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The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation

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Abstract

Mice with a targeted mutation in proSAAS have been generated to investigate whether peptides derived from this precursor could function as an inhibitor of prohormone convertase 1/3 (PC1/3) *in vivo* as well as to determine any alternate roles for proSAAS in nervous and endocrine tissues. Fetal mice lacking proSAAS exhibit complete, adult-like processing of prodynorphin in the prenatal brain instead of the incomplete processing seen in the brains of wild-type fetal mice where inhibitory proSAAS intermediates are transiently accumulated. This study provides evidence that proSAAS is directly involved in the prenatal regulation of neuropeptide processing *in vivo*. However, adult mice lacking proSAAS have normal levels of all peptides detected using a peptidomics approach, suggesting that PC1/3 activity is not affected by the absence of proSAAS in adult mice. ProSAAS knockout mice exhibit decreased locomotion and a male-specific 10-15% decrease in body weight, but maintain normal fasting blood glucose levels and are able to efficiently clear glucose from the blood in response to a glucose challenge. This work suggests that proSAAS-derived peptides can inhibit PC1/3 in embryonic brain, but in the adult brain proSAAS peptides may function as neuropeptides that regulate body weight or potentially other behaviors.

Keywords

proprotein; proSAAS; prohormone convertase 1/3; neuropeptide; body weight; hypothalamus

Introduction

Neuroendocrine hormones are frequently synthesized as inactive precursor proteins that require post-translational processing in order to be fully activated. Neuroendocrine proteins usually require limited endoproteolysis at multibasic consensus sequences during transit through the regulated secretory pathway. In nervous and endocrine tissues these initial cleavage events are typically carried out by prohormone convertases 1/3 and/or 2 (PCs) (Seidah & Chretien 1999). Endoproteolytic cleavage of precursor proteins frequently yields a peptide intermediate possessing C-terminal basic residues that must be further processed to

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confer full biologic activity. C-terminal basic residues are removed by members of the carboxypeptidase (CP) family such as CPE, which is present and active in dense core secretory granules (Fricker *et al.* 1996, Fricker & Leiter 1999). The peptide product generated by these sequential cleavage events may require additional posttranslational modifications such as amidation, sulfation, and/or phosphorylation for full activation (Bennet 1991, Czyzyk *et al.* 2005).

Protease activity is often regulated by endogenous inhibitors that prevent premature activation of the enzyme (Dubin 2005). The enzymatic activity of PC2 is regulated within the secretory pathway by the neuroendocrine protein 7B2, an endogenous chaperone and inhibitor of PC2. The C-terminal region of 7B2 is an extremely potent inhibitor of PC2 activity while the N-terminal region is required for PC2 maturation *in vivo* (Martens *et al.* 1994, Westphal *et al.* 1999). A potential endogenous inhibitor of PC1/3, named proSAAS, was identified in a screen of C-terminally extended peptide intermediates that accumulated in the brains of Cpe^{fat}/Cpe^{fat} mice (Fricker *et al.* 2000, Che *et al.* 2001). ProSAAS inhibits PC1/3 activity *in vitro* with an IC50 in the nM range, and over-expression of proSAAS in AtT-20 cells reduces the processing of pro-opio-melanocortin (POMC) (Fricker et al. 2000) at PC1/3-specific cleavage sites. An inhibitory hexapeptide, LLRVKR, was initially identified in a combinatorial peptide library screen for prospective PC1/3 inhibitors (Apletalina *et al.* 1998). The inhibitory region of proSAAS was subsequently localized to a processing intermediate peptide named PEN containing the hexapeptide sequence located on its C-terminus (Qian *et al.* 2000, Cameron *et al.* 2000, Basak *et al.* 2001).

During pre-natal and early post-natal development, a significant amount of unprocessed and/or incompletely processed high molecular proSAAS is detected (Morgan *et al.* 2005). The processing of proSAAS does not fully mature until adulthood at which point proSAAS is efficiently cleaved to produce fully processed peptide forms (Mzhavia *et al.* 2001), including big and little forms of PEN (proSAAS 221-242), LEN (proSAAS 245-260), GAV and SAAS (proSAAS 34-59) (Mzhavia et al. 2001, Sayah *et al.* 2001), in adult neurons and endocrine cells. Previous work showed that PEN-LEN (proSAAS 221-260), a C-terminal fragment of proSAAS with PC1/3 inhibitory potency is accumulated in e15.5 whole brain extracts (Morgan et al. 2005). Interestingly, this PEN-LEN accumulation occurs at a time during brain development when prodynorphin processing by PC1/3 does not occur. In contrast, in the adult brain, PEN-LEN is undetectable and prodynorphin is processed by PC1/3. One possible interpretation of these results is that proSAAS peptides inhibit PC1/3 activity in the embryonic brain when sufficient levels of such peptides are present.

Several noteworthy findings have suggested that proSAAS may have alternate functions in the nervous system that are independent of its potential role in the regulation of PC1/3 activity. First, while proSAAS and PC1/3 are both broadly expressed in neuroendocrine tissues, the relative mRNA expression level of proSAAS does not parallel that of PC1/3. Furthermore, proSAAS mRNA and protein are expressed in some neuroendocrine cells that lack PC1/3 expression (Lanoue & Day 2001, Feng *et al.* 2002, Feng *et al.* 2001, Morgan et al. 2005). Second, the region of proSAAS that inhibits PC1/3 is a small sequence present only in full-length proSAAS and transient processing intermediates; the mature form of PEN and the numerous other peptide products of proSAAS are not PC1/3 inhibitors suggesting that normal processing of proSAAS is sufficient to abolish its inhibitory effect on PC1/3 (Qian et al. 2000, Cameron et al. 2000). Third, these proSAAS-derived peptides are secreted from neuroendocrine cells via the regulated secretory pathway, raising the possibility that proSAAS peptides might serve as signaling molecules *in vivo* (Fricker et al. 2000, Hatcher *et al.* 2008). Fourth, transgenic mice overexpressing proSAAS show an increase in body weight but peptide processing by PC1/3 appears to be normal in these mice (Wei *et al.* 2004). Finally, one of the proSAAS-derived peptides, little SAAS, induces a phase delay in

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the circadian rhythm of the firing rate of suprachiasmatic nucleus neurons (Hatcher et al. 2008). Therefore, in the present study mice lacking proSAAS were produced to explore the possible role for proSAAS in the regulation of pre and early post-natal proprotein processing and also to investigate whether proSAAS-derived peptides perform additional roles independent of PC1/3.

Methods

Gene Targeting

All studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey. A targeting vector for disrupting proSAAS was constructed by subcloning two genomic fragments of proSAAS into a pKO Scrambler V919 targeting vector. A 2.5 kb XbaI/SacII fragment located 5′ of exon 1 was inserted into Xba I/Sac II sites in the targeting vector. A neomycin resistance cassette (NEO) and Herpes Simplex Virus thymidine kinase (HSV-TK) cassette were subcloned into Asc I (NEO) and Rsr II (HSV-TK) sites in the KO vector. A 5.5 kb Xba I genomic fragment downstream of exon 1 was subcloned into pBluescript, excised with Not I and Kpn I, and then subcloned into Bgl II (blunt) and Kpn I sites within the KO vector. Targeting vector (20 μg) was linearized and electroporated into AB2.2 ES cells (Lexicon Genetics, Woodlands, TX). Embryonic stem (ES) cells and mouse genotypes were determined by Southern blotting using a probe located outside (5′) of the genomic sequence used to construct the targeting vector.

Peptide Extraction for Radioimmunoassays

Brain and pituitary were homogenized and peptides extracted in buffer containing 10% acetic acid, 0.5 mg/ml BSA, and 0.3 mg/ml PMSF. Size exclusion chromatography was performed on a Superdex Peptide HR 10/30 gel-filtration column (Amersham Pharmacia, Piscataway, NJ) with 30% acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractions were collected and radioimmunoassays (RIAs) for PEN (mouse proSAAS 221-242; rabbit #141), ACTH (1-39) (Bachem, San Carlos, CA) and leucine enkephalin (Leu-Enk) were performed as previously described (Mzhavia et al. 2001, Morgan et al. 2005, Berman *et al.* 2001). RIAs for Leu-Enk were performed using a commercially available RIA kit (Bachem, San Carlos, CA). For the detection of larger Leu-Enk-containing peptides, fractions were lyophilized and re-suspended in 100 mM sodium phosphate buffer pH 7.5 and digested with 0.1 mg/ml trypsin for three hours at 37°C followed by digestion with 10ng/μl CPB at 37°C for one hour to release the Leu-Enk epitope from peptide intermediates. Proteolyzed samples were boiled for 20 minutes to inactivate all proteolytic activity and the RIA for Leu-Enk was performed as above.

Western Blotting

Brain proteins were extracted in 50 mM Tris-Cl, pH 7.4, 1% Triton X-100, and 10% glycerol supplemented with mammalian protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). PC1/3/PC3 antiserum directed against the N-terminal region of the enzyme corresponding to amino acids 84-100 was used at a dilution of 1:1,500 (Qian et al. 2000). For the detection of PC2, anti-PC2 polyclonal antiserum [directed against the C-terminal amino acid sequence (592-608) of the mouse enzyme] was used at a dilution of 1:1,000. The blots were normalized using tubulin as a marker of total protein detected with monoclonal anti-tubulin antiserum (Sigma, St. Louis, MO) at a dilution of 1:2,000. The blots were visualized using SuperSignal ELISA Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and OMAT AR film (Kodak, Rochester, NY).

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Measurement of Blood Glucose and Hormone Levels

Fasting blood glucose levels were determined in male mice between 4-6 months of age that were also used for body weight measurements (see below). Blood was sampled from the tail vein and glucose levels were measured using Accuchek Advantage Comfort Curve blood glucose strips and an Accuchek blood glucose meter (Roche, Indianapolis, IN). Glucose tolerance was examined by administering a 2mg/g of body weight i.p. D-glucose injection (Sigma, St. Louis, MO). Blood glucose levels were measured at 30, 60, and 120 minutes post-injection. Data for each time point was analyzed using an unpaired Student's t-test and error bars represent the SEM.

Body Weight Measurements

Body weight measurements were obtained from group housed male and female mice that were kept on 12:12 light dark cycle and were subjected to a standard diet that was made continually available (ad libitum). Measurements were taken in the afternoon every seven days for 20 weeks. Data for each time point was analyzed using an unpaired Student's t-test and error bars represent the SEM.

Locomotor Activity Test

Locomotor activity was obtained using an Accuscan locomotor activity boxes equipped with a Dell personal computer for data collection (Columbus, OH). Prior to testing, male mice were habituated to the procedure room for one hour. After habituation each mouse was placed in a novel open field chamber and locomotor activity data was collected for 60 minutes. Statistical analysis of open-field activity occurring during the initial 30 minutes of monitoring was performed using an unpaired student's t-test. Error bars represent the SEM.

Results

ES cells were transfected with a targeting vector designed to delete a portion of exon 1 from the proSAAS gene locus containing the transcription and translational start sites (Fig. 1A). Targeted ES cell clones were identified by Southern blotting using a ^{32}P radiolabeled 5′ external genomic DNA probe and were used for blastocyst microinjection. Injection of homozygous mutant ES cells produced a germline-transmitting proSAAS chimeric male that were used to establish the proSAAS colony. The X-linkage of proSAAS required that female mice carrying the mutant allele transmit this allele to male offspring. Interbreeding of wild type males with heterozygous proSAAS females produced the expected equal frequency of male mutants (N=38/83; 46%) and heterozygous females (N=29/68; 44%). Female proSAAS homozygous mice were also viable and fertile. The absence of proSAAS transcript was confirmed by Northern blot in whole brains from homozygous mutants (data not shown). To confirm that proSAAS mutant animals completely lacked proSAAS expression, adult whole brains were assayed for the proSAAS-derived peptide, PEN. PEN was not detected in the brains of either mutant male or female mice (Fig. 2a). Additionally, PEN levels were reduced by ~25% in female heterozygote brains relative to amount detected in wild-type female brains (although this difference was just outside the criteria for statistical significance (p=0.056), and over 50% in pituitary from adult female mice (Fig. 2b; P=0.022). Finally, a peptidomics approach was used to compare levels of brain neuropeptides in proSAAS knock out and wild-type mice. Seven proSAAS derived peptides were detected in wild type mouse hypothalami but none of these were detectable in the mutant mice (Table S1).

Previous work suggested that proSAAS might play a role regulating PC1/3 processing in a manner similar to the role of 7B2 in the regulation of PC2 (Fricker et al. 2000, Cameron et al. 2000). Since 7B2 is a chaperone for PC2 as well as an inhibitor of this enzyme, the loss

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of 7B2 causes a sharp reduction in PC2 levels. Western blot analysis of whole brain was performed to determine whether proSAAS deletion could cause a similar reduction in either PC1/3 or PC2 levels. Western blots using antisera selective for both PC1/3 and PC2 show that proSAAS deletion did not change the levels of either PC1/3 or PC2 protein in either brain (Fig. 3A, B) or pituitary (data not shown). Western blot images of PC1/3, PC2, and tubulin were subjected to densitometry analysis. The amounts of inactive (87 kDa) and active PC1/3 (67kDa) protein brain were normalized to the amount of tubulin. No differences in relative PC1/3 and PC2 levels were detected in the pituitaries (data not shown) and brains of adult proSAAS mutant and wild-type mice (Fig. 3).

Although we found no changes in PC1/3 protein levels, there could potentially still be functional alterations in PC1/3 activity. If PC1/3 activity were affected by proSAAS disruption, such an alteration would be expected to be reflected by altered levels of many hypothalamic peptides. To investigate this possibility in adult proSAAS KO mice, a quantitative peptidomics screen was used to compare levels of hypothalamic peptides in extracts of proSAAS mutant mice relative to wild-type mice. This analysis involved stable isotopic tags and mass spectrometry that allowed the precise molecular form of each peptide to be determined. Altogether, 40 peptides from secretory pathway proteins were identified in wild type hypothalamic extracts (Table S1); in addition to these 40 peptides, another 30 peptides were identified which were derived from cytosolic, nuclear, or mitochondrial proteins, and more peptides were detected that could not be identified by MS/MS sequence analysis (data not shown). As expected, none of the cytosolic, nuclear, and mitochondrial protein fragments showed major changes between wild type and proSAAS mutant mice (data not shown). Seven of the secretory pathway peptides detected in the wild type mice were proSAAS-derived peptides and none of these were detected in the proSAAS mutant hypothalamic extracts. The other 33 secretory pathway peptides represent 16 additional secretory pathway precursor proteins: cerebellin 1 precursor protein, chromogranin A, chromogranin B, prodynorphin, proenkephalin, prohormone convertase 2, promelanin concentrating hormone, proneurotensin, proopiomelanocortin, propeptidyl-amidating-monooxygenase, protachykinin A, protachykinin B, prothyrotropin releasing hormone, provasopressin, secretogranin II, secretogranin III. The average ratio of these 33 peptides in the proSAAS mutant versus wild type mice was very close to 1.00, indicating that they are not increased or decreased in the proSAAS mutant mice. Taken together, our peptidomics analysis strongly suggests that PC1/3 activity is normal in the hypothalami of adult proSAAS mutant mice.

Previous studies have also shown that 7B2 deletion causes hypersecretion of ACTH from the pituitary resulting in a sharp increase in the level of circulating corticosterone (Westphal et al. 1999, Laurent *et al.* 2002). This finding suggested that 7B2 could be a component of the secretory apparatus with the ability to tonically inhibit secretion. RIAs for ACTH and corticosterone were performed to investigate whether proSAAS deletion could alter the circulating levels of these peptides. The plasma levels of corticosterone and ACTH in proSAAS mutants were indistinguishable from the amount of these peptides detected in wild type littermates (Figure S1). This finding suggests that unlike 7B2, proSAAS does not appear to be involved in modulating ACTH secretion from pituitary corticotrophs, nor is it involved in its processing.

In addition to its activity in the hypothalamus, PC1/3 is needed to produce several pituitary peptides including ACTH. Extracts were fractionated on gel filtration columns and analyzed by RIA to investigate if the amount and molecular weight of ACTH, a POMC derived peptide produced by PC1/3, was similar in mutant and wild type pituitaries. Since the ACTH antibody used for RIAs is directed to the C-terminus of ACTH, this antibody also recognizes CLIP, a C-terminal cleavage product of ACTH. In assays of whole pituitary, the peak

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detected in fractions 21-26 corresponds to a combination of ACTH and CLIP (Fig. 4A) and suggests that processing of POMC to ACTH and CLIP occurs normally in the mutant pituitary. Dissection of the pituitary made it possible to examine production of ACTH and CLIP in the anterior and neurointermediate lobes of the pituitary, respectively. Production of ACTH in anterior pituitary was normal in proSAAS mutants relative to wild type littermates (Fig. 4B). Similarly, the amount of CLIP in the neurointermediate lobe of proSAAS mutants was similar to the level detected in wild type littermates (Fig 4C). The immunoreactive forms of ACTH and CLIP in the extracts of proSAAS mutant whole, anterior, and neurointermediate pituitaries had the same molecular weights as the forms of these peptides detected in pituitary tissues from wild type animals. This suggests that PC1/3 and PC2 processing of POMC into ACTH and CLIP is not affected by the absence of proSAAS which supports the findings of the peptidomic experiments showing normal levels of processed neuropeptides in mutant brains.

We previously determined that the processing of prodynorphin by PC1/3 is altered in embryonic brain tissues where the C-terminal part of proSAAS (PEN-LEN) that inhibits PC1/3 is accumulated (Morgan et al. 2005). In contrast to the developing brain, this inhibitory fragment of proSAAS is undetectable in adult mouse brain (Morgan et al. 2005, Mzhavia *et al*. 2002). These findings raised the possibility that proSAAS-mediated inhibition of PC1/3 might be temporally restricted to the developing tissues where accumulated PEN-LEN can be detected. This possibility was explored by examining prodynorphin processing in proSAAS mutant embryos. In e15.5 proSAAS mutant brain we detect accumulation of the high molecular weight prodynorphin intermediate (~8-10 kDa), corresponding to the 8 and/or 10 kDa intermediates produced by PC1/3 (Fig. 5). This finding is in contrast with e15.5 wild-type brains that express the inhibitory fragment of proSAAS. In the wild-type embryonic brain the high molecular weight prodynorphin intermediates are undetectable, likely due to the expression of the inhibitory forms of proSAAS (Fig. 5).

Finally, we have begun a global phenotypic analysis of proSAAS KO mice. Body weight measurements were taken weekly starting at four weeks of age and continued until mice were 20 weeks old (Fig 6A). Male proSAAS mutants have reduced body weights (~10-15%) relative to wild type littermates, which was first detected just after weaning with differences between genotypes being statistically significant at 7, 10-13, and 15-20 weeks of age ($p<0.05$). In contrast, no evidence for reduced body weight was observed in female proSAAS mutants. The body length of male proSAAS null mice is normal suggesting there is likely not a gross growth deficit (data not shown). Blood glucose homeostasis was examined by measuring fasting glucose levels as well as glucose levels in response to a 2 mg/g of body weight injection of D-glucose. Fasting blood glucose in male mutants was identical to the level measured in wild-type males (Fig. 6B). Administration of glucose (2 mg/g) causes a rapid increase in blood glucose, peaking around 30 minutes post-injection, and declining to pre-injection levels by 120-180 minutes following glucose administration. We find that glucose clearance in proSAAS mutant males was normal with mutant males having wild type levels of glucose at 30, 60 and 120 minutes following glucose injection (Fig. 6B).

Open-field locomotor activity was measured for 30 minutes in experimentally naive proSAAS mutant and wild type male mice. ProSAAS KO males exhibit decreased horizontal activity (Fig. 7A), number of total movements (Fig. 7B), number of rearing movements (Fig. 7C), and number of stereotypic movements (Fig. 7D). Cumulatively, these measurements suggest that proSAAS mutants are slightly less active than wild type littermates. Decreased activity in the context of a novel open-field raises the possibility that proSAAS mice might possess a proclivity towards anxiety-like behaviors.

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Discussion

Previous studies found that the gene expression of proSAAS is widespread during development relative to the more restricted pattern of PC1/3 transcript (Morgan et al. 2005). Similar observations have been made in nervous and endocrine tissues from adult mice where proSAAS was found to co-localize with PC1/3 in some but not all cells (Lanoue & Day 2001). These findings demonstrate that proSAAS and/or its derived peptides likely have roles unrelated to PC1/3 regulation. To address this possibility *in vivo*, mice lacking proSAAS have been produced. These knock-out mice lack detectable levels of proSAAS mRNA as wells as all proSAAS-derived peptides. ProSAAS knock-out mice are viable, fertile, and exhibit no major gross morphological abnormalities indicating that proSAAS is not an absolute requirement for normal development and survival.

We have provided several lines of *in vivo* evidence demonstrating that proSAAS is not involved in regulating PC1/3 maturation or activity. First, we found that the amount of PC1/3 protein in the brains of proSAAS mutant mice is normal, suggesting that proSAAS is not required for PC1/3 maturation. Second, the extent of PC1/3 mediated cleavages is similar between proSAAS mutant and wild-type mice, as measured using both RIAs as well as a quantitative peptidomics screen of hypothalamic peptides. Third, the phenotypes exhibited by proSAAS and PC1/3 mutant mice are different. While adult male proSAAS KO mice have a 10-15% reduction in body weight, this deficit was relatively mild compared to the phenotype of PC1/3 KO mice which are severely runted (40% decrease in body weight) (Zhu *et al*. 2002b, Zhu *et al*. 2002a). PC1/3 deficient mice are hypoglycemic due to hyper-proinsulinemia and exhibit significant pre-natal and early post-natal lethality (Zhu et al. 2002b, Zhu et al. 2002a) while proSAAS mutant mice have normal blood glucose homeostasis, indicating that insulin function is not grossly affected. Since PC1/3 is required for the production of insulin, this is further evidence that the absence of proSAAS does not affect PC1/3 activity.

In addition to eliminating PC2 activity, 7B2 disruption causes severe Cushing's disease due to hypersecretion of ACTH from the pituitary that leads to elevated circulating corticosterone (Westphal et al. 1999). ProSAAS and 7B2 exhibit remarkably similar patterns of transcript distribution in the adult and prenatal nervous system pituitary and each has been implicated as a regulator of prohormone convertase activity. We hypothesized that proSAAS might also control regulated secretion from the pituitary if there was additional functional similarity between these two proteins. This potential role for proSAAS was investigated by examining the amount of circulating corticosterone in proSAAS mutants. We find that plasma corticosterone is not changed in proSAAS mutant males suggesting that, unlike 7B2, proSAAS does not participate in regulating endocrine secretion of these proteins.

ProSAAS and/or proSAAS-derived peptides have previously been implicated in body weight regulation based on several observations. Although proSAAS can be detected in all post-mitotic neurons, it is expressed at a particularly high level in mouse hypothalamus nuclei that control feeding, such as the arcuate nucleus, the ventromedial hypothalamus, and other nuclei (Mzhavia et al. 2001, Fricker et al. 2000, Morgan et al. 2005). Second, proSAAS processing intermediates are up-regulated several fold in mouse hypothalamus after 2 days of food deprivation (Che *et al*. 2005b). Third, transgenic proSAAS animals that overexpress proSAAS are obese and exhibit increased fat deposition (Wei et al. 2004). Fourth, the C-terminal proSAAS-derived peptide is elevated in the hypothalamus, but not other brain regions, of $Cpe^{fat/fat}$ mice, implying that the production of proSAAS is up-regulated in a region-specific manner in these mice and suggests that it might be related to their obese phenotype (Zhang *et al*. 2008). In light of these previous studies, it was predicted

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that disruption of proSAAS would cause leanness. Indeed, our study found a 15% decrease in the body weight of male proSAAS mutants and provides the first *in vivo* evidence that proSAAS disruption specifically leads to a reduction in body weight. It is of interest that this alteration was restricted to male mice and was not observed in females. The knock-outs of neuropeptides NPB and NPW also produce body weight changes restricted to the male (Singh & Davenport 2006).

ProSAAS mutants were subjected to several behavioral tests, including locomotor activity in a novel open-field chamber. Mice lacking proSAAS exhibit a consistent trend to be less active when placed in activity chambers for 30 minutes (Fig. 7). Anxiety-like behavior in rodents is characterized by the tendency to exhibit a decrease in exploratory activity and the amount of time spent in the center of the open-field. In addition to being less active, proSAAS mice exhibit a decreased amount of time spent in the center of the open-field and anxiety-like behavior when placed in an elevated zero maze supporting the possibility that proSAAS mice might be prone to anxiety-like behavior (personal communication, R. Rodriguez and W. Wetsel) and is consistent with its expression in regions such as the amygdala and limbic system.

The ability to detect PEN-LEN, the putative PC1/3 inhibitor fragment of proSAAS, in embryonic brain but not in adult brain suggests that inhibition of PC1/3 by PEN-LEN may be temporally restricted to the developing nervous system (Morgan et al. 2005). Indeed, in wild-type prenatal brain extracts containing PEN-LEN, 8-10 kDa PC1/3-processed prodynorphin intermediates were not detected. Prodynorphin is normally processed by the coordinated action of PC1/3 and PC2 to generate smaller peptides such as neo-endorphin, dynorphin B-29, dynorphin A-8, dynorphin A-17, and Leu-Enk. It is believed that prodynorphin processing occurs sequentially with preliminary cleavages by PC1/3 generating processing intermediates (8 and 10 kDa) that are then subsequently cleaved by PC2 to generate smaller dynorphin peptides (Day *et al.* 1998, Dupuy *et al.* 1994). We hypothesized that proSAAS deletion might reverse the observed absence of high molecular 8-10 kDa prodynorphin processing intermediates occurring in wild-type prenatal brain. We find this is the case with prodynophin 8-10 kDa intermediates being detected in the brains of proSAAS mutant e15.5 embryos, while they are absent in wild-type embryos. (Fig. 5). The finding that prodynorphin is processed in an adult-like manner when proSAAS is absent supports our hypothesis that proSAAS inhibition of PC1/3 is temporally restricted to pre-natal and early post-natal development. This hypothesis is supported by previous work showing that the inhibitory PEN-LEN fragment is undetectable in adult mouse brain.

In this study we detect terminally processed POMC peptides in the pituitaries of proSAAS mutant mice suggesting that the physiological effects of proSAAS overexpression are not due to inhibition of PC1/3. This work provides direct evidence *in vivo* that proSAAS does not act as a chaperone for the maturation of PC1/3 activity. Additionally, proSAAS does not appear to be involved in mediating pituitary secretion as indicated by normal levels of ACTH and corticosterone in the plasma of proSAAS mutants (Fig. S1). Although proSAAS may inhibit PC1/3 in the context of the developing brain, this inhibitory role does not extend to the adult mouse, as is the case for 7B2. However, proSAAS deletion causes a 15% decrease in the body mass of male mice, consistent with the finding that over-expression of proSAAS leads to obesity. These manipulations of proSAAS levels raise the possibility that proSAAS is involved in controlling body weight, likely at the level of the hypothalamus. Future work on the mechanism of this role for proSAAS should prove valuable in identifying the possibility that proSAAS, or proSAAS-derived peptides, function as neuropeptides regulating body weight or other behaviors.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACTH	adrenocorticotropin
CP	carboxypeptidase
ES	embryonic stem cell
KO	knock-out
LC/MS	liquid chromatography mass spectrometry
Leu-Enk	leucine enkephalin
NEO	neomycin
PC	prohormone convertase
POMC	pre-opio-melanocortin
RIA	radioimmunoassay
SEM	standard error of the mean
TMAB	trimethylammoniumbutyrate-N-hydroxysuccinimide

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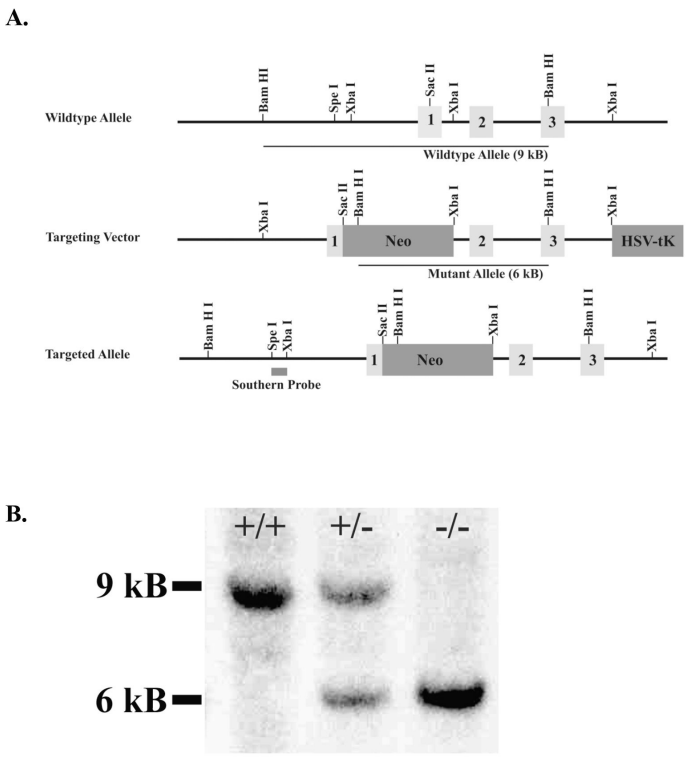

Figure 1. Production of proSAAS KO mice

A. A proSAAS genomic fragment was isolated from a murine Sw129/ReJ genomic library and genomic fragments upstream and downstream and of exon 1 were used to make the targeting vector. The targeting vector was made by subcloning these genomic DNA fragments, a neomycin (NEO) cassettee, and the HSV-*tk* gene into a pKO Scrambler V919 targeting vector. The NEO and HSV-TK cassettes were subcloned into Asc I (NEO) and Rsr II (HSV-TK) restriction sites. The 2.5 kb Xba I/Sac II genomic fragment, 5′ of exon 1 (A-arm) was inserted into Xba I/Sac II restriction sites within the targeting vector. A 5.5 kb Xba I genomic fragment located 3′ of exon 1 (B-arm) was sub-cloned into pBluescript, excised with Not I and Kpn I, and inserted into Bgl II (blunt) and Kpn I sites in the targeting vector. A 500 bp genomic fragment (Xba I/Spe I), just 5′ of the Xba I/Sac II A-arm fragment was used as a Southern blot screening probe. **B.** Male proSAAS chimeras were used to establish a colony of proSAAS knock-out (−/− or −/Y), female heterozygote (+/−), and wild type (+/+, +/Y) mice. Southern blotting of BamHI digested genomic DNA yields a 9 kB band from the wild type allele and a 6 kB band from the mutant allele.

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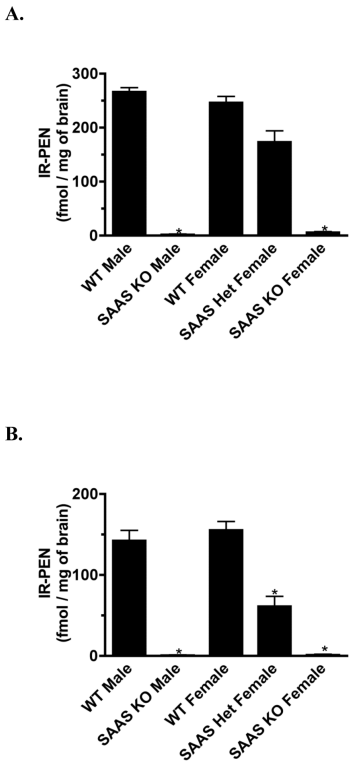

Figure 2. ProSAAS KO mice don't produce the proSAAS peptide PEN

A. Peptide extracts from the whole brain of adult wild type and proSAAS KO male mice as well as adult female wild type (+/+), heterozygote (+/−), and mutants (−/−) mice were assayed using RIA for the proSAAS derived peptide PEN. PEN was not detectable using our assay in brain of proSAAS male hemizygous and female homozygous KO mice, while female heterozygous mice possess a reduced amount of PEN relative to wild type controls. B. Peptide analysis of PEN in male and female proSAAS pituitaries. Error bars indicate the SEM and p-values were calculated using unpaired Student t-tests (* $p < 0.05$).

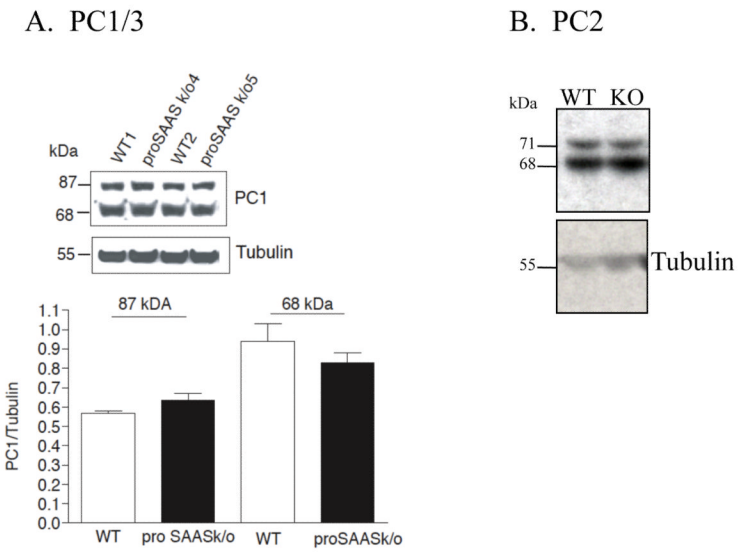

Figure 3. PC1/3 and PC2 protein levels are unaltered in the adult brain of proSAAS KO mice

The levels of PC1/3 (**A**) and PC2 (**B**) protein forms from whole brains of wild-type (**WT**) and proSAAS mutant (**KO**) animals were analyzed by Western blot. The blot for PC1/3 shows bands corresponding to the mature fully active 66 kDa form of PC1/3 as well as the inactive 87 kDa pro-PC1/3 form. The fully mature 68 kDa form of PC2 as well as an intermediate 71 kDa form of PC2 are shown. To ensure that the qualitative appearance of the PC1 blot accurately reflected the lack of effect of proSAAS mutation on either the inactive (68 kDA) or active PC1/3 isoforms (87 kDa), the ratio of these bands was determined in brains from wild-type (white bar) and proSAAS k/o (black bar) mice. Peptide sizes were estimated using Bio-Rad Kaliedoscope pre-stained standards (216, 132, 78, 45.7, 32.5, 18.4, and 7.6 kDa).

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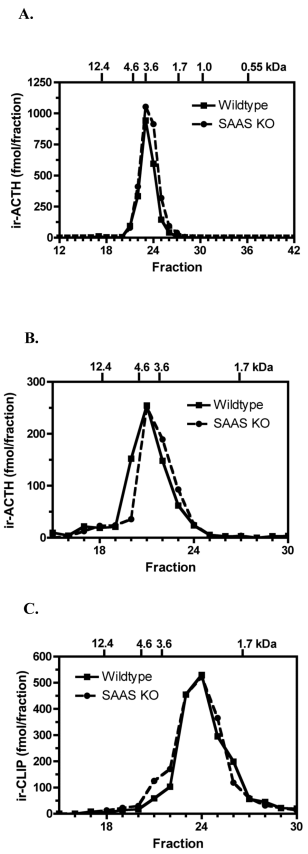

Figure 4. Processing of POMC into ACTH and CLIP is unaffected in AL and IL of proSAAS KO pituitary

The processing of POMC into ACTH was analyzed in pooled extracts of whole (**A**), anterior (**B**), or neurointermediate (**C**) pituitaries. Extracts from three proSAAS mutant (circles and dashed lines) or wild type (squares and solid lines) pituitaries were pooled and subjected to gel filtration chromatography. RIAs were performed using anti-sera that recognize ACTH (1-39). The following molecular weight calibration standards were used: cytochrome c, 12.4 kDa; ACTH, 4.6 kDa; β-endorphin, 3.5 kDa; α-MSH, 1.7 kDa, and Dyn A-8, 1.0 kDa. The void volume of the column occurs in fraction 12 and the salt fraction elutes in fraction 52.

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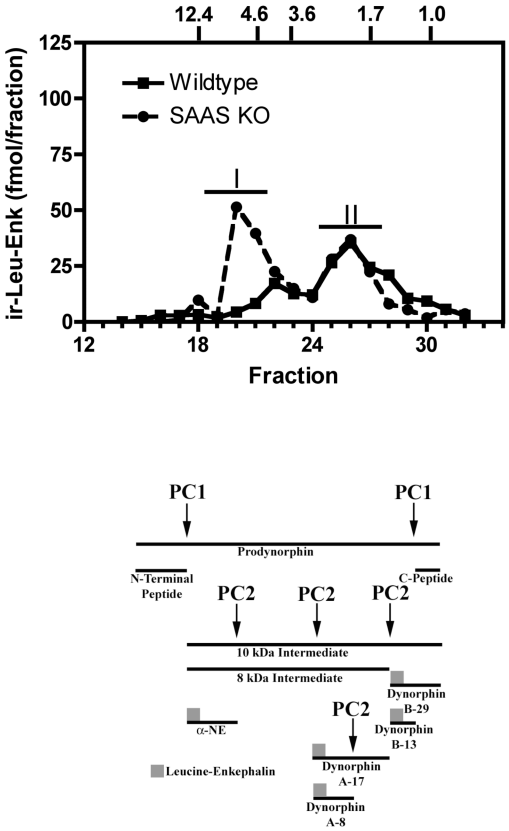

Figure 5. An adult rather than fetal processing pattern of prodynorphin is present in embryonic brain extracts lacking proSAAS

A. Extracts from brain of e15.5 mouse embryos (n=25-40) were pooled and separated by gel-filtration chromatography, treated with trypsin/carboxypeptidase and ir-Leucine enkephalin (ir-Leu-enk) was measured by RIA. High molecular weight prodynorphin intermediates were absent in wild type e15.5 whole brains (squares and solid line). A substantial amount of higher molecular weight prodynorphin characteristic of PC1/3 processing (seen in peak I) was detected in e15.5 brains lacking proSAAS (circles, dotted line) but not in e15.5 WT brains (square, bold line). Other lower molecular weight prodynorphin intermediates (Dynorphin A-17, B-13, and others in fractions 25-28; Peak II) were generated in mutant embryonic brains (circles, dotted line). The following molecular weight calibration standards were used: cytochrome c, 12.4 kDa; ACTH, 4.6 kDa; β-endorphin, 3.5 kDa; α-MSH, 1.7 kDa, and Dyn A-8, 1.0 kDa. The void volume of the column occurs in fraction 12 and the salt fraction elutes in fraction 52. **B.** Schematic diagram showing the processing of prodynorphin by PC1/3 into 8 and 10 kDa intermediates that are subsequently processed by PC2 to generate terminally processed dynorphin peptides (Dynorphin B-17, B-29, A-8, A-17, alpha-neo-endorphin (αNE), and leucine-enkephalin).

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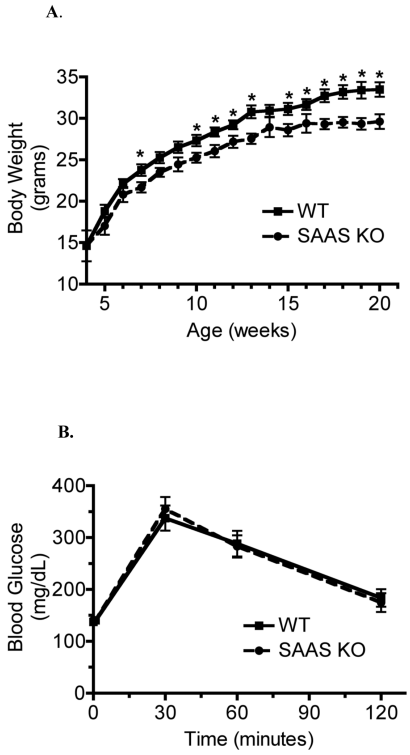

Figure 6. ProSAAS males have reduced body weight

A. The body weight of proSAAS male mutants (circles and dashed line) is plotted weekly and is shown relative to wild type littermates (squares and solid line). Mice were fed a standard rodent chow diet. Error bars correspond to the SEM and the differences at each time point were analyzed using Student's t-test (* $P<0.05$). **B.** Fasting blood glucose (t=0) and blood glucose levels at 30, 60, and 120 minutes following intraperitoneal administration of 2mg/g glucose were plotted for proSAAS mutants (circles and dashed line) and wild type littermates (squares and solid line).

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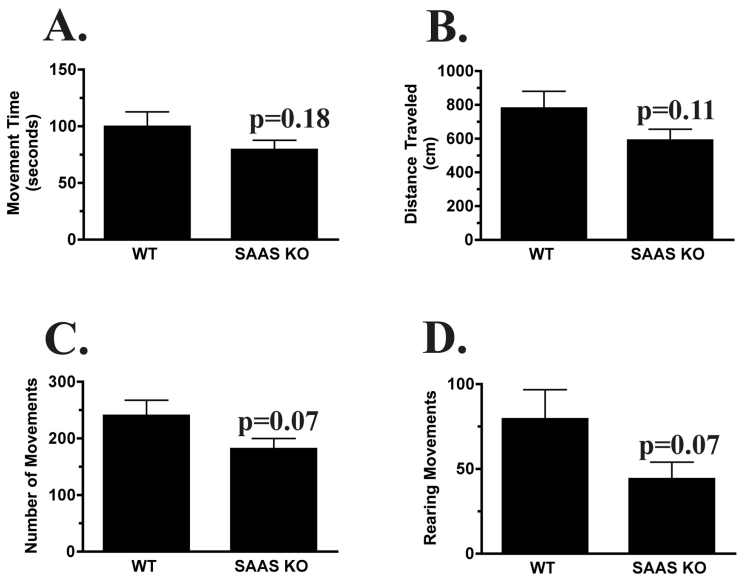

Figure 7. ProSAAS mutant mice are less active when placed in a novel open field

Locomotor activity was monitored for proSAAS male mutants (SAAS KO) and wild type littermates (WT) placed in a novel open-field for 30 minutes. Horizontal activity (**A**), number of movements (**B),** number of rearing movements **(C),** and the number of stereotypic movements **(D)** were monitored during the open field test. Error bars indicate the SEM and p-values calculated using unpaired Student t-tests (* $p < 0.05$).

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