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Proopiomelanocortin (POMC) sequencing and developmental delay: Preliminary evidence for a SNP in the 3' UTR region of the *POMC* gene—Possible relevance for biological risk and self-injurious behavior

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Abstract

The proopiomelanocortin (POMC) molecule has been implicated in models of self-injurious behavior (SIB) in neurodevelopmental disorders, but it has never been specifically sequenced in search of base specific polymorphisms. The empirical focus of this preliminary study was to sequence the *POMC* gene in 11 children (mean age = 41.8 months, range = 12–60 months; 73% male) with clinical concerns regarding global developmental delay, 5 with reported self-injury. Genomic DNA was extracted from blood samples, and the *POMC* gene was amplified by specific oligonucleotide primers via polymerase chain reaction. The amplified gene products were sequenced by the University of Minnesota Genomic Center, and the results were analyzed using Sequencher software. A single nucleotide polymorphism (SNP), 1130 C>T, was found in the 3' untranslated region (UTR) of two samples (one of whom had SIB). The program TargetScanHuman was used to predict the function of this mutation. Variant c.1130 C<T was predicted to be located in the target site of two microRNAs (miRNAs; hsa-mir-3715 and hsa-mir-1909), and the variant allele T may result in an increased minimum free energy for the two miRNAs. Further work with much larger samples is needed to continue the investigation of *POMC*'s possible function as a risk factor for the development of SIB in children with developmental delay/disability. The findings presented in this study show that the SNP found in the 3' UTR could alter the binding of miRNAs to *POMC* 3'UTR, thus, increasing *POMC* expression and affecting several biological systems with high relevance to the biology of self-injury. There was a significant difference in β -endorphin levels between SIB ($M = 169.25$ pg/mL) and no SIB ($M = 273.5$ pg/mL, $SD = 15.2$) cases ($p < .01$). Intervention implications are tied to prior observations of individual differences among SIB responders and nonresponders to treatment with the opioid antagonist naltrexone. Stratifying individuals with SIB by *POMC* mutation status may provide a potential tailoring-like variable to guide the selection of who is more (or less) likely

to respond to opiate antagonist treatment. Currently, opioid antagonistic treatment for SIB is empiric (trial and error).

Self-injurious behavior (SIB) is a devastating behavior disorder occurring at relatively high rates (>25%) among children with severe and profound intellectual and developmental disabilities and disorders (I/DD; Rojahn, Schroeder, & Hoch, 2008). The prevalence estimates vary depending, in part, on study methods but also on a number of putative risk factors including the severity of intellectual impairment and comorbid disability. The biological basis for SIB is not well understood, however, and our scientific knowledge of developmental pathways for the disorder is frustratingly limited. Among the various theoretical biological perspectives, the opioid model of SIB has been the most investigated and is complementary, perhaps, to more general theoretical perspectives related to stress/arousal and neuroendocrine mechanisms involving the hypothalamic–pituitary–adrenal axis system, which may be dysregulated among at least some SIB subgroups.

Specifically, the proopiomelanocortin (POMC) molecule has been implicated in SIB (Sandman, Spence, & Smith, 1999). In humans and many other species of vertebrates, POMC gene-derived peptides, synthesized by a series of post-translational proteolytic cleavages of POMC, carry out diverse and vital roles in life. POMC is a precursor to several active peptides, including ACTH (a stress mediator), β -endorphin (a pain/analgesia mediator), and melanotrophin (A, B, and Y-MSH), mainly expressed in both the anterior and intermediate pituitary gland in the hypothalamus, amygdala, and other regions of the central nervous system. One of the several POMC-derived end products, β -endorphin, composed of 31 amino acids, plays an important role in the descending pain inhibitory system in particular.

There are two general versions of the opioid model in SIB and I/DD. One perspective holds that individuals with SIB have an increased pain tolerance because of elevated opioid (i.e., β -endorphin) levels and do not “feel” pain, and therefore there is no natural “brake” on self-injury. The other theory presents the possibility of an addiction-like model of SIB, such that SIB is, in a sense, an addictive behavior maintained by dependence on endogenous opioid release and receptor binding. Within the addiction model, it is assumed that stress or pain associated with SIB induces the release of opioids (Sandman et al., 1999). In both models, the risk for SIB may be increased because of an irregularity in the *POMC* gene, or a different related opioid gene/molecule. There have been no empirical tests of such a risk model, however, with respect to the *POMC* gene and possible mutations.

Research specific to opioids and SIB among individuals with I/DD has generated the following observations. It appears that in some clinical samples, between 30% and 70% of individuals with SIB have deregulated POMC or opioid systems (Sandman et al., 1999). The evidence for this is in the form of response to treatment using opioid antagonists (Sandman et al., 1999) but also in more basic oriented research showing the relationship and processing of the POMC system are “uncoupled” in subgroups of self-injuring individuals resulting in different ratios of ACTH and opioids in the bloodstream, particularly under conditions of stress (normally ACTH and β -endorphin are released in a 1:1 ratio; Sandman, Touchette, Marion, Lenjavi, & Chicz-Demet, 2002). Although no generally effective pharmacological

treatment of SIB is available, subgroups of individuals exhibiting SIB have been found to respond to opioid antagonists (Symons, Thompson, & Rodriguez, 2004). However, there has been little to no work toward biologically stratifying patients prior to a trial to increase the predictive validity evidence to understand/differentiate responders from nonresponders.

To date, there has only been one investigation of the *POMC* gene in an I/DD sample with relevance to SIB. Almost two decades ago, Sandman, Spence, and Smith published a paper in which the *POMC* gene was examined from seven individuals (two participants were young brothers with autism and the other five were adult participants each with SIB and autism or autistic features; 1999). Three additional individuals were included in this study as “normal” controls. At the time of the study, the *POMC* gene was known to have three exons; however, it is now known that *POMC* has four exons (Wang, Gelernter, Kranzler, & Zhang, 2012). The current model for *POMC* includes a small exon, numbered today as two, that has 50 base pairs. Sandman et al. examined exons 1, 3 and 4 (current nomenclature shows that *POMC* includes four exons) and amplified each region using polymerase chain reaction (PCR). The products were run under heat denaturation and resolved on agarose gels. No differences were found in exon 3 between control and index samples. For exon 4, the heteroduplex analysis produced considerable differences between control and index samples. Sandman et al. noted this finding as being potentially caused by a mutation in the exon.

The specific genetic sequences of the samples were not investigated by Sandman et al. The goal, therefore, of this discovery-oriented “proof of concept” study was to conduct specific sequence analysis of all exons, and untranslated region (UTR) sequence of *POMC* in a high-risk sample (children with global developmental delay) with and without SIB. The sample size in this particular investigation precluded modeling risk through association, but the specific purpose here was to sequence the *POMC* gene in a small clinically selected group of children with global developmental delay. Using *POMC* as a theoretically grounded candidate gene, it was anticipated that doing so would further the work that Sandman et al. conducted in the search for a possible genetic basis for risk and the opioid biology relevant to SIB, and in turn provide the rationale for a larger population study, which would allow a more direct test of any specific mutations to further develop and define a biologically informed model of risk for SIB. For exploratory purposes, we also measured any emergent parent-reported child psychopathology and assayed circulating levels of ACTH and β -endorphin to examine in relation to SIB status and *POMC* sequence differences.

Method

Following institutional review board approval and informed consent, a clinical convenience sample was formed. Eleven children (mean age = 41.8 months, range = 12–60 months; 73% male) with clinical concerns regarding global developmental delay were consecutively enrolled and participated in the study. Children were recruited from Gillette Children’s Specialty Healthcare. All children were ambulatory.

To characterize global development, the Child Development Inventory (CDI) total score was used (Ireton, 1992). The CDI is a parent-report measure of development for children between the ages of 18 and 72 months. It contains 270 items yielding an overall General

Development Scale. Ireton and Glascoe (1995) reported acceptable levels of reliability and validity for the CDI in a sample that included young children with developmental delays. The mean total score for the sample was 31 (range = 16–45).

To characterize parent-reported concerns relevant to early developmental psychopathology, the Child Behavior Checklist (CBCL) was used (Achenbach & Rescorla, 2000). The CBCL preschool version (1.5–5) contains 99 specific child behaviors that parents/caregivers rate on a 3-point scale: 0 (*not true of the child*), 1 (*somewhat or sometimes true*), or 2 (*very true or often true*) over the past month. *T* scores between 60 and 63 are considered subclinical, while scores above 63 are considered to be in the clinical range. Achenbach and Rescorla reported extensive psychometric data in support of the CBCL 1.5–5. The mean total CBCL *t* score in this sample was 65 (range = 51–73).

Self-injury was measured via parent-proxy report using the Repetitive Behavior Scale—Revised (RBS-R; Bodfish, Symons, Parker, & Lewis, 2000). The RBS-R was chosen for the study because it is used in clinical settings and is used widely in measuring repetitive behavior in developmental disability and related research (Mirenda et al., 2010). For the purpose of the study, the SIB subscale was used. The SIB subscale consists of eight questions regarding actions that have the potential to cause redness or bruising to the body, and that are repeated in a similar manner (e.g., bangs head on the floor, bites hand, or picks at the skin). The subscale items are rated on a scale of 0 to 3 (0 = *behavior does not occur*, 1 = *behavior occurs and is a mild problem*, 2 = *behavior occurs and is a moderate problem*, and 3 = *behavior occurs and is a severe problem*). Prior research with comparable groups of young children with developmental concerns has yielded scores that appear to be reliable and valid (Lam & Aman, 2007).

Procedures

Briefly, the research protocol was “piggy-backed” onto a developmental pediatric standard of care protocol in which each child was anesthetized for imaging (magnetic resonance) and labs (i.e., blood draw) at a tertiary healthcare center. An anesthesiologist sedated and drew blood samples from each child. As a partial healthy control, blood samples were obtained from two adults with no developmental or intellectual disability. After blood was drawn (avg. volume of 10 ml, range 5–10 ml), the samples were centrifuged and the white blood cell layer, or buffy coat, was extracted.

Each child’s genomic DNA (gDNA) was extracted from his or her white blood cells using a Blood/Tissue Genomic DNA extraction kit (Qiagen, Inc.), following manufacturer standard protocol. Standard PCR amplification and sequencing protocols were used to amplify and sequence the *POMC* gene in each of the 11 study samples as well as in both controls. Oligonucleotide primers were designed using Primer3 software by aligning *POMC* sequences from human (GenBank accession No. NG_008997.1; see Table 2) Specific primer details are available from the first author. Reaction components for each 12-μL reaction included 10 × PCR buffer, 200 mmol/L dNTP mix, gene-specific primer pair (1.2 μmol/L each primer), template (50 ng genomic DNA), 1.5 units HotStar Taq polymerase (QIAGEN, Inc.), and dH₂O to final volume. Cycling conditions were 95 °C for 15 min, then 35 cycles of the following: 95 °C for 30 s, 58 °C for 30 s, and 72 °C variable extension time, with a 72 °C

10-min final extension. Amplified products were resolved on agarose gels and purified for sequencing with ExoSap-IT (Affymetrix) according to the manufacturer's protocol. Templates were sequenced in both directions with the original primers on an ABI automated DNA sequencer at the Advanced Genetic Analysis Center, University of Minnesota. DNA sequences were groomed and edited with Sequencher software (Gene Codes Corp.). Contigs were assembled using the human *POMC* sequence (GenBank accession No. NG_008997.1) as a scaffold. The team focused on *POMC* exonic data and both UTR regions. Intronic data between exons was trimmed before sequence analysis occurred.

The *POMC* transcript includes 4 exons, with exons 1 and 2 being noncoding. The complete coding sequence of *POMC* in human (804, bp) begins in exon 3 and ends in exon 4. The team was unable to attain cDNA, so it was necessary to utilize gDNA for the sequencing analysis. To predict the function of the identified variant in the 3' UTR region of *POMC*, microRNAs (miRNAs) putatively bound to the sequence containing 3' UTR variants were identified by the program TargetScanHuman (Wang, et al., 2012). The minimum free energy for hybridization of miRNAs to target mRNA sequences was predicted using the program RNAhybrid (Wang et al., 2012).

Samples extraction protocol for peptide assays.—Samples were extracted 1:2.5 in acetonitrile, vortexed 5 s, and incubated at room temperature for 10 min. After incubation, the samples were centrifuged at 2400 RPM's for 5 mi. The supernatant was removed and dried by using a Speed Vacuum at the highest vacuum setting. The dried samples were reconstituted and assayed immediately.

Peptide assay methods—Luminex.—Samples were assayed according to the manufacturer's instructions. Fluorescent color-coded beads precoated with antihuman (β -endorphin) capture antibody were added. After incubation, and washing, biotinylated antihuman detection antibody was added followed by phycoerythrin-conjugated streptavidin. The beads were read on a Luminex instrument (Bioplex 200), which is a dual-laser fluidics-based instrument. One laser determines the analyte being detected via the color coding; the other measures the magnitude of the PE signal from the detection antibody, which is proportional to the amount of analyte bound to the bead. Samples were run in duplicate, and values were interpolated from 5-parameter fitted standard curves generated on each 96-well plate.

Results

Sequence analysis

Sequence analysis for exons 1, 2, and 3 yielded no sequence variation among all samples, both control and study members, with respect to the *POMC* reference sequence. The coding portion of exon 4 also yielded no genetic variations for all samples. One single nucleotide polymorphism (SNP) was found 63 bp after the stop codon for exon 4. This SNP, located in the 3' UTR, 1130 C>T, was found and of the 11 samples, 7 showed the same base, C, as the reference sequence at this position. Two of the 11 samples were heterozygous for this SNP, and the final 2 samples showed a different base, T, than the reference sequence (Table 1). One of the adult control samples had the same base at this position as did the reference

sequence, but the other control sample was heterozygous at this location. Variant c.1130 C<T was predicted to be located in the target site of two miRNAs (hsa-mir-3715 and hsa-mir-1909), and the variant allele T may result in an increased MFE for these two miRNAs.

Self-injurious and developmental psychopathology relevant behavior ratings.

—Overall, 5 children were reported by parents to exhibit SIB (RBS-R was not completed for 1 case). When SIB was considered by *POMC* genotype: 1 of the 2 homozygous T cases was reported to exhibit SIB; neither of the two heterozygous C/T cases were reported to exhibit SIB, and 4 of the six homozygous C cases were reported to exhibit SIB. The mean CBCL externalizing subscale scores were 62.1 (48–88) for the C genotype, 57 (56–58) for T genotype, and 55.5 (47–64) for C/T genotype cases. With such small sample sizes, behavioral data were reported for descriptive purposes only.

Genotype, SIB, and POMC circulating protein products.—For descriptive purposes, we also investigated two of the major protein products in blood from the POMC molecule with relevance to SIB (ACTH, beta-endorphin [β -endorphin]). There were no apparent differences (see Table 1) among the three *POMC* sequence genotypes for either ACTH or β -endorphin (one case [a C/C] was omitted because of an outlier ACTH value (>3 *SD*). We also explored blood peptide levels by SIB status (we use the term “explored” rather than “tested” because the small sample size precludes robust inferential modeling). There was a significant difference in β -endorphin levels between SIB ($M = 169.25$ pg/mL, $SD = 50.1$) and no SIB ($M = 273.5$ pg/mL, $SD = 15.2$) cases ($t = -3.42$, $p < .01$; Cohen’s $d = 2.82$). Given that ACTH and opioids in the bloodstream are typically released in a 1:1 ratio; Sandman et al., 2002), we also checked the correlations among the peptides between the SIB and no SIB groups. ACTH and β -endorphin were negatively correlated ($r = -.44$) in the SIB cases and positively correlated in the no SIB cases ($r = .49$).

Discussion

Mutations occurring in the 3' and 5' UTRs of a gene can have detrimental effects on a protein. It is known that genetic mutations in the untranslated regions of genes can potentially yield significant damage for an individual. MicroRNAs are small noncoding RNAs that repress protein synthesis by binding to target messenger RNAs (Long et al., 2007). A mutation affecting miRNAs can alter the minimum free energy of hybridization (Wang et al., 2012). One single alteration can be responsible for the altered expression of many genes. UTR mutations can also cause down modulation of translation efficiency (Signori et al., 2001). Transcriptionally, a mutation may affect only the allele and genes that are physically linked (Chatterjee & Pal, 1999). However, since 3'-UTR binding proteins also function in the processing and nuclear export of mRNA, a mutation can also affect other unrelated genes (Chatterjee & Pal, 2009). There is also the possibility that a mutation in these regions yields no quantifiable or noticeable change.

When considering further Sandman’s investigation of *POMC* in a developmental disability sample, postulations arose about the possibility of finding some genetic variations in our samples, specifically in exon 4. However, no variations in the coding region of exon 4 were found. The observation of no *POMC* coding region mutations could potentially be explained

by sample differences between this sample and Sandman's. The current sample was composed of very young children with global developmental delay with early emergent SIB in some but not all cases. Sandman's work was with adults with significant intellectual impairments and associated developmental disorders with long histories of severe self-injury. It seems entirely plausible that various levels of impairment and risk for SIB could have different underlying genetic components. This possibility has been noted by research groups investigating preclinical models of SIB. Chen et al. (2010) found that rhesus monkeys with different forms of SIB (biters vs. scratchers, etc.) had different polymorphisms in their *TPH2* gene. Consistent polymorphisms were observed among their samples for different SIB forms/topographies. In the current sample, 1 of 2 of the homozygous T genotypes were reported to exhibit SIB, none of the heterozygous C/T cases were reported to exhibit SIB, and 4 out of 6 homozygous C genotypes were reported to exhibit SIB.

The variant (c.1130 C<T) in the 3' UTR was predicted to be located in the binding site of two miRNAs (hsa-mir-3715 and hsa-mir-1909). The variant allele T potentially increases the minimum free energy for hybridization of these two miRNAs (hsa-mir-3715: from 225.0 kcal/mol to 222.1 kcal/mol; hsa-mir-1909: from 226.7 kcal/mol to 224.9 kcal/mol) to the target sequence in *POMC* 3'UTR, thus reducing the binding of miRNAs to *POMC* 3'UTR and increasing *POMC* expression (Wang et al., 2012). In other areas of *POMC* investigation (e.g., substance abuse and obesity), various mutations in both the coding region and UTR regions of *POMC* have been documented (Wang et al., 2012). The same 3'UTR variant 1130 C<T SNP was found in the current study. It is worth noting the possibility of *POMC* mutations associated with substance dependence as that would be highly relevant to one of the SIB opioid models.

From a different line of work, Hosoi, Ozawa, and Granstein (1999) examined skin, more specifically, Langerhan cells (LC), and the effects of the production of POMC in skin and regulation of melanocytes and LC by its product, especially α MSH. Their work demonstrated that β -endorphin, either from central or local sources, modulates skin immune function. They found that LC have specific receptors for β -endorphin on their surface. The binding potentials shown to exist with a *POMC* 1130 C<T SNP has the potential to affect how β -endorphin binds to LC cells and thus impact the function of LC cells. The relevance to SIB and I/DD is worth noting. There is work with I/DD samples showing differences in peripheral innervation (epidermal nerve fiber densities) and related cutaneous/skin differences in subgroups of individuals with self-injury and corresponding abnormal sensory/nociceptive behavioral response properties (Symons, 2011). Whether this could relate to *POMC* effects and LC cells is unknown but worth investigating from a "sensory risk marker" perspective.

Specific to nociception, Seo et al. (2008) investigated changes to the *POMC* gene and β -endorphin in the hypothalamus using various mouse pain models. They explored the molecular mechanism involved in hypothalamic *POMC* gene expression induced by noxious stimulation. They found the *POMC* mRNA expression was significantly higher during periods of noxious stimulation and concluded that nociceptive stimulation may differentially affect the regulation of the hypothalamic *POMC* mRNA, in which the inflammatory

nociceptive stimuli may play an important role in the expression of *POMC*. These findings lead to the intriguing possibility that the 1130 C<T mutation that is predicted to effect miRNA binding could have an impact in how an individual with this mutation processes pain, determined, in part, by *POMC* mRNA binding function. The foregoing was speculative, but there is relevance here to prior work in self-injury and individuals with I/DD such that reports documenting hypothalamic–pituitary–adrenal axis differences in subgroups of self-injury (Symons, 2011; Symons, Wolff, Stone, Lim, & Bodfish, 2011) as well as nociceptive/pain expression differences between individuals with comparable levels of I/DD with and without SIB with the SIB subgroup documented to be more reactive/expressive in relation to nociceptive relevant stimuli (Symons Shinde, Clary, Harper, & Bodfish, 2010).

Further work is clearly needed to continue the investigation and clarify *POMC*'s possible function as a risk factor for SIB in children with developmental delays at risk for intellectual disability. It is important to note that a much larger sample would be needed to say anything definitive with regard to genotype–behavior phenotype associations. The purpose of this work was as a preliminary investigation to specifically sequence the *POMC* gene in a high-risk sample. Obviously there is not a direct link between a *POMC* SNP and SIB. What may be likely, though, is a *POMC* “dosage” effect. The findings presented in this study show that the SNP found in the 3' UTR could alter the binding of miRNAs to *POMC* 3'UTR, thus, increasing *POMC* expression. Other lines of inquiry have found potentially significant clinical results relevant to addiction, skin function, and nociceptive processing indicating *POMC* variations could lead to complications for problems in each of these areas. There is no reason to think that *POMC* mutations among children with neurodevelopmental disorders would not have similar effects that interfere with some normative aspect of nociception, peripheral immune function, or central reward circuitry/processing that collectively could increase risk for the development of self-injury. The observation, albeit preliminary, of different amounts of circulating β -endorphin (no SIB >SIB) and different directions in the correlation between ACTH and β -endorphin between the SIB (negative) and no SIB (positive) groups may be an additional marker for dysregulated *POMC* regulation. In addition to risk, there are intervention implications tied to prior observations of individual differences among SIB responders and nonresponders to treatment with the opioid antagonist naltrexone. Currently, opioid antagonistic treatment for SIB is empiric (trial and error). Stratifying individuals with SIB $\times$ *POMC* Mutation status may provide a potential tailoring-like variable to guide the selection of who is more (or less) likely to respond to opiate antagonist treatment.

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Table 1.

SNP distribution across groups and circulating POMC-product peptide levels

SNP (c.1130C<T)	Study group	Control group	Total count	SIB case ^a	ACTH ^b (pg/mL)	β-END(pg/mL)
C/C	7/11	1/2	8/13	4/6	<i>M</i> = 33.5 (<i>SD</i> = 34.4)	<i>M</i> = 188.7 (<i>SD</i> = 58.1)
C/T	2/11	1/2	3/13	0/2	<i>M</i> = 40.4 (12.9)	<i>M</i> = 249.6 (47.2)
T/T	2/11	0/0	2/13	1/2	<i>M</i> = 35.2 (8.6)	<i>M</i> = 191.6 (91.0)

Note: SNP, single nucleotide polymorphism.

^a *N* = 10, RBS-R was not completed for one of the C/C study cases; ACTH LLOQ = 3.0 pg/ml; β-END LLOQ = 20.0 pg/mL

^b ACTH, a C/C ACTH outlier case (>3 *SD*) was dropped from mean/*SD* calculations.

Table 2.

Primers for sequencing four POMC exons

		Forward primers (5'–3')	Reverse primers (5'–3')
Polymerase chain reaction primers	Exon 1	GGCAGGACAGTGCTAATTCC	GCGCAGAAAGTTTGTCCGAG
	Exon 2	CCCACCTGGTTAAGGAAAT	CGTCACGTGTCAGATGTCTTG
	Exon 3	TGCTGCTGTCCTTAAAAATGC	GAATTTCCCTTTCCCCACAC
	Exon 4 (1/2)	GAGTGCCAAAGAAAGCAGGTG	CATGGAGTAGGAGCGCTTG
	Exon 4 (2/2)	GTCTTCCCCCAGGAGTGC	GAAGCACATTTTCCCTTTCACG
Sequencing primers	Exon 1	GGCAGGACAGTGCTAATTCC	GCGCAGAAAGTTTGTCCGAG
	Exon 2	CCCACCTGGTTAAGGAAAT	CGTCACGTGTCAGATGTCTTG
	Exon 3	TGCTGCTGTCCTTAAAAATGC	GAATTTCCCTTTCCCCACAC
	Exon 4–1	GAGTGCCAAAGAAAGCAGGTG	CATGGAGTAGGAGCGCTTG
	Exon 4–2	GTCTTCCCCCAGGAGTGC	GAAGCACATTTTCCCTTTCACG

Note: Primers were designed according to genomic sequence from the Ensemble database (*POMC*, ENST00000380794).