Pituitary

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Significant advances have been made in the field of pituitary development, pituitary tumor biology and pituitary signaling, as well as the interaction between signaling, development and tumor formation. Numerous new mutations and their functional implications for pituitary function have been identified. In addition, continuing progress is being made to uncover the functions of oxytocin, especially its role in behavior and an exciting development is the pharmacological use of oxytocin to affect behavior [1].

New mutations

Heterozygous orthodenticle homeobox 2 mutations are associated with variable pituitary phenotype

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J Clin Endocrinol Metab 2010;95:756–764

Background: Orthodenticle homeobox 2 (OTX2) is a transcription factor that appears to be critical for normal forebrain and eye development. A number of heterozygous mutations in the gene encoding this protein, OTX2, have been identified in association with ocular malformations such as anophthalmia or microphthalmia. Recent studies have suggested a positive role of OTX2 in pituitary as well as ocular development and function, but there appears to be considerable variability in the phenotype, e.g. some mutations do not appear to be associated with eye phenotypes [2]. Detailed pituitary phenotypes in patients with OTX2 mutations and OTX2 target genes for pituitary function other than HESX1 and POU1F1 remain to be determined.

Methods: The authors studied 94 Japanese patients with various ocular or pituitary abnormalities, and sequenced all patients for mutations in OTX2. Multiplex ligation-dependent probe amplification (MLPA) was performed for OTX2 intragenic mutation-negative patients. Transient transfection assays were performed using luciferase reporters containing IRBP, HESX1, POU1F1 and GNRH1 promoters. Human cDNA from a variety of tissues was screened for the presence of OTX2 transcripts.

Results: The authors identified the following heterozygous mutations: p.K74fsX103 in case 1 with isolated GH deficiency (IGHD), anophthalmia/microphthalmia, and anterior pituitary hypoplasia (APH) and an ectopic/undescended posterior pituitary (EPP); p.A72fsX86 in case 2 with normal pituitary function and microphthalmia; p.G188X in 2 unrelated cases (case 3 with microphthalmia, combined pituitary hormone deficiency (CPHD) and APH with an EPP, and case 4 with microphthalmia and normal pituitary function), and a 2.86-Mb microdeletion including OTX2 in case 5 with IGHD and APH associated with anophthalmia/microphthalmia. Wild-type OTX2 protein transactivated the GNRH1 promoter as well as the HESX1, POU1F1, and IRBP (interstitial retinoid-binding protein) promoters, whereas the p.K74fsX103-OTX2 and p.A72fsX86-OTX2 proteins led to loss of transactivation and the p.G188X-OTX2 protein demonstrated reduced (approx. 50%) transactivation function for the four promoters, with no dominant-negative effect. cDNA screening identified positive OTX2 expression in the hypothalamus. Two additional missense mutations, p.T178S and p.A245V, were also identified in 2 further patients; these were not shown to compromise the function of the protein and may reflect rare sequence variants.

Conclusions: The results imply that *OTX2* mutations are associated with variable pituitary phenotypes, with no genotype-phenotype correlations, and that *OTX2* can transactivate *GNRH1* as well as *HESX1*, *POU1F1* and *IRBP* promoters.

Orthodenticle homeobox 2 (*OTX2*) is critical for normal forebrain and eye development and heterozygous mutations in *OTX2* have been associated with anophthalmia or microphthalmia. Since transcriptional regulation of the development of the hypothalamus, pituitary, eyes and optic nerves overlap, this interesting study has screened a sizeable cohort of patients with ocular and/or pituitary abnormalities for mutations in *OTX2*. The authors report considerable variability in the phenotypes associated with the mutations with no clear genotype-phenotype correlations, although it appears likely that GH deficiency is the commonest endocrine manifestation. This is reminiscent of mutations in *HESX1*, which is believed to be a target of *OTX2*. The exact role of *OTX2* in both murine and human hypothalamo-pituitary development is unclear. Additionally, the authors state that since *OTX2* transactivates the *GNRH1* promoter, *GNRH1* may also be a target of *OTX2*. However, one needs to apply caution with respect to this statement. In vivo studies are needed in animals that are conditionally deleted for *Otx2* in the hypothalamo-pituitary region. Similarly, one cannot extrapolate the binding of *OTX2* to the *POU1F1* promoter with subsequent transactivation to a direct genetic interaction between *OTX2* and *POU1F1* – the co-expression of the two genes needs to be shown, as does loss of expression of *Pou1f1* expression in animals that are conditionally deleted for *Otx2*. It is therefore clear that *OTX2* is an important player in hypothalamo-pituitary development in humans, but much remains to be learnt with respect to its role in normal development.

A novel mutation in the LIM homeobox 3 gene is responsible for combined pituitary hormone deficiency, hearing impairment, and vertebral malformations

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Background: The LIM homeobox 3 (*LHX3*) LIM-homeodomain transcription factor gene, found in both human and mouse, is required for development of the pituitary and motor neurons, and is also expressed in the auditory system. Mutations in the gene are associated with combined pituitary hormone deficiency (CPHD) in association with a short stiff neck, and variable sensorineural hearing impairment. To date, homozygous recessive mutations have been identified in 9 unrelated families.

Methods: The objective of this study was to determine the cause of, and further explore, the phenotype in six patients (aged 6 months to 22 years) with CPHD, restricted neck rotation, scoliosis, and congenital hearing impairment. Three of the patients also have mild autistic-like behavior. The association of previously described *LHX3* mutations with CPHD and restricted neck rotation led to the adoption of a candidate gene approach, and the gene was sequenced. Neck anatomy was explored by computed tomography and magnetic resonance imaging, including three-dimensional reformatting.

Results: A novel recessive splice-acceptor site mutation was found. The predicted protein encoded by the mutated gene would lack the homeodomain and carboxyl terminus of the normal, functional protein. Genealogical studies revealed a common gene source for all six families dating back to the 17th century. Anatomical abnormalities in the occipito-atlantoaxial joints in combination with a basilar impression of the dens axis were found in all patients assessed. Cervical lordosis and thoraco-lumbar hyperlordosis were observed in all children by the time the children could stand. Scoliosis was identified in 5 of the children, as was significant sensorineural hearing loss. MRI revealed severe anterior pituitary hypoplasia with a cystic structure identified in patient 5.

Conclusions: This study extends both the mutations known to be responsible for *LHX3*-associated syndromes and their possible phenotypic consequences. Previously reported traits include CPHD, restricted neck rotation and variable hearing impairment; patients examined in the present study also show a severe hearing defect. In addition, the existence of cervical vertebral malformations are revealed, and believed to be responsible for the rigid neck and the development of scoliosis.

Initial studies suggested that *LHX3* mutations were associated with CPHD excluding ACTH deficiency in association with a short stiff neck and limited rotation [3]. However, the phenotypic spectrum has recently expanded to include variability in the presence of the short stiff neck [4], the presence of a pituitary microadenoma [5] and the presence of ACTH deficiency with sensorineural hearing impairment [6]. This study is fascinating as it reports the occurrence of a founder mutation in *LHX3* in a northern Swedish population, and confirms the presence of hearing deficit in affected patients, thereby confirming a role for *LHX3* in human auditory development. It is important to note that the presence of hypoglycemia led to cortisol replacement in 3 of the 6 individuals. Additionally, patient 2 had a low concentration of cortisol in the neonatal period, suggesting a diagnosis of ACTH deficiency. This paper nicely describes for the first time the occipito-atlantoaxial abnormalities observed in this cohort of patients. Additionally, it describes the presence of progressive scoliosis from 5 to 6 years of age in the majority of patients. It is important to note that skeletal abnormalities were also noted in 4 patients described by Rajab et al. [6], and must now be considered to be a component of the wider *LHX3* mutant phenotype.

New genes – pituitary development

A role of the LIM-homeobox gene *Lhx2* in the regulation of pituitary development

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Dev Biol 2010;337:313–323

Background: The mammalian pituitary gland originates from two separate germinal tissues during embryonic development. The anterior and intermediate lobes of the pituitary are derived from Rathke's pouch, a pocket formed by an invagination of the oral ectoderm. The posterior lobe is derived from the infundibulum, which is formed by evagination of the neuroectoderm in the ventral diencephalon. Previous studies have shown that development of Rathke's pouch and the generation of distinct populations of hormone-producing endocrine cell lineages in the anterior/intermediate pituitary lobes is regulated by a number of transcription factors expressed in the pouch and by inductive signals from the ventral diencephalon/infundibulum. However, little is known about factors that regulate the development of the posterior pituitary lobe.

Methods: In this study, the authors investigated the role of the LIM homeobox gene *Lhx2* in hypothalamo-pituitary development in mice. *Lhx2* null mutants (*Lhx2*^{−/−}) were generated and the phenotype analyzed. The expression of a number of genes encoding signaling molecules and transcription factors was studied in embryonic sections. BrdU assays were performed to assess cell proliferation and TUNEL assays performed to detect apoptosis.

Results: *Lhx2* is extensively expressed in the developing ventral diencephalon, including the infundibulum and the posterior lobe of the pituitary, in addition to other tissues such as the retina, forebrain, midbrain, hindbrain and spinal cord. Deletion of *Lhx2* resulted in persistent cell proliferation, a complete failure of evagination of the neuroectoderm in the ventral diencephalon, and defects in the formation of the distinct morphological features of the infundibulum and the posterior pituitary lobe. Rathke's pouch is formed and endocrine cell lineages are generated in the anterior/intermediate pituitary lobes of the *Lhx2* null mutant. However, the shape and organization of the pouch and the anterior/intermediate pituitary lobes are severely altered due to the defects in development of the infundibulum and the posterior lobe, with a large mass of cells occupying the area between the third ventricle and the anterior/intermediate lobes of the pituitary. After E15.5, the mutant mice die in utero due to a severe defect in definitive erythropoiesis. BrdU assays revealed a dramatic increase in cell proliferation in the mutants as compared with wild-type animals at E11.5, E12.5, E13.5 and E14.5. Expression of infundibular markers such as *Nkx 2.1*, *Sox3*, *Six3*, and the cellular retinoic acid binding protein CRABP2 was unaffected in the mutants whereas expression of posterior lobe markers such as calbindin and vasopressin was

absent in the region corresponding to the posterior lobe. An increased number of TUNEL-positive apoptotic cells were present in the large abnormal mass occupying the ventral diencephalon of *Lhx2*^{-/-} mutants. The expression domain of Fgf8 was expanded rostrally, overlying the pouch that failed to grow dorsally in the mutants.

Conclusion: This study thus reveals an essential role for *Lhx2* in the regulation of posterior pituitary development and suggests a mechanism whereby development of the posterior lobe may affect the development of the anterior and intermediate lobes of the pituitary gland.

The normal development of the pituitary gland is a carefully orchestrated process that is dependent on the expression of a number of signaling molecules and transcription factors in a coordinated manner [7]. Early tissue recombination studies have suggested that the neuroectoderm of the ventral diencephalon, leading to neurohypophysis generation, provides inductive signals that are required for the growth and differentiation of Rathke's pouch. This exciting work has led to the identification of a further piece of the complex jigsaw. Deletion of *Lhx2* resulted in persistent cell proliferation, a complete failure of evagination of the neuroectoderm in the ventral diencephalon, and defects in the formation of the distinct morphological features of the infundibulum and the posterior pituitary lobe. The importance of *Lhx2* lies in the fact that it is one of the few genes that have been identified to be critical for normal development of the infundibulum and posterior pituitary. It also reiterates the importance of the inductive interaction between the neuroectoderm and the oral ectoderm. Whether it will be implicated in the etiology of hypopituitarism in humans remains to be seen, although one would predict that the phenotype in affected patients would be extensive given the presence of severely defective erythropoiesis and embryonic death in the *Lhx2* mutants.

Genetic regulation of pituitary gland development in human and mouse

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Endocr Rev 2009;30:790–829

Normal hypothalamo-pituitary development is closely related to that of the forebrain and is dependent upon a complex genetic cascade of transcription factors and signaling molecules that may be either intrinsic or extrinsic to the developing Rathke's pouch. These factors dictate organ commitment, cell differentiation, and cell proliferation within the anterior pituitary. Abnormalities in these processes are associated with congenital hypopituitarism, a spectrum of disorders that includes syndromic disorders such as septo-optic dysplasia, combined pituitary hormone deficiencies, and isolated hormone deficiencies, of which the commonest is GH deficiency. The highly variable clinical phenotypes can now in part be explained due to research performed over the last 20 years, based mainly on naturally occurring and transgenic animal models. Mutations in genes encoding both signaling molecules such as Sonic Hedgehog and transcription factors such as HESX1, SOX2, SOX3, LHX3, LHX4, PROP1 and POU1F1 have been implicated in the etiology of hypopituitarism, with or without other syndromic features, in mice and humans. To date, mutations in known genes account for a small proportion of cases of hypopituitarism in humans. However, these mutations have led to a greater understanding of the genetic interactions that lead to normal pituitary development. This comprehensive review attempts to describe the complexity of pituitary development in the rodent, with particular emphasis on those factors that, when mutated, are associated with hypopituitarism in humans. It is clear that normal hypothalamo-pituitary development is a highly complex phenomenon, and features such as phenotypic variability and variability of penetrance remain to be explained.

This review describes the current state of knowledge in the field of pituitary development in mouse and human. The review describes key processes in murine pituitary development and also the known genetic causes of hypopituitarism in humans, and attempts to correlate phenotypes in both mouse and human. It describes in great detail the development of the pituitary and regulation of development by genes encoding signaling molecules such as Sonic Hedgehog and transcription factors such as HESX1, SOX2, SOX3, LHX3, LHX4, PROP1 and POU1F1. It continues to describe the consequences of mutations of these genes in murine and human development of the hypothalamic-pituitary axis and

the implication of these mutations in the etiology of hypopituitarism, with or without other syndromic features in humans.

New mechanisms – pituitary development

The notch target gene *HES1* regulates cell cycle inhibitor expression in the developing pituitary

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Endocrinology 2009;150:4386–4394

Background: Growth of the pituitary during development and adulthood is a tightly regulated process. Hairy and enhancer of split (*HES1*), a transcription factor whose expression is initiated by the Notch signaling pathway, is a repressor of cell cycle inhibitors. The authors hypothesized that with the loss of *Hes1*, pituitary progenitors are no longer maintained in a proliferative state, but chose to exit the cell cycle instead.

Methods: Expression of cell cycle regulators, proliferation and apoptosis in wild-type and *Hes1*-deficient pituitaries was assessed using immunohistochemistry.

Results: Changes in phosphohistone H3 expression in cells in Rathke's pouch in early pituitary development [8] indicate decreased proliferation in *Hes1*-deficient mutants. Furthermore, pituitaries lacking *Hes1* have increased cell cycle exit, shown by significant increases in the cyclin-dependent kinase inhibitors, p27 and p57, from e10.5 to e14.5. Additionally, *Hes1* mutant pituitaries have ectopic expression of p21 in Rathke's pouch progenitors, an area coincident with increased cell death.

Conclusions: These data indicate a role for *HES1* in the control of cell cycle exit and in balancing proliferation and differentiation, allowing for the properly timed emergence of hormone secreting cell types.

This study is not only of importance for the understanding of pituitary development, but may also be important for the understanding of pituitary tumor formation, emphasizing the close relationship between embryonic development and tumor formation. We first became interested in *HES1* when we were studying regulation of chondrocyte and adipocyte proliferation and differentiation – Ptf1 (*DLK1*) functions as a receptor in the Notch signaling system and needs down-regulation for terminal adipocyte differentiation to occur [9] but is also present in the growth plate and pituitary. Notch signaling dictates cell fate and influences cell proliferation, differentiation, and apoptosis in multiple tissues. Factors at each step – ligands, receptors, signal transducers and effectors – play critical roles in executing the effects of Notch signaling. Ligand binding to *DLK1* or other Notch receptors results in regulation of Notch effectors and *HES1* is one of these Notch effectors. Recent studies postulated that Notch signaling in the pituitary maintains a proliferative zone of cells lining the Rathke's pouch. In line with a role for Notch effectors in the regulation of proliferation and differentiation, *HES1* is a repressor of cell cycle inhibitors. *HES*^{−/−} mice have hypomorphic pituitaries with reductions in all hormone cell types and absence of α -MSH-producing cells that normally appear at a more differentiated stage of pituitary development. This study further clarifies its role in pituitary progenitors located in Rathke's pouch, which normally express *HES1*. Absence of *Hes1* leads to dysregulation of cyclin-dependent kinase inhibitors like p57, p27 and p21. When mutant *Hes1* is present, pituitary progenitors show an increased cell cycle exit, increased cell death and a reduction in cell proliferation resulting in a depletion of the progenitor pool. This suggests a role for *HES1* in the maintenance of a balance between proliferation and differentiation, allowing cell cycle exit for differentiation to hormone-secreting cells at an appropriate time. Inappropriate regulation of this process results in a hypomorphic pituitary gland. Further work is needed to establish whether increased activation of Notch-*HES1* signaling in the pituitary may lead to tumor formation.

Co-repressors TLE1 and TLE3 interact with HESX1 and PROP1

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Mol Endocrinol 2010;24:754–765

Background: Pituitary hormone deficiency causes short stature in 1 in 4,000 children born and can be caused by mutations in a number of genes encoding transcription factors such as HESX1, LHX3, LHX4, SOX2, SOX3, PROP1 and POU1F1. HESX1 interacts with a member of the groucho-related gene family, TLE1, through an engrailed homology (eh1) domain and represses PROP1 activity. Mice with Prop1 deficiency exhibit failed differentiation of the POU1F1 lineage, resulting in lack of TSH, GH, and prolactin, in addition to ACTH and gonadotrophin deficiency. In addition, these mutants exhibit profound pituitary dysmorphology and overexpress Hesx1 and Tle3. Tle3 is structurally related to Tle1, and is expressed in the developing pituitary in an overlapping yet distinct pattern. The ability of HESX1 to interact with TLE3 has not been explored previously.

Methods: The authors tested the ability of TLE3 to enhance HESX1-mediated repression of PROP1 activation at the *POU1F1* promoter in cell culture using 293T cells (human embryonic kidney cells). In vivo studies using transgenic mice tested the functional consequences of ectopic TLE3 and HESX1 expression by driving constitutive expression in pituitary thyrotrophs and gonadotrophs using the *Cga* promoter.

Results: In the cell culture experiments, both TLE3 and TLE1 repressed PROP1 in conjunction with HESX1 with similar efficiencies, and the eh1 domain appears to be critical for this repression. TLE1 and TLE3 could each repress PROP1 in the absence of HESX1, probably via a protein-protein interaction. In vivo, terminal differentiation of thyrotrophs and gonadotrophs was suppressed by HESX1 alone and by TLE3 and HESX1 together but not by TLE3 alone. Interestingly, whereas HESX1 alone did not affect the expression of endogenous *Cga*, the *Tg(Cga-Tle3)*, *Tg(Cga-Hesx1)* double transgenic embryos showed a dramatic reduction in the concentration of endogenous CGA protein, suggesting that the presence of the co-repressor TLE3 in addition to HESX1 was critical for the repression of the endogenous *Cga*.

Conclusion: This work presents evidence that HESX1 is a strong repressor that can be augmented by the co-repressors TLE1 and TLE3. In vitro studies suggest that TLE1 and TLE3 might also play roles independent of HESX1 by interacting with other transcription factors like PROP1.

Although mutations in *HESX1* were first identified in 1998 [10], its function remains largely unknown. Its role as a repressor is undisputed, yet its partners and targets have not as yet been clearly established. HESX1 interacts with a member of the groucho-related gene family, TLE1, through an engrailed homology domain and represses PROP1 activity and TLE1, and the similar gene TLE3, are expressed in a pattern overlapping that of HESX1. This paper reports that TLE3 and TLE1 are potential co-repressors, as has been previously established for TLE1 [11, 12]. It is interesting that the in vivo studies report enhancement of HESX1 function by TLE3, although HESX1 can also act as a repressor independently of TLE proteins. This suggests that other co-repressors such as NCoR1 [13] may also enhance the function of HESX1 as a repressor. Much remains to be understood about normal pituitary development and the mechanisms involved, and this is underlined by our inability to identify a genetic cause in the vast majority of children with congenital hypopituitarism.

Use of the desmopressin test in the differential diagnosis of pseudo-Cushing state from Cushing's disease

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J Clin Endocrinol Metab 2010;95:1115–1122

Background: The diagnosis of Cushing disease is generally based upon a combination of urinary free cortisol (UFC), midnight serum cortisol and serum cortisol after dexamethasone suppression. However, the diagnosis can be difficult to make. The desmopressin (DDAVP) test has been proposed to discriminate Cushing's disease (CD) from pseudo-Cushing states (PC); however, current information on its value is limited and contradictory.

Methods: The authors aimed to study the ability of the DDAVP test to distinguish between CD and PC, with emphasis on subjects with mild hypercortisolism. They conducted a retrospective/prospective study that included 52 subjects with CD, 28 with PC, and 31 control subjects (CT) with simple obesity in whom Cushing syndrome had previously been excluded using standard diagnostic tests. The DDAVP test was performed and compared with standard diagnostic procedures for the diagnosis of Cushing's syndrome. The diagnosis/exclusion of CD was measured.

Results: Interpretation of the DDAVP test based on percentage and absolute increment of cortisol and ACTH did not in itself give acceptable values of both sensitivity (SE) and specificity (SP). CD diagnosis based on simultaneous positivity for basal serum cortisol >331 nmol/l and absolute ACTH increment >4 pmol/l and its exclusion in subjects negative for one or both measures yielded an SE of 90.3% and an SP of 91.5%. The approach was also highly effective in distinguishing PC from: (1) CD with moderate values of urinary free cortisol (SE 86.9%, SP 92.8%); (2) CD with moderate values of serum cortisol after dexamethasone suppression (SE 86.6%, SP 92.8%), and (3) CD with moderate values of midnight serum cortisol (SE 100%, SP 92.8%).

Conclusion: Interpretation of the DDAVP test through a combination of parameters allowed effective discrimination of Cushing disease from pseudo-Cushing, even in subjects with mild hypercortisolism.

Pseudo-Cushing state is caused by conditions (e.g. depression, alcoholism, polycystic ovary syndrome, severe obesity) that can activate the hypothalamic-pituitary-adrenal axis and is characterized by clinical and biochemical signs typical of Cushing's syndrome. The overlapping clinical features with Cushing disease and the similar values frequently determined in tests such as urinary free cortisol, serum cortisol after dexamethasone suppression, and midnight serum cortisol in the respective patients make it difficult to distinguish subjects with these two entities. This study has examined the usefulness of the DDAVP test in diagnosing Cushing disease in a large cohort of adult patients and suggests that assessment of percentage and absolute increment of cortisol and ACTH concentration in the DDAVP test allows for discrimination between Cushing disease and pseudo-Cushing. Whether the test can be a useful adjunct to the standard tests in a pediatric population remains to be proven.

Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat

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Endocrinology 2010;151:2276–2286

Background: Variations in maternal behavior among lactating rats associate with differences in estrogen-oxytocin interactions in the medial preoptic area and in dopamine concentrations in the nucleus accumbens. Individual differences in pup licking/grooming (LG) are abolished by oxytocin receptor blockade or treatments that equalize dopamine signal in the nucleus accumbens. In this paper, novel evidence is provided for a direct effect of oxytocin at the level of the ventral tegmental area in the regulation of accumbens dopamine levels.

Methods: In vivo study of normal rats divided in high LG (>1 SD) or low LG (<-1 SD) dependent on frequency scores for licking/grooming. Retrograde tracing of projections from the ventral tegmental area by stereotactic fluorogold injection into ventral tegmental area followed by oxytocin and fluorogold immunohistochemistry. In situ dopamine concentration was measured using electrochemical probes implanted in the nucleus accumbens. Drugs were injected directly into the ventral tegmental area. Histology was used to verify proper location of electrodes and cannulae.

Results: Mothers that exhibit consistently increased pup LG (i.e. high LG mothers) by comparison with low LG mothers show increased oxytocin expression in the medial preoptic area and the paraventricular nucleus of the hypothalamus and increased projections of oxytocin-positive cells from both medial preoptic area and paraventricular nucleus of the hypothalamus to the ventral tegmental area. Direct infusion of oxytocin into the ventral tegmental area increased the dopamine signal in the nucleus accumbens. High compared with low LG mothers show greater increases in dopamine signal in the nucleus accumbens during bouts of pup LG, and this difference is abolished with infusions of an oxytocin receptor antagonist directly into the ventral tegmental area.

Conclusions: These studies reveal a direct effect of oxytocin on dopamine release within the mesocorticolimbic dopamine system. This supports a role for oxytocin-dopamine interactions in the establishment and maintenance of social bonds.

Promoting social behavior with oxytocin in high-functioning autism spectrum disorders

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Proc Natl Acad Sci USA 2010;107:4389–4394

Background: Social adaptation requires specific cognitive and emotional competences. Individuals with high-functioning autism or with Asperger syndrome cannot understand or engage in social situations despite preserved intellectual abilities. Recently, it has been suggested that oxytocin, a hormone known to promote mother-infant bonds, may be implicated in the social deficit of autism. This study investigated the behavioral effects of oxytocin.

Methods: Thirteen subjects with high-functioning autism spectrum disorder were entered in a simulated ball game where participants interacted with fictitious partners and received inhaled oxytocin. Decision behavior in the game, visual examination of faces and emotional response using a self-rated scale was assessed.

Results: After oxytocin inhalation, patients exhibited stronger interactions with the most socially cooperative partner and reported enhanced feelings of trust and preference. Also, during free viewing of pictures of faces, oxytocin selectively increased patients' gazing time on the socially informative region of the face, namely the eyes.

Conclusions: During oxytocin treatment, patients respond more strongly to others and exhibit more appropriate social behavior and affect, suggesting a therapeutic potential of oxytocin through its action on a core dimension of autism.

The first of the papers above reports on the mechanism whereby oxytocin may affect maternal licking and grooming of pups. Within a population of normal rats, the authors were able to differentiate high and low grooming mothers by the number of oxytocin neurons in several hypothalamic nuclei projecting to the ventral tegmental area and showed that oxytocin acts directly on dopamine release in the nucleus accumbens. In addition, the authors mention submitted work that associates activity of dopamine-sensitive pathways (by functional MRI) to maternal responsiveness to infant-related stimuli in humans. This work opens doors to the therapeutic use of oxytocin, especially since oxytocin has been implicated in the etiology of autism. Normal subjects that receive oxytocin are more inclined to trust other players and to even send them money in simulated investment games [14], confirming a pharmacological effect of oxytocin on human behavior. The second paper describes a study in which patients with high-functioning autism spectrum disorders (HF-ASD) received inhaled oxytocin. Patients with high-functioning autism spectrum disorders have normal language and intellectual abilities but avoid eye contact, have less spontaneous interaction with people and show impairments in understanding intentions of others. Indeed, these patients had reduced oxytocin plasma concentrations compared to normal subjects. In a simulated game, oxytocin-treated subjects scanned the eye region of the face more often and reported greater trust. These results are promising for the development of pharmacological strategies to increase social interaction and adaptation in patients with autism.

New paradigm

Functional amyloids as natural storage of peptide hormones in pituitary secretory granules

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Science 2009;325:328–332

Background: Amyloid fibrils are highly organized cross- β -sheet-rich protein or peptide aggregates that are associated with pathological conditions including several neurodegenerative diseases such as Alzheimer's disease and other conditions including type 2 diabetes. However, amyloids may also have a normal biological function, when they are termed 'functional amyloids' as demonstrated by fungal prions, which are involved in prion replication, and the amyloid protein Pmel17, which is involved in mammalian skin pigmentation. The authors hypothesized that peptide and protein hormones in secretory granules may adopt an amyloid-like structure, and that this could explain most of their properties.

Methods: 42 peptide and protein hormones from multiple species and organs and with a variety of different three-dimensional structures were selected. They were assayed for their capacity to form amyloids by the amyloid-specific dyes thioflavin T and Congo Red using luminescent conjugated polyelectrolyte probes, by the conformational transition into β -sheet-rich structure measured by circular dichroism spectroscopy, and by the presence of fibrils in electron microscopy images. Furthermore, x-ray fiber diffraction was measured for a subset of hormones.

Results: Ten of the 42 hormones showed considerable formation of amyloids. Given the possible involvement of glycosaminoglycans (GAGs) in the formation of both secretory granules and amyloid fibrils, the amyloid formation of all 42 peptides and proteins was monitored in the presence of low molecular weight heparin as a representative of GAGs. Most hormones ($n = 31$) formed amyloid fibrils after 2 weeks of incubation in the presence of heparin. Adrenocorticotrophic hormone (ACTH) could not form amyloid-like aggregates on its own but did so in the presence of β -endorphin, which is also processed from pro-opiomelanocortin and secreted together with ACTH in a regulated secretory pathway. These data were

confirmed in the mouse pituitary tumor neuroendocrine cell line Aft20. Within the mouse pituitary, immunohistochemistry revealed the abundant presence of amyloids in the anterior and posterior pituitary. Co-localization of the amyloid-specific dye Thio S and the hormones ACTH, β -endorphin, prolactin and GH in the anterior lobe, ACTH in the intermediate lobe, and oxytocin and vasopressin in the posterior lobe, were indicative that their storage in the secretory granules was extensively amyloid-like.

Conclusion: This study reports that peptide and protein hormones in secretory granules of the endocrine system are stored in an amyloid-like cross- β -sheet-rich conformation. Thus, functional amyloids in the pituitary and other organs can contribute to normal cell and tissue physiology encompassing processes such as granule formation including hormone selection, membrane surrounding and inert hormone storage, and subsequently the release of hormones from the granules.

Secretory proteins and peptides are synthesized in the endoplasmic reticulum and Golgi and then stored in vesicles or secretory granules ready for release into the extracellular space. This paper presents a highly novel concept, and suggests the association of amyloid with hormones, which then form secretory granules. The authors suggest that, although amyloid has been associated with disease processes in the past, it may play a critical functional role in hormone storage and secretion. They propose that in the Golgi, amyloid aggregation of the prohormone is initiated spontaneously above a critical prohormone concentration and/or in the presence of helper molecules such as GAGs in parallel to a possible prohormone processing. Because the prohormone may aggregate less into an amyloid entity than its hormone counterpart, prohormone processing at critical hormone concentrations may initiate the aggregation. Amyloid aggregation thereby sorts the protein/peptide hormones into secretory granule cores, concentrates them to the highest density possible, and excludes non-aggregation prone constitutively secreted proteins. During the aggregation process, the hormone amyloids become surrounded by membrane, separate from Golgi, and form mature granules, leading to long-term storage. On signaling, secretory granules are secreted and the cross β -sheet structure of the amyloid enables a controlled release of monomeric functional hormone. Whether the presence of functional amyloid impacts on disease processes such as autosomal dominant GHD and autosomal dominant diabetes insipidus, where hormone secretion is abnormal, remains to be established. The authors suggest a possible functional amyloid state of many endocrine hormones in secretory granules of the hypothalamus (e.g. CRF) and pancreas (e.g. somatostatin).

New mechanism – pituitary tumors

Use of the metallothionein promoter-human growth hormone-releasing hormone (GHRH) mouse to identify regulatory pathways that suppress pituitary somatotrope hyperplasia and adenoma formation due to GHRH-receptor hyperactivation

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Endocrinology 2009;150:3177–3185

Background: Hyperactivation of the GHRH receptor or downstream signaling components is associated with hyperplasia of the pituitary somatotrope population, and may result in the formation of adenomas relatively late in life. This study aimed to assess the role of GHRH and somatostatin in pituitary tumor formation.

Methods: Hyperplastic and adenomatous pituitaries of metallothionein promoter-human GHRH transgenic mice (4 and > 10 months, respectively) were used to identify mechanisms that may prevent or delay adenoma formation in the presence of excess GHRH.

Results: In hyperplastic pituitaries, expression of the late G1/G2 marker Ki67 increased, whereas the proportion of 5-bromo-2'-deoxyuridine-labeled cells (an S-phase marker) did not differ from age-matched controls indicating that cell cycle progression was blocked. Further evidence suggested that

enhanced p27 activity may contribute to this process. Adenoma formation was associated with loss of p27 activity. Increased endogenous somatostatin (SST) tone may slow the conversion from hyperplastic to adenomatous tissue since mRNA levels for SST receptors, *sst2* and *sst5*, were elevated in hyperplastic pituitaries, whereas adenomas were associated with a decline in *sst1* and *sst5* mRNA. Also, SST-knockout Tg pituitaries were larger and adenomas formed earlier compared with those of SST-intact Tg mice. Unexpectedly, these changes were independent of changes in proliferation rate within the hyperplastic tissue, suggesting that endogenous SST controls GHRH-induced adenoma formation primarily via modulation of apoptotic and/or cellular senescence pathways, consistent with the predicted function of some of the most differentially expressed genes (Casp1, MAP2K1, TNFR2) identified by membrane arrays and confirmed by quantitative real-time RT-PCR.

Conclusions: A block in cell cycle progression is responsible for hyperplasia of pituitaries under continuous GHRH signaling, but loss of p27 is needed for adenomatous transformation. Reduction of somatostatin tone plays a role in adenoma formation by modulation of apoptosis and senescence rather than proliferation.

This paper and a paper mentioned later in this chapter investigate mechanisms involved in tumor growth, this one assessing the role of GHRH-somatostatin balance, and the next one the role of the less well known pituitary tumor transforming gene (PTTG1). GH producing adenomas are often found to overexpress GHRH and this study therefore focused on the role of GHRH in pituitary tumor formation. These tumors however often develop late in life and do not have 100% penetrance, suggesting that other factors are needed to allow for uncontrolled growth. This study aimed to find genes that allow for or halt pituitary tumor formation despite continuous GHRH stimulation. Pituitaries of mice overexpressing GHRH were used and these mice were crossed with mice deficient in somatostatin to study the contribution of somatostatin in slowing tumor formation. It was not too unexpected that loss of somatostatin tone enhanced tumor formation induced by GHRH overexpression. GHRH however seemed to act by affecting cell cycle and cyclin-dependent kinase inhibitors (p27), whereas action of somatostatin was proliferation independent and may be through modulation of apoptotic and senescence pathways. Array analysis comparing hyperplastic and adenomatous pituitaries showed differential regulation of genes involved in, amongst others, proliferation, apoptosis and microtubule formation (mitogen-activated protein kinase kinase1, adenomatosis polyposis coli binding protein (Mapre1), tumor necrosis factor receptor 1b (TNFR2)). Subsequent studies will undoubtedly assess the role of these genes in pituitary tumor formation in more detail.

New mechanisms – signaling in the pituitary and hypothalamus

ERK signaling in the pituitary is required for female but not male fertility

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Background: Males and females require different patterns of pituitary gonadotropin secretion for fertility. The mechanisms underlying these gender-specific profiles of pituitary hormone production are unknown but there is evidence to suggest that ERK1 and 2 are essential modulators of hypothalamic GnRH-mediated regulation of pituitary gonadotropin production and fertility.

Methods: Mice with a pituitary-specific depletion of ERK1 and 2 were generated and a range of physiological parameters including fertility was assessed.

Results: ERK signaling was found to be required in females for ovulation and fertility, but male reproductive function was unaffected. The effects of ERK pathway ablation on LH biosynthesis underlined this gender-specific phenotype, LHβ expression being dependent on ERK-dependent up-regulation of the transcription factor Egr1.

Conclusion: These findings help to elucidate the molecular basis of gender-specific regulation of the hypothalamic-pituitary-gonadal axis and sexually dimorphic control of fertility.

This paper aims to further our understanding of the sexually dimorphic control of reproductive function at the molecular level. The mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) signaling system comprises a three-level phosphorylation cascade (MAPK-kinase-kinase Raf1, MAPK kinases MEK1 and MEK2, and MAPKs ERK1 and ERK2) that is activated upon a range of extracellular stimuli and is activated in gonadotrophs by GnRH. However, up until now, the *in vivo* function of this pathway has not been assessed. Mice with P-lox elements surrounding the *ERK2* locus were crossed with α -*GSU*-Cre mice resulting in deletion of ERK2 in gonadotrophs and thyrotrophs, and then crossed to *ERK1* null mice, rendering gonadotrophs and thyrotrophs ERK1/2 deficient. Although thyrotrophs and ovaries were also ERK1 deficient, the authors argue that this is unlikely to have affected the phenotype of the mice. Ablation of the ERK signaling pathway in pituitary gonadotrophs led to infertility in female but not male mice. Female mice did not have estrous cycle activity and did not mate. Ovaries contained follicles of various maturation, but lacked luteal tissue. LH β synthesis was reduced in gonadotrophs and ovulation occurred in response to exogenous LH β , implicating LH deficiency as the primary cause of infertility. In males, LH β expression was only slightly decreased and fertility was unaffected, reflecting the lower levels of LH that are required for Leydig cell function. This work implies ERK signaling as the sexually dimorphic link between gender-specific GnRH pulsatility and LH β synthesis. In addition, it suggests that FSH synthesis is less dependent on ERK signaling than LH synthesis.

Hypothalamic and pituitary c-Jun N-terminal kinase 1 signaling coordinately regulates glucose metabolism

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Proc Natl Acad Sci USA 2010;107:6028–6033

Background: c-Jun N-terminal kinase (JNK) 1-dependent signaling plays a crucial role in the development of obesity-associated insulin resistance.

Methods and Results: This work demonstrates that JNK activation not only occurs in peripheral tissues, but also in the hypothalamus and pituitary of obese mice. To resolve the importance of JNK1 signaling in the hypothalamic/pituitary circuitry, the authors generated mice with a conditional inactivation of *JNK1* in nestin-expressing cells (*JNK1(DeltaNES)* mice). *JNK1(DeltaNES)* mice exhibit improved insulin sensitivity both in the CNS and in peripheral tissues, improved glucose metabolism, as well as protection from hepatic steatosis and adipose tissue dysfunction upon high-fat feeding. Moreover, *JNK1(DeltaNES)* mice also show reduced somatic growth in the presence of reduced circulating growth hormone and insulin-like growth factor 1 concentrations, as well as increased thyroid axis activity.

Conclusion: These experiments reveal an unexpected, critical role for hypothalamic/pituitary JNK1 signaling in the coordination of metabolic/endocrine homeostasis.

Recent yearbooks discussed in length the new concept of glucose homeostasis and body weight being under control of hypothalamic circuits regulating food intake, energy expenditure, and hepatic glucose production. Obesity causes an increased production of cytokines and inflammatory and stress signaling which results in activation of c-jun terminal kinase (JNK) and thereby insulin resistance. Indeed, *JNK1*^{−/−} mice are protected from insulin resistance and obesity-induced hyperglycemia and are leaner. However, obesity does not only result in a reduction of insulin sensitivity in classical peripheral tissues, but also in the central nervous system thereby causing neuronal insulin and leptin resistance. In this work, mice were generated that have an ablation of *JNK1* specifically in neurons. The authors started by showing that high fat feeding not only promotes JNK1 activation in peripheral tissues and CNS but also in the pituitary. Since Nestin is expressed not only in neurons but also in pituitary stem cells, use of a Nestin-Cre mouse will allow for ablation of genes in all pituitary cell lines. The authors therefore generated mice in which *JNK1* was ablated in Nestin-expressing cells and confirmed ablation of *JNK* in both the hypothalamus and pituitary. Central *JNK1* ablation resulted in increased insulin sensitivity with an increased Akt activation in response to insulin, and protection of diet induced insulin resistance. However, the mice also had reduced growth (approx. 20%) without increased adiposity as a result of a reduction in pituitary GHRH receptors, pituitary GH and peripheral

IGF1. Unexpectedly the thyroid axis was also affected, with an increase in TRH, TRH receptors, TSH β , and T3, a phenotype that is reminiscent of healthy ageing (increased insulin sensitivity accompanied by reduced glucose, insulin, and GH levels). This work therefore revealed an unexpected role of hypothalamic and pituitary JNK1 in the regulation of GH secretion and growth, combined with an increase in central insulin sensitivity and increased thyroid activity.

New mechanisms – GH deficiency

Growth hormone (GH)-releasing hormone increases the expression of the dominant-negative GH isoform in cases of isolated GH deficiency due to GH splice-site mutations

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Endocrinology 2010;151:2650–2658

Background: IGHD II, the autosomal dominant form of isolated GH deficiency, can be due to heterozygous splice site mutations that weaken recognition of exon 3 leading to aberrant splicing of *GH-1* transcripts and production of a dominant-negative 17.5-kDa GH isoform. Previous studies suggested that the extent of mis-splicing varies with different mutations and the level of GH expression and/or secretion.

Methods: To study the functional relation between mutations and GH secretion, wt-hGH and/or different hGH-splice site mutants (*GH-IVS+2*, *GH-IVS+6*, *GH-ISE+28*) were transfected in rat pituitary cells expressing human GHRH receptor (GC-GHRHR).

Results: As expected, upon GHRH stimulation, GC-GHRHR cells coexpressing wt-hGH and each of the splice site mutants displayed reduced hGH secretion and intracellular GH content when compared with cells expressing only wt-hGH, confirming the dominant-negative effect of 17.5-kDa isoform on the secretion of 22-kDa GH. Furthermore, increased amount of 17.5-kDa isoform produced after GHRH stimulation in cells expressing GH-splice site mutants reduced production of endogenous rat GH, which was not observed after GHRH-induced increase in wt-hGH.

Conclusion: The severity of IGHD II depends on the position of the splice site mutation and the production of increasing amounts of 17.5-kDa protein, which reduces the storage and secretion of wt-GH in the most severely affected cases. Due to the absence of GH and IGF-I-negative feedback in IGHD II, a chronic up-regulation of GHRH would lead to an increased stimulatory drive to produce more 17.5-kDa GH from the severest mutant alleles, thereby accelerating autodestruction of somatotrophs in a vicious cycle.

Splice site mutations in the *GH-1* gene, like *GH-IVS+1*, *GH-IVS+2*, *GH-IVS+6* and *GH-ISE+28*, result in deletion of exon 3, resulting in production of a 17.5-kDa GH isoform. This isoform is retained in the ER, disrupts the Golgi apparatus and prevents normal production and secretion of the 22-kDa isoform, so having a dominant-negative effect. Significant variation in severity and age of onset exists in patients with IGHD type II [15], with some patients showing progressive hypopituitarism, depending on the *GH-1* gene alterations, but the exact reason is unclear. This study is a good example of bedside to bench work, aiming to study this clinical phenomenon using basic research tools. Mice expressing a high-copy number of a transgene mimicking the *GH-IVS+1* mutation exhibit a dwarfed phenotype, and show disruption of somatotrophs and invasion of macrophages in the pituitary, and also have deficits of other pituitary hormones, the phenotype being less severe in low-copy number mice. Since the main driver of GH production is GHRH, which is overexpressed in IGHD, this study aimed to mimic the in vivo situation in vitro by transfecting wt-GH and three different exon 3 splice site mutations (*GH-IVS+2*, *GH-IVS+6*, and *GH-ISE+28*) which cause variable IGHD II in human patients, in a rat pituitary cell line stably expressing GHRH receptors. The results show that the three hGH splice site mutants produce different amounts of 17.5-kDa hGH after GHRH stimulation and exert

different degrees of a dominant negative effect on endogenous wt-GH production. There is no dominant effect at the transcriptional level, but accumulation of the 17.5-kDa protein in the proteosomal degradation pathway results in impaired cell function and storage and secretion of wt-GH and finally leads to somatotroph destruction. The GH-IVS+2 mutation, which results in the most severe clinical presentation of the three, results in the most severe dominant negative effect on endogenous wt-GH production and secretion. Up-regulation of GHRH likely results in a vicious circle of increased 17.5-kDa GH production and somatotroph destruction and further up-regulation of GHRH. This study demonstrates how carefully designed in vitro experiments may not only be able to explain the mechanism of a disease, but also predict severity and progress of disease.

Follow-up on Yearbook 2009 – pituitary tumorigenesis

E2F1 induces pituitary tumor transforming gene (PTTG1) expression in human pituitary tumors

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Mol Endocrinol 2009;23:2000–2012

Background: Rb(retinoblastoma protein)/E2F is dysregulated in murine and human pituitary tumors. Pituitary tumor transforming gene (PTTG1), a securin protein, is required for pituitary tumorigenesis, and PTTG1 deletion attenuates pituitary tumor development in *Rb*+/- mice. This paper examines the regulation of PTTG1 by E2F1.

Methods: Immunofluorescence, transient transfection of cultured cells, chromatin immunoprecipitation, RNA down-regulation with siRNA.

Results: E2F1 and PTTG1 were concordantly overexpressed in most of 46 *Rb*+/- murine pituitary tissues and also in over half of 80 human pituitary tumors. E2F1 specifically bound the *hPTTG1* promoter, indicating that *hPTTG1* may act as a direct E2F1 target. Transfection of E2F1 and its partner DP1 dose-dependently activated *hPTTG1* transcription up to 3-fold in p53-devoid H1299 cells but not in p53-replete HCT116 cells. E2F1 overexpression enhanced endogenous *hPTTG1* mRNA and protein levels up to 3-fold in H1299 cells. The presence of endogenous p53/p21 constrained the induction, whereas decreasing either p53 or p21 in HCT116 cells restored E2F1-induced *hPTTG1* transactivation and expression. Moreover, suppressing Rb by small interfering RNA concordantly elevated E2F1 and *hPTTG1* protein levels. In contrast, transfection of E2F1 small interfering RNA lowered *hPTTG1* levels 24 h later in HCT116 than in H1299 cells, indicating that p53 delays E2F1 action on *hPTTG1*.

Conclusion: These results elucidate a mechanism for abundant tumor *hPTTG1* expression, whereby Rb inactivation releases E2F1 to induce *hPTTG1*.

Pituitary tumors account for approximately 15% of intracranial tumors in adults. As is true for tumor formation of most tissues, aberrant cell cycle regulation plays a major role in pituitary tumor formation. Retinoblastoma protein (Rb) controls G1/S phase cell phase transition and *Rb*+/- mice spontaneously develop pituitary tumors. PTTG is a proto-oncogene, essential for proper chromatoid separation, and facilitates cell cycle progression. Overexpression of PTTG in mice facilitates pituitary tumor development and *hPTTG1* overexpression in human tumors correlates with tumor invasiveness, recurrence and prognosis. Quite a lot is known about downstream targets and action of PTTG (for example up-regulation of bFGF, VEGF and c-myc) (see *Yearbook 2009*, Pituitary [16, 17]), but little is known about proximal regulatory mechanisms. This paper now convincingly identifies E2F1 as a direct regulator of *hPTTG* in several ways, including identification of potential binding sites in the *hPTTG1* promoter. E2Fs are key interacting factors for Rb proteins and are universal regulators of G1/S transition and cell cycle progression [18]. This paper enhances our knowledge of Rb-E2F1-*hPTTG1* signaling and the requirement of *hPTTG1* for pituitary tumorigenesis.

Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice

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Proc Natl Acad Sci USA 2010;107:7467–7472

Background: Ghrelin is able to enhance food intake and growth hormone secretion from pituitary cells, but its essential function is obscure since elimination of the gene encoding ghrelin or its receptor produces only mild phenotypes in mice. Ghrelin is an octanoylated peptide, and Ghrelin O-acyltransferase (GOAT) attaches octanoate to proghrelin, which is processed to ghrelin. Here, the authors eliminate the *Goat* gene in mice, thereby eliminating all octanoylated ghrelin from blood.

Methods: Goat knockout mice (*Goat*^{−/−}) were generated and used in physiological experiments.

Results: *Goat*^{−/−} mice grew and maintained the same weights as wild-type (WT) littermates on normal or high fat diets. When subjected to 60% calorie restriction, both WT and *Goat*^{−/−} mice lost 30% of body weight and 75% of body fat within 4 days. In both lines, fasting blood glucose initially declined equally. After 4 days, glucose stabilized in WT mice at 58–76 mg/dl. In *Goat*^{−/−} mice however, glucose continued to decline, reaching 12–36 mg/dl on day 7. At this point, WT mice showed normal physical activity, whereas *Goat*^{−/−} mice were moribund. GH rose progressively in calorie-restricted WT mice and less in *Goat*^{−/−} mice. Infusion of either ghrelin or GH normalized blood glucose in *Goat*^{−/−} mice and prevented death.

Conclusion: An essential function of ghrelin in mice is elevation of GH levels during severe calorie restriction, thereby preserving blood glucose and preventing death.

Ghrelin was identified more than a decade ago as the endogenous ligand for the growth hormone secretagogue receptor, located in the hypothalamus and pituitary, which on stimulation led to release of growth hormone from the pituitary. Pharmacological studies have since shown ghrelin's potential to increase food intake, gut motility and growth hormone release. It has been much harder to identify the physiological role of ghrelin since deletion of ghrelin, its receptor or both, has no significant impact on growth or appetite, at least in mice [19]. Results of this study suggest that ghrelin is important for the maintenance of the blood-glucose concentrations needed for survival during prolonged nutrient restriction. Ghrelin is unique in that its third amino acid, serine, is modified by the attachment of a medium chain fatty acid, octanoate, and this modification is necessary for its action. In this paper, the authors take a new approach to show the physiological role of ghrelin by generating mice that lack the enzyme GOAT, which is required for the octanoylation of ghrelin. The enzyme is located in the stomach and small intestine and lacks, as far as is known, any other function. *Goat*^{−/−} mice indeed did not have detectable acyl-ghrelin. Mice fed a normal ad libitum diet did not show any abnormalities, much like *ghrelin* or *Ghs-r* null mice, but experiments in this paper went one step further to expose the mice to prolonged severe nutrient restriction. Normal mice were able to cope and maintain normoglycemia in contrast to the *Goat*^{−/−} mice that became hypoglycemic and died. In addition, serum GH concentration was much further increased when glucose concentration declined in WT mice, compared to *Goat*^{−/−} mice, and GH treatment of *Goat*^{−/−} mice prevented hypoglycemia without raising IGF1 or ghrelin. These data suggest a role for ghrelin in the release of GH during times of calorie restriction to preserve normoglycemia likely through the effect of GH on gluconeogenesis. The evidence presented reminds us also that GH is pivotal in preserving normal blood-glucose levels. This is supported by the lower concentrations of GH observed in the GOAT knockout mice and the rescue of blood glucose by infusion of GH or ghrelin.

Use of mutant mouse lines to investigate origin of gonadotropin-releasing hormone-1 neurons: lineage independent of the adenohypophysis

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Endocrinology 2010;151:766–773

Background: Mutant mouse lines have been used to study the development of specific neuronal populations and brain structures as well as behaviors. GnRH is essential for vertebrate reproduction, with either GnRH-1 or GnRH-3 controlling release of gonadotrophins from the anterior pituitary, depending on the species. It is clear that the neuroendocrine GnRH cells migrate from extra-central nervous system (CNS) locations into the forebrain. However, the embryonic origin of GnRH-1 and GnRH-3 cells is controversial and has been suggested to be nasal placode, adenohypophyseal (anterior pituitary) placode, or neural crest, again dependent on the species examined. In two zebrafish knockouts, *you-too* (*Gli2*^{-/-}) and *detour* (*Gli1*^{-/-}), it was reported that when the adenohypophysis was missing or reduced, so were the hypothalamic GnRH neurons, although olfactory structures were intact [20].

Methods: In this report, single [(*Gli2*^{-/-}), (*Gli1*^{-/-}) and (*Lhx3*^{-/-})] and double (*Gli1*^{-/-} *Gli2*^{-/-}) mutant mice were used to examine the lineage of GnRH-1 cells. Standard immunohistochemistry was performed and GnRH-1 cells counted in embryos in three areas: nasal region, nasal-forebrain junction, and forebrain. For a subset of embryos, forebrain GnRH-1 cells were further characterized as being in the olfactory bulb/rostral forebrain region, the preoptic area, or the hypothalamus.

Results: Mutant mice with either missing or disrupted anterior pituitaries (*Gli2*^{-/-}, *Gli1*^{-/-}/*Gli2*^{-/-} and *Lhx3*^{-/-}) exhibited a normal GnRH-1 neuronal population and these cells were still associated with the developing vomeronasal organ. At E15.5, the proportion of the GnRH-1 population in nasal areas versus the forebrain was the same in controls (30 and 70%), single knockouts (KOs) and double knockouts (35 and 65% respectively). At E18.5, in *Gli* single and double KOs, GnRH-1 cells within the forebrain showed a similar distribution. There was no difference in the brain distribution among genotypes. Within the CNS, cells were detected in the olfactory bulb/rostral forebrain, preoptic area and hypothalamus. This distribution was similar to control mice. Analysis of the mutants revealed that several diencephalic regions were normal in both single- and double-*Gli* KOs. The vomeronasal organ was reduced in size in *Gli2*^{-/-}, *Gli1*^{+/-}/*Gli2*^{-/-}, and double KOs, but was structurally normal. The pituitary was normal in *Gli1*^{-/-} mice but there was a missing adenohypophysis in 4 of 5 *Gli2*^{-/-} and *Gli1*^{+/-}/*Gli2*^{-/-} mice, and all *Gli1*^{-/-}, *Gli2*^{-/-} mice had complete loss of the adenohypophysis. *Lhx3*^{-/-} mice were characterized by the presence of an undifferentiated anterior pituitary that was still connected to the oral ectoderm; however there was no difference in GnRH-1 cell number or distribution.

Conclusion: These results indicate that in mice, GnRH-1 cells develop independent of the adenohypophyseal placode and are associated early with the formation of the nasal placode.

The origin of GnRH-1 cells has been the subject of much debate. In mammals, the GnRH-1 cells are first identified in the nasal placode which also gives rise to the nonsensory respiratory epithelium, the olfactory epithelium and the vomeronasal organ. In the mouse, it has been suggested that the GnRH-1 progenitor cells are located in an intermediate area between the anterior respiratory cells and the OE sensory cells. In the chick, GnRH-1 transcripts were identified in the primitive streak and later in the anterior neural ridge which gives rise to the anterior pituitary, with subsequent localization to the presumptive nasal cavity and olfactory placode. Ablation experiments suggested that the GnRH-1 lineage was developmentally associated with the respiratory area of the nasal placode. However, in two zebrafish KOs in the Sonic Hedgehog pathway, *you-too* and *detour*, it was reported that absence or hypoplasia of the adenohypophysis was associated with absence or reduction of GnRH neurons, yet with intact olfactory structures. The authors of this study have used these mutants in the mouse to try and establish the lineage of GnRH-1 neurons. Their data seem to confirm that the origin of the GnRH-1 cells may be independent of the adenohypophysis. However, it is important to note that the murine genetic models that they have used may not be associated with GnRH-1 cell abnormalities as the genes that have been mutated may be implicated at later stages of pituitary

development; hence, one cannot rule out a very early common origin of GHRH-1 and pituitary progenitor cells. Additionally, whereas *Gli1*^{-/-} mutants are not associated with an abnormal pituitary in the mouse, in zebrafish, the mutants are associated with a small pituitary. Hence, species differences may account for some of the discrepant results. Many unanswered questions remain, for example mutations of *SOX2* in humans are associated with hypogonadotrophic hypogonadism in association with a small anterior pituitary gland, whereas the rest of the pituitary hormones are largely unaffected apart from occasional GH deficiency. However, in the mouse, there is a general reduction in all the anterior pituitary hormones [7].

New image

Cellular in vivo imaging reveals coordinated regulation of pituitary microcirculation and GH cell network function

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Proc Natl Acad Sci USA 2010;107:4465–4470

Background: Growth hormone (GH) exerts its actions via coordinated pulsatile secretion from a GH cell network into the bloodstream. The pulsatile release is dependent upon a carefully orchestrated pattern of GHRH and somatostatin secretion. Practically nothing is known about how the network receives its inputs in vivo and releases hormones into pituitary capillaries to shape GH pulses.

Methods: The authors have developed in vivo approaches using transgenic GH-eGFP mice to measure local blood flow, oxygen partial pressure, and cell activity at single-cell resolution in mouse pituitary glands in situ. They developed optical imaging methods that can monitor directly in vivo the relationship between the blood vasculature and GH cell network function. These methods involved the modification of a fluorescent stereomicroscope with long working distance objectives to image at wide field and single cell resolution an exposed pituitary gland deep in its in vivo environment.

Results: Single capillaries were identified in close proximity to structural GH cell network motifs. When secretagogue (GHRH) distribution was modeled with fluorescent markers injected into either the bloodstream or the nearby intercapillary space, a restricted distribution gradient evolved within the pituitary parenchyma. Injection of GHRH led to stimulation of both GH cell network activities and GH secretion, which was temporally associated with increases in blood flow rates and oxygen supply by capillaries, as well as oxygen consumption. The authors also report a time-limiting step for hormone output at the perivascular level; macromolecules injected into the extracellular parenchyma moved rapidly to the perivascular space, but were then cleared more slowly in a size-dependent manner into capillary blood.

Conclusion: These data suggest that GH pulse generation is not simply a GH cell network response, but is shaped by a tissue microenvironment context involving a functional association between the GH cell network activity and fluid microcirculation, with corresponding changes in oxygen supply and oxygen consumption.

In this work, in vivo techniques in transgenic GH-eGFP mice to measure local blood flow, oxygen partial pressure, and cell activity at single-cell resolution in mouse pituitary glands in situ, were exploited to monitor directly the relationship between the blood vasculature and GH cell network function in vivo. These elegant studies have begun to shed light on the highly complex, yet poorly understood processes leading to pulsatile GH secretion. It is clear that the secretion of GH by the GH cell network is dependent upon the fine regulation of hypothalamic inputs by the pituitary microcirculation, the supply and consumption of oxygen by the vasculature and GH cells, and the dynamic uptake of secreted products by the efferent blood capillaries. The study of these processes in an in vivo setting will revolutionize our understanding of GH secretion, and will probably impact on our understanding

of the pathophysiology associated with GH deficiency and related disorders, e.g. in children with tumors, congenital GH deficiency and those who have impaired GH secretion as a result of traumatic brain injury and radiotherapy.

Reviews

Minireview: the melanocortin 2 receptor accessory proteins

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Mol Endocrinol 2010;24:475–484

The melanocortin 2 receptor (MC2R) accessory protein, MRAP, is one of a growing number of G-protein-coupled receptor accessory proteins that have been identified in recent years. GPCR accessory proteins modulate GPCR function, direct receptor trafficking and targeting, moderate signaling intensity, and modify receptor structure and ligand binding. MRAP interacts directly with MC2R and is essential for its trafficking from the endoplasmic reticulum to the cell surface, where it acts as the receptor for the pituitary hormone ACTH. In addition, MRAP2, a newly described homolog of MRAP, is also able to support the cell surface expression of MC2R. The mechanism of MRAP action is only beginning to be understood although it is clear that MRAP is required for MC2R function. Recent work has started to reveal which MRAP domains are involved in MC2R functional expression, and new data have shown a potential role for both MRAP and MRAP2 in the regulation of the other melanocortin receptors. This is an excellent review that highlights the importance of the various components of melanocortin receptor (MCR) signaling. MCRs act as receptors for α -, β -, and γ -MSH and ACTH and have a diverse range of functions – MC1R in skin pigmentation, MC2R as the receptor for ACTH, MC3R and MC4R in energy homeostasis. MRAPs act as accessory proteins for MC2R and are required for MC2R to travel to the cell surface and for ACTH signaling, and mutations cause ACTH resistance, i.e. familial glucocorticoid deficiency. The review covers the similarities and differences of MRAP and MRAP2, their structure and function in ACTH signaling, and also discusses their MC2R independent function and ability to modulate function of other MCRs. This review facilitates the understanding of MCR signaling and its relation with disease.

Food for thought – curcumin

Growth suppression of mouse pituitary corticotroph tumor AtT20 cells by curcumin: a model for treating Cushing's disease

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PLoS One 2010;5:e9893

Background: Pituitary corticotroph tumors secrete excess adrenocorticotrophic hormone (ACTH) resulting in Cushing's disease. Standard treatment includes surgery and, if not successful, radiotherapy, both of which have undesirable side effects and frequent recurrence of the tumor. Pharmacotherapy using PPAR γ agonists, dopamine receptor agonists, retinoic acid or somatostatin analogs is still experimental. Curcumin, a commonly used food additive in South Asian cooking, has potent growth inhibitory effects on cell proliferation, possibly by inhibiting constitutively activated NF κ B, and selectively targets

tumor cells. Increased expression of the pro-survival protein Bcl-2 is commonly observed in pituitary tumors. The pro-survival Bcl-2 family of proteins (Bcl-2, Bcl-xL, and Mcl-1) are target genes of NF κ B. The authors recently demonstrated that curcumin inhibited growth and induced apoptosis in prolactin- and growth hormone-producing tumor cells. Subsequently, Schaaf et al. [21] confirmed these data and also showed the *in vivo* effectiveness of curcumin in suppressing pituitary tumorigenesis. However, the molecular mechanisms that mediate this effect of curcumin are still unknown.

Results: Using the mouse corticotroph tumor cells, AtT20 cells, the authors report that curcumin had a robust, irreversible inhibitory effect on cell proliferation and clonogenic property; significant inhibition of colony formation was observed with concentrations of 5 μ M, and complete inhibition was observed with 20 μ M. The curcumin-induced growth inhibition was accompanied by a dose-dependent decrease in constitutive NF κ B activity. Further, curcumin down-regulated the pro-survival protein Bcl-xL, depolarized the mitochondrial membrane, and increased PARP cleavage, which led to increased apoptosis. Finally, curcumin had a concentration-dependent suppressive effect on ACTH secretion from AtT20 cells.

Conclusion: The ability of curcumin to inhibit NF κ B and induce apoptosis in pituitary corticotroph tumor cells suggests that it might be used as a novel therapeutic agent for the treatment of Cushing's disease.

The treatment of Cushing syndrome is not straightforward and often needs a combination of medical treatment, surgery and radiotherapy. Although rare in children, it can be associated with life-long complications and impact on normal growth and development and also lead to diabetes mellitus, osteoporosis and hypertension. Hence, any effective alternative therapies need to be explored. In this study, the authors have investigated the use of the potent proliferation inhibitor curcumin, which has previously been used in Indian cooking, as an anti-tumor agent. In the Indian population, it is estimated that the average daily consumption of curcumin is 60–100 mg. The results are certainly interesting, although the *in vivo* effects have not as yet been established in humans. Additionally, although no toxic effects are known at present, potential side effects will need to be considered, especially before use in children and adolescents; *in vitro*, curcumin decreased secretion of ACTH and its effects are irreversible. The proposed mechanism of action of curcumin is interesting. It suppresses the TNF α -induced activation of IKK that leads to the inhibition of TNF-dependent phosphorylation and degradation of I κ B α and subsequent nuclear translocation of the p65 subunit of NF κ B to regulate expression of genes implicated in cell cycle progression (e.g. cyclin D family), apoptosis (*bcl-2*), and cell migration and invasion (e.g. *MMP2* and *MMP9*). Constitutive activation of NF κ B has been reported in cell lines as well as tumor samples, and may be linked to tumor progression as well as drug resistance. Bcl-xL plays a central role in pituitary cell survival and apoptosis, and these studies suggest that Bcl-xL plays a major role in regulating cell survival in pituitary corticotrophs. Curcumin may therefore modify Bcl-xL levels and hence act as a tumor suppressor, offering new hope to the treatment of Cushing disease.

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