amino acid pairs, Arg–Lys/Arg-Arg, seem to be the preferred recognition site for endoproteases. The prohormone convertases (PCs) are members of a family of subtilisin-like enzymes that are tissue specific (i.e., they reside in certain tissues and their actions result in specific peptide sequences). Two of these PCs (PC1 and PC2) convert the biologically inactive POMC molecule into bioactive peptides [Seidah and Chretien, 1992; Seidah et al., 1991]. PC2 is more broadly distributed in the central nervous system than PC1 and is primarily expressed in the intermediate lobe of the pituitary gland. PC1 is expressed both in the anterior and intermediate lobes. PC1 has relatively limited processing properties compared with the wider processing properties of PC2, but both liberate several interesting smaller peptides from the large biologically inactive precursor POMC molecule (Fig. 1). Among the active peptides processed from POMC by PC1 are ACTH (39 amino acids) and β-LPH (91 amino acids), whereas PC2 cleaves β-endorphin (βE, 31 amino acids) in addition to several smaller fragments (MSH (13 [α] and 18 [β] amino acids and CLIP [21 amino acids]). The structure of enkephalin also is contained in the POMC molecule, but because of the tissue specificity of the PCs, it is not expressed (it is expressed from proenkephalin). Both convertases are present in the fetus by midgestation, but the great differences in distribution of PC1 and PC2 that are evident prenatally begin to disappear as organisms reach adulthood [Zheng et al., 1994]. This developmental pattern, in *normal circumstances*, results in the concentrations of POMC products, particularly ACTH and βE, being perfectly correlated in adults [Bertagna, 1994; Strand, 1999].

In the fetal mouse, the CRF gene is activated in basal diencephalon at about the same time as POMC and PC1 and 2 [Japon et al., 1994]. This suggests important roles for POMC, CRF, and PCs in neuronal development. Uncoupling (perturbed release patterns) of these peptides in any group is evidence of abnormal processing of the POMC molecule and

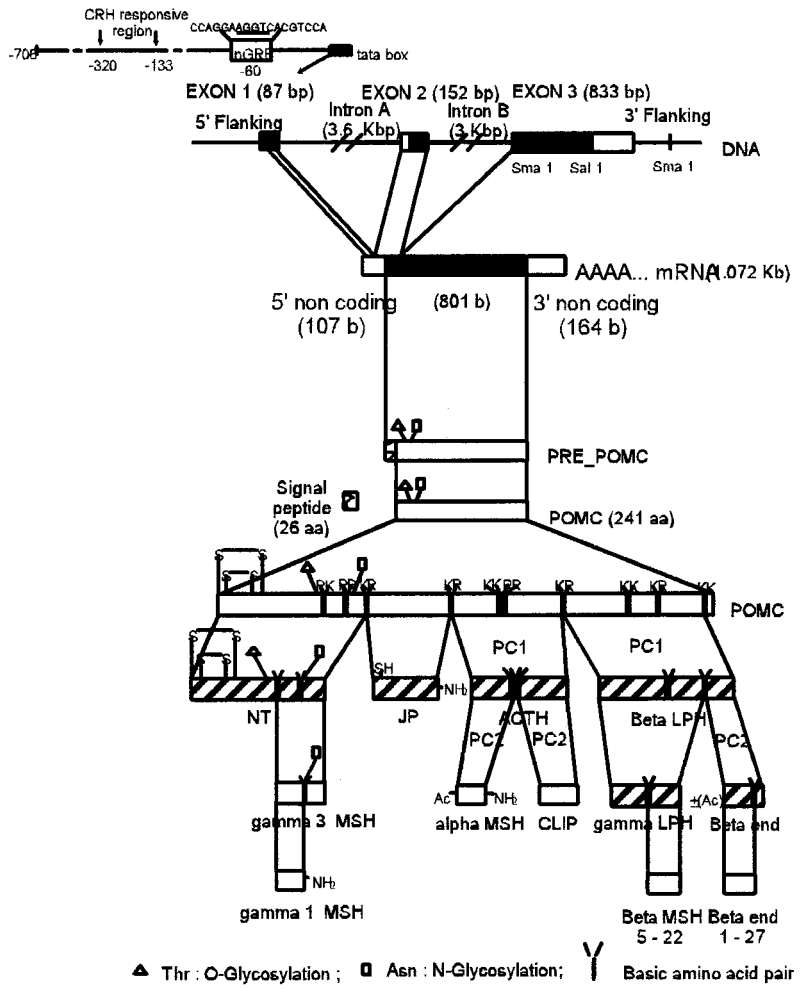

Fig. 1. The proopiomelanocortin (POMC) system illustrates the relationship among the CRH gene, the POMC gene, prohormone convertases (PCs), and bioactive products such as ACTH and the endogenous opioids. Protein coding regions of the gene (solid black segments) and peptides found in the anterior pituitary (cross-hatched segments). Adapted from Bertagna [1994] and Boutillier et al. [1995].

perhaps early disturbance of this delicately balanced system. There is evidence that POMC is expressed differently during development because of the existence of several variants of the gene (a normal variant, a long variant, and a deletion variant) [Bicknell et al., 1996]. It is relevant to the study of autism that the POMC alleles elicited different cleavage products (i.e., peptide structures) in the C-terminal of the molecule, suggesting that POMC alleles influence the activity of the PCs [Bicknell et al., 1996]. Thus, uncoupling of POMC fragments, such as ACTH and βE, is evidence for variants of the POMC gene [Bicknell et al., 1996; Gen et al., 1994]. Because all elements of this CRF–POMC–PC-peptide system unfold early in fetal life, uncoupling of POMC products or abnormal levels of POMC products would be evidence of genetic disturbance expressed during the prenatal period with potentially profound influences on development.

The POMC gene is highly conserved. It has three exons and two introns. The large exon 3 contains nucleotide coding for all of the biologically active peptides of POMC. The gene is assigned to human chromosome band 2p23.3 [Satoh and Mori, 1997]. This region in humans contains at least two other regulatory genes, one for controlling serum leptin levels and another for glucokinase regulatory protein [Comuzzie et al., 1997].

Opiate blockers and SIB

Biological and pharmacological studies conducted during the past 10 years have generated estimates that between 30% and 70% of patients with SIB have disregulated POMC or opioid systems. Two POMC or opiate hypotheses of SIB—increased pain tolerance and addiction to endogenous opioids—have been reviewed extensively [Sandman and Hetrick, 1995; Sandman et al., 1998; Sandman, 1990/1991; 1988; Cataldo and Harris, 1982; Deutsch, 1986; Farber, 1987]. Apparent insensitivity to pain generated the hypothesis that SIB was a symptom of general sensory depression, including hypoalgesia [Cataldo and Harris, 1982; Davidson et al., 1983; Farber, 1987; Sandman et al., 1983; Sandman, 1988]. In this case, opiate blockers might attenuate SIB by increasing pain (hyperalgesia) during the abusive act. Consistent with the possibility that blocking the opioid system reversed chronic ''hypoalgesia'' or sensory depression in patients with SIB, it was reported that the opiate blockers naloxone and naltrexone (NTX) reversed congenital insensitivity to pain [Dehen et al., 1977], reversed hypothalamic dysfunction coexisting with elevated pain threshold [Dunger et al.,1980], and increased brain sensory-evoked potentials [Arnsten et al., 1983].

The addiction hypothesis presumed that patients engaged in self-injury because they developed dependence on their own endogenous opioids. This argument reasoned that stress or pain-induced release of opioids [Forman et al.,1990; Giuffre et al., 1988; Holson et al., 1988; Knigge et al., 1989; Oltras et al., 1987; Recher et al., 1988; Shutt et al., 1988] during SIB produced a ''fix'' for tolerant, downregulated opiate receptors [Belluzzi and Stein, 1977; Lal, 1975; Madden et al., 1977; Wei and Loh, 1976]. Consistent with this reasoning, it was postulated that opioid receptor blockade attenuated SIB by eliminating the ''euphoria'' associated with the release of opiates after the self-abusive act [DeMet and Sandman, 1991].

Early support for both of the opiate hypotheses derived from the successful treatment of SIB with pharmacological agents, naloxone and naltrexone, which blocked opiate receptors. Because these opiate receptor blockers have few effects in the absence of opiates [Reisine and Pasternak, 1996], it was reasoned that effective treatment of SIB with these agents must engage the endogenous opioid system. More than 20 studies have now been conducted examining the effects of NTX on SIB and recently have been reviewed [Sandman et al., 1998]. The consensus is that about half of all patients treated acutely with NTX reduce their SIB [Sandman et al., 1993, 1998; Thompson et al., 1994; Casner et al., 1996]. The percentage is highest for patients with severe SIB, those who

damage their head and those who engage in self-biting [Sandman et al., 1993; Thompson et al., 1994]. Moreover, it is important to acknowledge that the significant effects of NTX especially are apparent in studies that directly observe SIB rather than rely on global clinical judgments [Willemsen-Swinkels et al., 1995].

The persisting effects on SIB of opiate blockers and the response to continued treatment might be more complex. There may be subgroups of Patients with SIB who manifest long-term sensitivity after acute exposure to opiate blockers [Barrett et al., 1989; Walters et al., 1990; Crews et al., 1993; Panksepp and Lensing, 1991; Smith et al., 1995; Casner et al., 1996; Sandman et al., in press]. Furthermore, the results suggest that there may be an interaction between acute sensitivity to NTX and subsequent response to the drug. Independently, there may be patients who respond to continued or long-term exposure to NTX. These patterns could describe subgroups of patients with unique profiles of endogenous opioids or receptors that control their response to challenge [Sandman et al., 1997].

The precise mechanisms responsible are unknown for the observed relationships between the persisting influence of acute exposure NTX in some patients. NTX and its metabolites are cleared from the body within 48–96 hours [Gonzalez and Brogden, 1988]. However, animal studies indicate that there are important differences between intermittent and chronic exposure to NTX. Daily administration of NTX upregulates opiate receptors and increases the reinforcing effects of opioids. These effects disappear within days of cessation of treatment [Bardo and Neisewander, 1987; Brunello et al., 1984; Marley et al., 1995; Zukin et al., 1982]. However, intermittent (weekly) exposure to NTX results in long-lasting supersensitivity (at least 10 weeks after treatment subsides) and generates a more complex receptor binding pattern in the brain. Chronic treatment results only in upregulation, but intermittent NTX upregulates opioid receptors in the hindbrain and downregulates midbrain receptors.

To compound the issue, the effects of NTX are regulated by the level of exposure to opioids. There is a dual-modulation of cellular response to opioids [Shen and Crain, 1992; Crain and Shen, 1995], and the same receptors may mediate both excitatory and inhibitory effects [Smart and Lambert, 1996]. Chronic exposure to opioids results in tolerance to the inhibitory effects of high doses of opiates but in supersensitivity to the excitatory effects of extremely low doses of opiates and to the effects of opiate antagonists [Shen and Crain, 1992; Crain and Shen, 1995]. This is consistent with our previous findings that patients with high levels of endogenous opiates were the most responsive to treatment with NTX [Sandman et al., 1997].

Neurochemical Phenotype Predicts Response to Opiate Blockers

If response to opiate receptor blockers is interpreted as evidence of opioid involvement among patients with SIB, then it is apparent that the opiate hypotheses either do not pertain to all forms of SIB [Thompson et al., 1994] or to all patients exhibiting SIB [Sandman et al., 1993]. Early attempts to classify patients with SIB by measurement of either basal or resting levels of a variety of opioids in plasma or cerebrospinal fluid generated inconclusive results. This is not surprising, because there is little consistency among studies regarding the rigor of diagnosis (phenotype), the peptides measured, or the conditions assessed [Coid et al.,1983; Ernst et al., 1993; Gillberg et al., 1985; Gillberg et al., 1990; LeBoyer et al.,1994; Ross et al., 1987; Sandman, 1988; Sandman et al., 1990, 1991; Weizman et al., 1984]. Except for our recent study [Sandman et al., 1997] there are no direct studies of opioid concentration during or immediately after an SIB episode.

Equally interesting in this study was the uncoupling of the adenohypophyseal POMC products βE and ACTH.

In studies from our current project [Sandman et al., 1997] plasma levels of highly specific immunoreactive β-endorphin (βE) activity were elevated relative to the coreleased peptide, ACTH, minutes after an SIB episode. Moreover, levels of βE after SIB predicted subsequent response (i.e., changes in frequency of SIB) to challenges with NTX. Equally interesting in this study was the uncoupling of the adenohypophyseal POMC products βE and ACTH. Disruption of the corelease of βE and ACTH in the plasma is uncommon and is not evident after a variety of physical and psychological stresses [Recher et al., 1988; Shutt et al., 1988; Knigge et al., 1989; Holson et al., 1988; Giuffre et al., 1988; Forman et al., 1990; Oltras et al., 1987].

Leboyer et al. [1994] reported a massive difference in resting levels between N-terminal BE ($_{1–23}$) and C-terminal BE ($_{20–29}$) POMC fragments in patients with autism. They found that the $BE_{20–29}$ fragment was elevated in plasma resting levels of patients with autism but that the $BE_{1–23}$ fragment was depressed or not different compared with controls. This research team [Bouvard et al., 1995] subsequently reported that $BE_{20–29}$ decreased in plasma after treatment with NTX in patients categorized as positive responders. The findings for the BE fragments were found to be reliable by retesting at a 1-year interval. Moreover, they reported that other fragments of POMC (e.g., ACTH) were not abnormal in their patients (further evidence of uncoupling of this system). Recently, this group [Leboyer et al., 1999] reported elevations in both whole blood serotonin and plasma C-terminal BE among autistic probands. Of central interest, they reported that siblings, mothers, and fathers of the autistic probands had whole blood serotonin levels significantly elevated compared to controls. They also reported that C-terminal BE was elevated significantly in mothers of autistic children. These findings confirm the familiality of hyperserotoninemia and are the first to suggest a maternal influence for POMC fragment variations. The authors concluded that these differences may be evidence of abnormal processing of the POMC gene in autism.

The dissociation among POMC fragments has been discovered with assays that measured different segments, strongly implicating a basic defect in the POMC molecule. As presented in Table 1, our assay is highly specific and does not cross-react with $LPH_{1–91}$ or with shorter fragments in the 31-amino-acid βE chain. Our findings of dissociation are between ACTH and the $\beta E_{1–31}$ fragment. The antibodies used in the Leboyer et al. studies for the C- and N-terminals had profiles that were different from each other, and were different from the highly specific two-site opioid $\beta E_{1–31}$ antibody complex used in our studies. The C-terminal antibody that detected activity of the $\beta E_{20–29}$ fragment was highly cross-reactive (94.4%) with β-$LPH_{1–91}$, and the N-terminal antibody was cross-reactive with several shorter opioids (alpha $\beta E_{1–16}$ and gamma $\beta E_{1–17}$). Thus, these assays, in addition to measuring $\beta E_{1–31}$ activity, each measured a substan-

Table 1. Cross-reactivity (percent) with Proopiomelanocortin (POMC) Fragments for Beta-Endorphin (βE) Directed Antisera for POMC-Peptide Assays for the Present Proposal

	βE Immunoreactive Region		
Peptide	βLP/C-terminal[a]	N-terminal[b]	Specific βE_{1-31} Assay[c]
βE_{1-31}	100	100	100
β-Lipotropin$_{1-91}$	100	<1.0	16 at 500 pg/ml
βE_{6-31}	43	2	N/A
βE_{1-27}	92	43	0.007 at 5 μg/ml
βE_{1-17}	0	N/A	0.007 at 5 μg/ml
βE_{1-16}	0	69	0.005 at 5 μg/ml
Leu-enkephalin	N/A	<0.04	<0.001 at 5 μg/ml
Metenkephalin	0	9	<0.001 at 5 μg/ml

[a]Directional insert; Peninsula Laboratories, Belmont, CA.
[b]Directional insert; Euro-Diagnostica AB, Malmo, Sweden.
[c]Directional insert; Nichols Institute Diagnostics, San Juan Capistrano, CA.

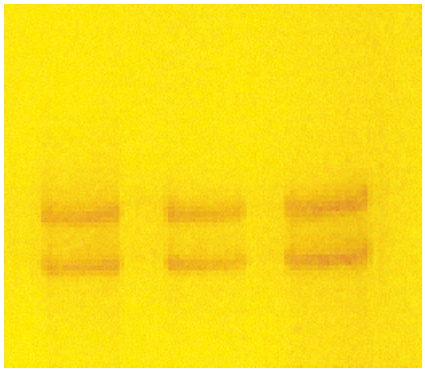

Fig. 2. An example of SSCP analysis of POMC, exon 2 (MSH bands). The two bands detected are not different between controls and patients.

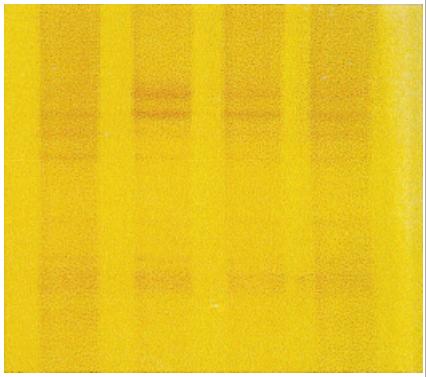

Fig. 3. Heteroduplex (more complex) analysis of the same region in Figure 2 generated by heat denaturation. Even though a more complex pattern emerged, there were no differences between controls and patients.

tial level of other peptides. Collectively, these studies suggest that there are significant differences in subgroups of patients with autism in processing of the POMC molecule and that these differences may confer sensitivity to treatment with opiate blockers and may yield highly specific phenotypes necessary for careful genetic studies.

POMC Gene: Defects Among Adults With Autism?

We present preliminary results below, collected in a small pilot sample, which suggest that the fragment of the gene that codes for melanocyte-stimulating hormone (MSH) fragment of POMC is constant in our patients but that the opioid region of POMC located between nucleotides 7424 and 7843 is highly polymorphic. Results of PCR analysis of the sequence encoding opioid activity revealed that in one patient, a deletion or mutation occurred. This is the first evidence that we are aware of that the opioid fragment of the POMC gene may be defective in any patient. These findings complement the very recent report [Krude et al., 1998] of the first defect related to deletion in the MSH region of the POMC gene defining a new monogenic disorder with early onset. This disorder is characterized by severe early-onset obesity, adrenal insufficiency, and red hair pigmentation. The early onset of these symptoms/characteristics that are related to mutations in the MSH region of the POMC gene suggests that this region of chromosome 2 contains candidate genes for developmental and regulatory function. It is also of interest that mutations in the PC1 gene are associated with obesity and defects or uncoupling of peptide expression [Jackson et al., 1997]. It is reasonable to conclude that POMC is implicated in specific subtypes (phenotypes) among individuals with autism exhibiting SIB.

We tested our hypothesis that deficits in the POMC gene could account for opioid disregulation among individuals with autism. For these pilot data, two young brothers with autism and five adult subjects (with SIB/agitation and with either the diagnosis of autism or autistic features) comprised the sample. Three normal controls were included.

Examination of POMC exon 2

Primers used for examination of exon 2 were those described by Krude et al. [1998], discussed above. The region amplified by these primers extends from nucleotide 4396 to 4753 in the POMC gene sequence (documented in Genbank, emb V0151OHSACTH). The polymerase chain reaction (PCR) product derived using the POMC exon 2 primers electrophoresed as a single band on acrylamide gel (Fig. 2). On these gels, two bands were detected. No differences were noted between samples from individuals with autism and those from controls. Because the size of the PCR product derived using these primers is larger than is optimal for demonstrating sequence differences between allelic DNA strands, we also ran PCR product on gels as described by Ganguly and Prockop [1990] to demonstrate heteroduplexes generated by heat denaturation. Under these conditions, the exon 2 POMC PCR product generated a more complex series of bands, illustrated in Figure 3. The pattern of migration of bands was the same in subjects and controls. Thus, as expected, no difference between subjects and controls was detected in POMC exon 2 region.

Analysis of POMC exon 3

We analyzed the exon 3 sequence using the primers described by Krude et al. [1998]. In this article, two sets of primers were described for exon 3. One set of primers amplified the region between nucleotides 7424 and 7843 of POMC that encode portions of the corticotropin lipoprotein precursor (aa 27–102, melanotropin gamma [aa 77–87]). The amino acids between 87 and 134 have sites for amidation and glycosylation, whereas amino acids 105 to 134 encode a processed peptide. The second set of primers generated PCR products that included a small portion of the corticotrophin ACTH region. Because the product size was considerably larger than is optimal for analysis by single stranded conformational polymorphism (SSCP), the product was analyzed using heteroduplex analysis [Ganguly and Prockop, 1990]. Results of heteroduplex analysis on POMC exon 3 (5′ region) are

illustrated in Figure 4. The third sample from the left is a control sample; the remaining samples are from individuals with autism. There are considerable differences in the heteroduplex patterns observed in the subjects. It is interesting to note that the heteroduplex patterns in the first and second samples from the left are derived from PCR of genomic DNA from two brothers.

Results of these studies led to the conclusion that differences may exist in one of the sequences; however, a different series of primers would need to be designed to examine the 3′ POMC exon 3 region. To initiate analysis of this region, we designed primers to amplify the βE region of POMC.

Analysis of the βE region of POMC

The βE region of POMC comprises 31 amino acids encoded by nucleotides 8089–8181 in the H. sapiens ACTH and beta-LPH precursors (HSACTH, Genbank abbreviation) sequence. Two distinct forms of βE are derived from this sequence. One peptide contains the first 23 amino acids (corresponding to Leboyer's N-terminal) and a second peptide contains amino acids 20–31 (roughly similar to Leboryer's C-terminal). We needed to select primers that amplified the 145-base pair region between nucleotides 8041 and 8186 of the HSACTH sequence. This region encodes 15 amino acids of βLP in addition to 31 amino acids comprising βE.

Results of PCR amplification indicated that there was one subject whose DNA failed to yield a product with these primers (Fig. 5). From right to left, the lanes 1–4 contain PCR reaction using the END (βE) primers, lanes 5–8 contain POMC exon 2 products derived from DNA from the same four individuals, and lanes 9–12 contain PCR products from the same four individuals using END primers (repeat of reactions in lanes 1–4). Molecular weight marker DNA is present in lane 13. Note that there is no PCR product generated from DNA of the individual in lane 4. The presence of DNA in the reaction mix is revealed by the stained high-molecular-weight band present under the wells. When the PCR stringency conditions were lowered (i.e., the hybridization temperature was decreased) and the reaction from the individual in lane 4 was overloaded on the gel, a weak PCR band was seen (Fig. 6). By overloading the PCR sample in the gel lane (note that overloading is evidenced by intense residual DNA close to the wells in lanes 4 and 5, right to left), we demonstrated a very weak band in the subject that coincided with the normal band. In addition, a slightly smaller lower-molecular-weight band occurred. This band was present in all samples, and it shows up more clearly on lanes 4 and 5 because of overloading. On this same gel, we demonstrate that this same DNA sample gave a normal band with the POMC exon 2 primers, lanes 6–9.

One possible interpretation of these findings is that there is a sequence alteration in DNA that causes the primers to bind less efficiently to the DNA. To investigate this possibility, primers will need to be selected outside this region (select primers from a more 5′ region and from a more 3′ region) and the DNA will need to be reamplified, sized, and sequenced. However, these preliminary data represent the first report to our knowledge of a mutation in the opioid region of the POMC gene. Further studies are needed to determine the functional significance of the detected genetic variation of the opioid region of POMC. We view these encouraging findings as a small first step in characterizing a genetic anomaly associated with self-injuring individuals with autism.

CONCLUSION

Our program of research has evolved from pharmacological studies of the opioid system to the search for a gene that may be associated with an autistic phenotype and may determine response to opiate blockers. Initially, our studies were focused on the effects of opiate blockers on the behavior of autistic adults who exhibited self-injury. In general, the results indicated that NTX was an effective treatment for some individuals [Sandman et al., 1998]. However, the effects of NTX on SIB were not simple. We and others [Leboyer et al., 1994, 1999; Bouvard et al., 1995] have found that background POMC fragments, specifically opioid fragments, contributed to the symptoms of autism and to the response to treatment. The intact BE fragment is uncoupled from ACTH during self-injury and the C-terminal BE fragment is significantly increased at rest among individuals with autism. Both of these findings are examples of POMC disregulation and both predicted efficacy of NTX treatment. Uncoupling of POMC fragments suggests an underlying basic, perhaps genetic, mechanism. The findings of a maternal influence on the C-terminal BE fragment among individuals with autism [Leboyer et al., 1999] supported this possibility. Our preliminary findings of a mutation in the opioid region of the POMC gene in an autistic individual were encouragingly consistent with the prospect that a subgroup of patients will be identified who share this genetic defect.■

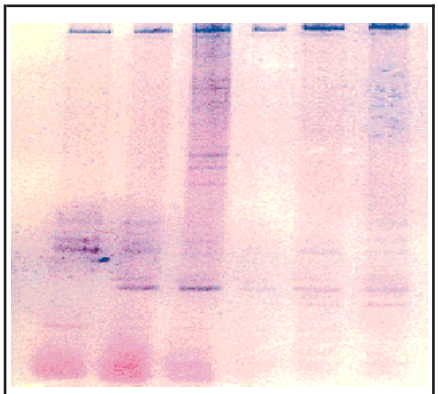

Fig. 4. Heteroduplex analysis of exon 3 produces considerable differences among the subjects. The third band from the left is a control and the others are patients. The first and second samples from the left are from brothers diagnosed with autism.

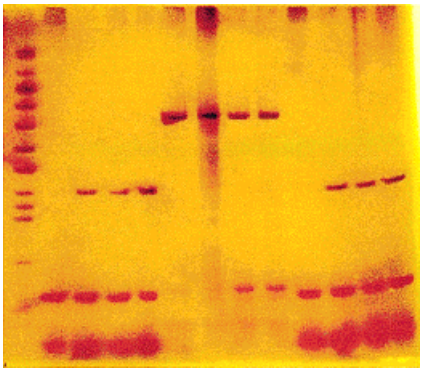

Fig. 5. Polymerase chain reaction (PCR) amplification indicated that one subject (lane 4) failed to yield a product with primers yielding products in the endogenous opioid region. Lanes 1–4 (from the right) are PCR reactions to BE primers, lanes 5–8 are exon 2 products, and 9–12 are repeats of 1–4.

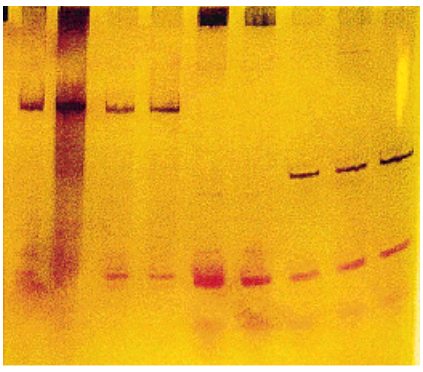

Fig. 6. A weak polymerase chain reaction band is evident in the target subject when the gel is overloaded.

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