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Identification of differentially expressed genes in the zebrafish hypothalamus - pituitary axis

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Abstract

The vertebrate hypothalamic-pituitary axis (HP) is the main link between the central nervous system and endocrine system. Although several signal pathways and regulatory genes have been implicated in adenohypophysis ontogenesis, little is known about hypothalamic and neurohypophysial development or when the HP matures and becomes functional. To identify markers of the HP, we constructed subtractive cDNA libraries between adult zebrafish hypothalamus and pituitary. We identified previously published genes and ESTs and novel zebrafish genes, some of which were predicted by genomic database analysis. We also analyzed expression patterns of these genes and found that several are expressed in the embryonic and larval hypothalamus, neurohypophysis, and/or adenohypophysis. Expression at these stages makes these genes useful markers to study HP maturation and function.

Keywords

Adenohypophysis; cell differentiation; hormone; hypothalamic-pituitary axis; hypothalamus; neurohypophysis; organ maturation; pituitary; subtractive cDNA library

1. Results and Discussion

The hypothalamic-pituitary axis (HP) is the main link between the central nervous system and endocrine system. The HP regulates hormone secretion during homeostasis, growth, reproduction, and several other physiological functions. Under control of the hypothalamus, the adenohypophysis (anterior pituitary lobe) produces and secretes hormones involved in these functions. This control occurs through the production of hypothalamic hypophysiotropic hormones that stimulate or inhibit synthesis and release of adenohypophysial hormones. The hypothalamus also secretes hormones such as vasotocin and oxytocin (in mammals), and

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isotocin and vasopressin (in fish) through its neuronal projections into the neurohypophysis (Bentley, 1998; Unger and Glasgow, 2003).

Several genes regulate induction, specification, survival, patterning, and cell differentiation in the adenohypophysis (reviewed in (Dasen and Rosenfeld, 2001; Pogoda and Hammerschmidt, 2007; Zhu et al., 2005). However, little is known about the genetic regulation of later hypothalamic and neurohypophysial development or even when the HP matures and becomes physiologically functional.

To identify developmental markers of the HP, we constructed subtractive cDNA libraries between adult hypothalamus and pituitary and examined expression of these genes in embryos and larvae. We report the construction of these subtractive libraries and the identification of genes represented in them. We also present the expression patterns of some of these genes in the HP during the first 5 days of development. Some of these genes are previously known expressed sequence tags (ESTs), with undocumented or incompletely documented HP expression patterns. Other genes were previously unreported in zebrafish, some of which are novel and some of which are homologous to genes in other species.

1.1. Library construction and clone identification

To identify genes expressed in the HP, we constructed subtractive cDNA libraries between adult zebrafish hypothalamus and pituitary. The hypothalamic cDNA library was obtained by subtracting pituitary cDNAs from hypothalamic cDNAs: 448 clones were isolated (Table 1). Conversely, the hypothalamic cDNAs were subtracted from pituitary cDNAs to construct the pituitary cDNA library: 558 clones were isolated (Table 1). We eliminated clones that contained the most redundant sequences by membrane hybridization. We then characterized the remaining clones by sequencing and comparison with NCBI nucleotide and protein databases and the Sanger Institute *Danio rerio* genomic database.

The subtraction process enriched cDNAs that are differentially expressed in hypothalamus or adenohypophysis. Our initial analysis suggested that the proportion of house keeping genes, such as mitochondrial and ribosomal genes, is low (Table 1). With the exception of the *spermatogenesis associated protein 2* gene that we found in both libraries (1 sequence in the pituitary library, 2 similar sequences in the hypothalamic library), no other overlap was observed between the libraries, including mitochondrial and ribosomal sequences (not shown).

The sequences we identified consisted of previously published genes (known genes) and ESTs or novel genes (genes not cloned or identified in zebrafish yet or predicted only by genomic database analysis). Based on studies in other species and analysis of conserved domains, these genes can be classified into functional categories that include structural and physiological functions, cell metabolism, and protein synthesis.

We identified only one transcription factor in the pituitary library: the *POU domain class1 transcription factor 1 (pou1f1)* that plays a role in somatotrope, lactotrope, and thyrotrope development (Table 2; Gage et al., 1996; Nica et al., 2004). Because transcription factors are expressed at a lower frequency compared to many other mRNAs, the PCR amplification and subtraction method we used might not have been sensitive enough to identify genes expressed at these low levels. Alternatively, it is possible that many transcription factors are expressed in both tissues and were subtracted during the construction of the libraries. Also, some tissue specific transcription factors are probably no longer expressed in differentiated adult tissues.

We also identified genomic DNA sequences that do not apparently correspond to genes. Some might represent non-coding RNA. Others that correspond to intronic sequences might have resulted from unspliced mRNA variants, although we did not find corresponding exon

sequences of these genes. We can also not entirely exclude the possibility that some of these sequences might derive from genomic DNA contamination. Because our goal was to isolate genes expressed in the HP, we did not analyze these sequences further.

The pituitary library (supplemental Table 1) contained genes encoding most of the adenohypophysial hormones including: *follicle stimulating hormone beta-subunit (fshb)*, *glycoprotein hormones alpha polypeptide (cga)*, *growth hormone (gh)*, *luteinizing hormone beta-subunit (lhb)*, *prolactin (prl)*, *proopiomelanocortin (pomc)*, *somatolactin beta (smtlb)* and *thyroid stimulating hormone beta subunit (tshb)*. One exception was *somatolactin alpha (smtla)* that we did not detect. Together, these represent 68.5% of all identified sequences.

Although *pomc* is expressed in both the pituitary and hypothalamus and should therefore have been subtracted, *pomc* was represented abundantly (36.4%) in the pituitary library, and was absent from the hypothalamic library. Presumably, this results from very high expression levels of *pomc* in the pituitary compared to the hypothalamus at the time of tissue dissection. In spite of our precautions to limit stress by MS-222 anesthesia and chilling on ice, *pomc* encoded ACTH biosynthesis might have been elevated in response to stress (Engelmann et al., 2004).

In the hypothalamic library (supplemental Table 2), *ependymin (epd)* was the most frequently represented gene (36.7%). This gene encodes a specific CNS glycoprotein, an adhesion molecule involved in memory consolidation (Schmidt, 1995; Schmidt et al., 1995; Shashoua, 1991). We found only one gene that has been reported to regulate pituitary hormone production and/or secretion: *adenylate cyclase activating polypeptide 1b (adcyap1b)*, 1.4%. This gene, also known as *pituitary adenylate cyclase activating peptide (pacap)*, functions in various species as a hormone releasing factor (Gracia-Navarro et al., 2002; Zhou et al., 2005). We also found genes corresponding to neuropeptides, such as *proenkephalin-like* (1.7%) and *prodynorphin* (0.3%) that belong to the opioid gene family (Dores et al., 2002), and *pro-melanin concentrating hormone-like* encoding the hypothalamic hormone, Melanin concentrating hormone (Mch; 0.6%; manuscript in preparation).

1.2. Gene expression patterns during the first 5d of development

The genes represented in these libraries were expressed in a tissue-specific manner in the mature, functional HP of adults. Therefore, the onset of expression during development of the hypothalamus, adenohypophysis, and neurohypophysis would make these genes interesting potential markers for the functional maturation of the HP.

To test whether the genes isolated in the adult pituitary and hypothalamic libraries are expressed in embryos and larvae, we analyzed their expression patterns between Prim-5 (24h) stage and day 5 (5d) of development. We analyzed whole-mount embryos by mRNA *in situ* hybridization, if the length of the isolated sequence permitted such analysis (45 genes out of 116 total; 39% genes), or consulted the ZFIN gene expression database (<http://zfin.org>, 57 genes out of 116 total; 49% genes).

Because we subtracted cDNAs exclusively between hypothalamic and pituitary tissues and not from the entire fish, several genes were also expressed in other tissues, for example in the CNS, or throughout the animal (Suppl. Fig. 1–21). In addition, because we constructed the libraries from adult fish, some genes were not expressed between Prim-5 (24h) stage and 5d. Probably, these are expressed later during development and adulthood (see section 1.3).

Our analysis indicates that, among the genes with expression pattern data, 23.5% (24 out of 102 total) are expressed in the HP during the first 5 days of development (Table 2). 63.7% (65 out of 102 total) are expressed outside of the HP and 12.7% (13 out of 102 total) are not expressed during the first 5d of development. Because we analyzed gene expression by whole

mount *in situ* hybridization, it is possible that the sensitivity of this assay was insufficient to detect low expression levels. For instance, some gene expression not detected by *in situ* hybridization was successfully detected by PCR (see section 1.3; Suppl. Fig. 22).

1.2.1. Genes expressed in the adenohypophysis—All the genes expressed in the adenohypophysis during the first 5d of development were isolated from the pituitary library.

Adenohypophysial hormones and *pou1f1* were previously shown to be expressed in the adenohypophysis during the first 5 days of development (Table 2; (Herzog et al., 2003; Lopez et al., 2005; Nica et al., 2004; Pogoda et al., 2006).

secretogranin III (scg3) belongs to the granin family (chromogranin, secretogranin), a group of acid secretory proteins found in the secretory granules of endocrine cells. Even though their precise role is unclear, proteins of this family are thought to be involved in secretion (Holthuis et al., 1996; Holthuis and Martens, 1996). Consistent with this postulated role, *scg3* is found in zebrafish in endocrine organs such as adenohypophysis (Fig. 1A), pancreas, and epiphysis (not shown) at Prim-5 (24h) stage. Additional weak expression is observed in the telencephalon and midbrain. Expression in these domains continues through protruding mouth (72h) stage. *scg3* expression also appears in the retina and epiphysis by 5d (ZFIN, <http://zfin.org>). In the adenohypophysis, cells in the most anterior part of the gland, called the rostral pars distalis (RPD; Fig. 1A), express *scg3*.

hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase (hsd3b7) plays a role in steroid biosynthesis. It was identified in zebrafish using microarrays to analyze global hematopoietic and vascular endothelial gene expression (Covassin et al., 2006). In addition, *hsd3b7* is expressed in the adenohypophysis (Fig. 1C) and in the telencephalon (Suppl. Fig. 3) from Prim-5 (24h) stage to protruding mouth (72h) stage. We find that all adenohypophysial cells express the gene. Additional expression of *hsd3b7* outside of the HP is shown in Suppl. Fig. 3.

solute carrier family 16 (monocarboxylic acid transporters) member 9a (slc16a9a) belongs to the monocarboxylic acid transporter family. *slc16a9a* is expressed from Prim-5 (24h) stage to 5d in the RPD (Fig. 1B), which contains lactotrope, corticotrope, and melanotrope cells. Additional expression outside of the HP is visible from 72h and later (Suppl. Fig. 6).

adrenomedullin 2 (adm2) is a predicted gene that belongs to the *calcitonin/calcitonin related gene* family. In rat, it is also known as *intermedin (imdn)* and is expressed in the intermediate lobe of the pituitary and in specific adenocorticotrope cells in the anterior lobe (Lin Chang et al., 2005). Lin Chang et al. showed that PRL release increases in a dose dependent manner in response to Intermedin treatment. In female rats, these results suggested a role of Intermedin in the regulation of PRL release during reproduction.

In zebrafish, *adm2* is weakly expressed in the adenohypophysis at 24h. *adm2* expression intensifies by long-pec (48h) stage and is reduced at 72h (Fig. 1D). We observed no expression at 4d or 5d. *adm2* expressing cells in the adenohypophysis coexpress *pomc* in the RPD and the posterior part of the adenohypophysis called pars intermedia (PI). Lactotrope, corticotrope, and melanotrope cells are located in these subdomains of the adenohypophysis.

uo:ion002 is a novel gene. A peptide domain of the acyltransferase family was recognized as a putative conserved domain in the protein encoded by this gene, indicating a potential role of this gene in lipid metabolism. Expression of *uo:ion002* starts at 48h and is maintained until 5d. It is exclusively expressed in the PI which contains melanotrope cells (Fig. 1E).

1.2.2. Genes expressed in the hypothalamus—Genes expressed in the hypothalamus during the first 5d of development were found in the hypothalamic library except for *serpin peptidase inhibitor, clade I (neuroserpin), member 1* which was found in the pituitary library.

cytochrome P450 family 19 subfamily A polypeptide 1b (Lassiter and Linney, 2007) and *pro-melanin concentrating hormone like (pmchl)* are expressed in the hypothalamus during the first 5 days of development.

adenylate cyclase activating polypeptide 1b (adcyap1b), also known as *pituitary adenylate cyclase-activating peptide (pacap)* was originally discovered in hypothalamic extracts and was shown to stimulate cAMP accumulation in pituitary cells (Miyata et al., 1989; Miyata et al., 1990). Among several postulated roles, *pacap* is known as a releasing factor for hormones, including adeno-hypophysial hormones (Rawlings and Hezareh, 1996). *pacap* was shown to increase the secretion of Growth Hormone (Gracia-Navarro et al., 2002; Wong et al., 2000; Zhou et al., 2005), Gonadotropin (Petersen et al., 1996; Wong et al., 2000), Thyrotropin (TSH; Okada et al., 2006), and Prolactin (Rawlings and Hezareh, 1996).

In zebrafish, *adcyap1b* is expressed in the telencephalon at Prim-5 (24h) stage (Fig. 2A). At 48h (Fig. 2B) and 72h (Fig. 2C), the expression pattern expands throughout the anterior CNS, including the telencephalon, hypothalamus, ventral mesencephalon, and cerebellum. Double *in situ* hybridization with *pomc* mRNA probe indicates that the hypothalamic expression is located close to the adeno-hypophysis (Fig. 2D). This proximity is consistent with a role in pituitary hormone regulation. We did not observe *adcyap1b* expression in the neurohypophysis.

potassium channel tetramerization domain containing 4 (kctd4) is an EST. *kctd4* is expressed in the telencephalon and hypothalamus from Prim-5 (24h) stage through 5d. The hypothalamic expression at 24h is located close to the adeno-hypophysis (Fig. 2E). Later, this expression domain extends throughout the anterior hypothalamus, close to the adeno-hypophysis although not in the neurohypophysis (Fig. 2F–H). Weak hindbrain expression starts at 48h (Fig. 2F). *kctd4* is an interesting marker because its expression pattern resembles that of the hypophysiotropic gene *adcyap1b*.

calcium/calmodulin-dependent protein kinase 1D (camk1d) was known as an EST. The calcium/calmodulin-dependent protein kinase best documented in the literature is *camkII*. It is a multifunctional serine/threonine kinase acting by phosphorylating proteins which functions are Ca²⁺ dependent. CamkII is involved in a variety of functions, such as cell division, differentiation, synaptic plasticity and transmission, dendritic morphology plasticity, and cardiac contraction (Colbran and Brown, 2004; Griffith, 2004; Hudmon and Schulman, 2002).

In zebrafish, *camk1d* expression starts at 48h in the hypothalamus, telencephalon and midbrain (Fig. 2I). This expression persists at 72h (Fig. 2J–K). No expression was found in the neurohypophysis (Fig. 2K).

stathmin 1/ oncoprotein 18 b (stmn1b) was known as an EST. It plays a role in microtubule destabilization. In vertebrates, it is expressed in neurons and in cells with a potential to proliferate. The expression is reduced in terminally differentiated cells (Cassimeris, 2002).

In zebrafish, *stmn1b* is expressed in the hypothalamus, telencephalon and neural crest at 24h (Fig. 2L). *stmn1b* is expressed in the telencephalon, hypothalamus and mesencephalon at 48h (Fig. 2M) and 72h (Fig. 2N,O). Expression in the hypothalamus is near the adeno-hypophysis. Some, but not all neurohypophysial cells express *stmn1b* (Fig. 2O).

pleckstrin and Sec7 domain containing 3 like (psd3l) is a novel gene. According to conserved domains, it is a guanine nucleotide exchange factor for small GTPases. The Sec7 domain is the central domain.

psd3l is expressed in the hypothalamus and telencephalon at 24h (Fig. 2P). This expression is maintained through 48h (Fig. 2Q) and 72h (Fig. 2R,S). We found no expression in the neurohypophysis (Fig. 2S).

serpin peptidase inhibitor, clade I (neuroserpin), member 1 (serpini1) is a novel gene. It is a member of the serine proteinase inhibitor (serpin) gene family. It was shown in other species that it reacts preferentially with plasminogen activator in the brain. It is released from neurons and is involved in synaptic plasticity (Yepes and Lawrence, 2004).

serpini1 was found in the pituitary library, however we detected no expression in the adenohypophysis during the first 5d of development. Expression in the hypothalamus is visible from 24h (Fig. 2T). At 48h (Fig. 2U) and 72h (Fig. 2V,W), we observed additional expression in telencephalon and midbrain (Fig. 2U–W). Expression in the hypothalamus is close to the adenohypophysis but not in the neurohypophysis (Fig. 2W).

1.2.3. *uo:ion003* is expressed in both adenohypophysis and hypothalamus—

Genes represented in the subtractive libraries were expected to be expressed exclusively in the hypothalamus or in the pituitary. However, one gene, *uo:ion003* (from the pituitary library), was expressed both in adenohypophysis and hypothalamus during the first 5d of development and in adult brain (Fig. 3 and 4). *uo:ion003* is a novel gene. No putative conserved domains were detected. At 24h, *uo:ion003* is expressed in both hypothalamus and adenohypophysis (Fig. 3A). This expression persists through 48h (Fig. 3B) and 72h (Fig. 3C–D and Fig. 1F). In addition, *uo:ion003* is expressed in the telencephalon, midbrain and hindbrain at all stages. In the adenohypophysis, *uo:ion003* is expressed in the posterior pars distalis (Fig. 1F and Fig. 3D), but not in the area that corresponds to the neurohypophysis (Fig. 3D).

1.2.4. *crabp1a* is expressed in the neurohypophysis—

In teleosts, neurohypophysis and adenohypophysis do not form morphologically distinct lobes. Hypothalamic neurons project and interdigitate with the adenohypophysis. This interdigitation can be observed morphologically and is defined as the neurohypophysis (Chapman et al., 2005). During dissection, it was not possible to separate the neurohypophysis from the hypothalamus or the adenohypophysis. Consequently, neurohypophysial cDNAs contributed to hypothalamic and pituitary cDNA pools and might have been subtracted. However, depending on the location and expression level of particular mRNAs within the neurons contributing to the neurohypophysis (in the cell body, axon, or dendrites), the corresponding cDNAs might have been abundantly represented in either of the cDNA pools.

We identified *cellular retinoic acid binding protein 1a (crabp1a)* in the pituitary library. *Crabp1a* belongs to the intracellular lipid-binding protein family that binds fatty acids, retinoids, and steroids. In mammals, *Crabp1* function is not fully understood. Based on its expression in sites of ongoing plasticity that respond to retinoic acid, *Crabp1* has been proposed to play a role in retinoic acid targeting, metabolism, and presumably also in neurogenesis (Thompson Haskell et al., 2002; Zetterstrom et al., 1999; Zetterstrom et al., 1994; Zhou and Wei, 2001). Two orthologs of *crabp1* exist in the zebrafish genome: *crabp1a* that we identified in the present study and *crabp1b* (Liu et al., 2005; Sharma et al., 2003).

crabp1a is expressed at 24h in hypothalamic cells, near the adenohypophysis (Fig. 3E), and in the neurohypophysis at 48h (Fig 3F) and 72h (Fig 3G). Saggital sections of the adenohypophysis confirmed that *crabp1a* is expressed in the neurohypophysis, dorsal to the

adenohypophysis (visualized by *pomc* expression; Fig. 3H). In addition, *crabp1a* is also expressed in the telencephalon, in the tegmentum, and in the anterior spinal chord from 48h until 4d (Fig. 3F,G).

1.3. Gene expression in adults

Because we subtracted cDNAs from adult hypothalamus and pituitary tissues, genes we identified that were not expressed in the HP during the first 5d of development might presumably be expressed later during development and adulthood. To analyze expression in the adult, we performed PCR on adult hypothalamus and pituitary cDNAs and *in situ* hybridized mRNA probes for a subset of genes to adult brain.

We performed PCR on adult hypothalamus and adult pituitary cDNAs using primers for 20 genes represented in each library. Primers were designed to amplify a fragment between 350 and 500 bp (not the full length cDNA). In each library, 80–85% of the genes (17 genes from the pituitary library, 16 genes from the hypothalamic library) were amplified from both adult pituitary and hypothalamic cDNA. 15% of the genes (3 genes) were amplified only from the adult pituitary cDNA (genes from the pituitary library) or from the adult hypothalamic cDNA (genes from the hypothalamic library). Only one gene (from the hypothalamic library) was not amplified from either cDNA set. However, we cannot rule out a failure of the PCR or primers used (Suppl. Fig. 22). Apparently, these results are independent of the number of clones represented in the library for each gene. For example, genes that are represented in a large number of clones in the libraries (for example *epd* in the hypothalamic library or *pomc* in the pituitary library) were amplified from both sets of cDNAs, whereas genes represented by 1–3 clones were amplified from only one set of cDNA (Suppl. Fig. 22).

At first sight, these results appear inconsistent with the theoretically predicted outcome of the subtraction method, however, they are probably due to a higher sensitivity to gene expression differences than anticipated. When most of the genes analyzed were detected by PCR from both hypothalamic and pituitary cDNAs, there was almost no identical sequence represented in both libraries (including mitochondrial and ribosomal). Only *spermatogenesis associated protein 2* was represented in both hypothalamus and pituitary library, corresponding to 1 sequence in the pituitary library, 2 identical sequences in the hypothalamic library, out of 840 total sequences.

It is possible that hypothalamus and pituitary express different isoforms of the same gene. If this is true, the sequences represented in each library would be specific for the particular isoform. However, no identical genes were represented in both hypothalamic and pituitary library by different sequences. Interestingly, for some of the genes analyzed, we amplified a fragment of different size from hypothalamic cDNAs and from pituitary cDNAs. In some cases, two fragments of different sizes were amplified from one set of cDNAs, when a single fragment was amplified from the other set of cDNAs (Suppl. Fig. 22).

The fact that most of the analyzed genes were amplified by PCR from both sets of cDNAs can also be explained by a difference in the level of expression in the hypothalamus compared to the pituitary. Accordingly, genes represented in each library are specifically expressed or expressed at higher level in one tissue compared to the other. For example, *pomc* was represented abundantly only in the pituitary library, even though it is expressed in both pituitary and hypothalamus.

Even though the intensity of the amplified fragment obtained by PCR is obvious in some cases, we based our analyses on the presence or absence of amplification, without taking the intensity of the amplified product into account. Quantitative PCR would be the best way to address a difference in gene expression levels between hypothalamus and pituitary. However, because

the goal of this study was to identify useful markers for the HP, we instead analyzed the expression of some genes in adult brain by *in situ* hybridization.

Consistent with a differential expression level, some cDNAs (6 out of 13) amplified from both tissues were detected by *in situ* hybridization in only one of the tissues. *kctd4*, *psd3l*, *natriuretic peptide precursor C-like* and *uo:ion006* (from the hypothalamic library) expression was found only in hypothalamus and *uo:ion002* and *neuromedin s* (from the pituitary library) was found only in the adenohypophysis (Fig. 4). Three genes (out of 13) were detected by *in situ* hybridization in both hypothalamus and adenohypophysis (*proline-rich nuclear receptor coactivator 2*, *uo:ion001* (not shown) and *uo:ion003*, from the pituitary library; Fig. 4). The other 4 genes analyzed (*interleukin 1 receptor accessory protein-like 2* from hypothalamic library; *adrenomedullin 2*, *serpini1* and *uo:ion005* from the pituitary library) had no expression detectable by *in situ* hybridization.

Our subtractive library analysis from adult hypothalamus and pituitary identified genes expressed in the embryonic, larval, and adult HP and serves as the basis for a more extended analysis of HP development. Specifically, we will use these genes to analyze development, maturation, and function of the HP.

2. Experimental procedures

2.1. Tissue Dissection and Library Construction

We dissected 50 hypothalami and 179 pituitaries from adult zebrafish (see Suppl. Fig. 23) and isolated total RNA from each tissue. We synthesized and amplified cDNA from 1.5 µg of total RNA using the BD SMART™ PCR cDNA Synthesis Kit (BD Biosciences). The subtraction of pituitary cDNAs from hypothalamic cDNAs, and vice versa, was performed according to the Clontech PCR-Select™ cDNA Subtraction Kit (Clontech) protocol. We cloned the subtracted pituitary and hypothalamic cDNAs into pCRII-TOPO TA vector (Invitrogen) and stored the libraries as glycerol stocks of individual bacterial clones.

2.2. Clone identification and analysis

We screened the libraries by sequencing each clone and analyzing the resulting sequences by comparison against nucleotide and protein databases at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and against the Sanger Institute zebrafish genome database (Zebrafish Genome Annotation Browser v5 and v6, http://vega.sanger.ac.uk/Danio_rerio/).

After sequence analysis of the first 100 clones, we identified the most redundant sequences: *ependymin* was the most redundant clone in the hypothalamic library and *pomc* was the most redundant clone in the pituitary library. To eliminate additional copies of these clones before further sequencing of the libraries, we identified *pomc/ependymin* positive clones by membrane hybridization.

Glycerol stocks were spotted onto nylon membranes, DNA was UV cross-linked to membranes and hybridized with *ependymin* and *pomc* DNA digoxigenin probes (synthesized with PCR dig probe synthesis kit, Roche). Positive clones were detected using Alkaline Phosphatase mediated NBT/BCIP color reaction.

2.3. Expression patterns

The expression patterns of genes identified in the screen were determined between Prim-5 (24h) stage and 5d. We either consulted the ZFIN expression database or analyzed whole-mount embryos or larvae by mRNA *in situ* hybridization is the size of the fragment allowed

(~350bp). We also confirmed whether *adrenomedullin 2*, *kctd4*, *interleukin 1 receptor accessory protein-like 2*, *psd3l*, *nppcl*, *pncr2*, *serpini1*, *nms*, *uo:ion001*, *uo:ion002*, *uo:ion003*, *uo:ion005* and *uo:ion006* were expressed in adult HP (Fig. 4).

Embryos were fixed and hybridized with one or two mRNA probes according to previously described protocols (Hauptmann and Gerster, 1994; Varga et al., 1999). We used antisense mRNA probes for the gene fragment contained in each library clone and used *pomc* as a reference for the adenohypophysis. When doubly labeling embryos, we used NBT/BCIP to detect one mRNA probe and Fast Red to detect the other probe. For more detailed morphological analysis, several embryos were cryosectioned at a thickness of 15µm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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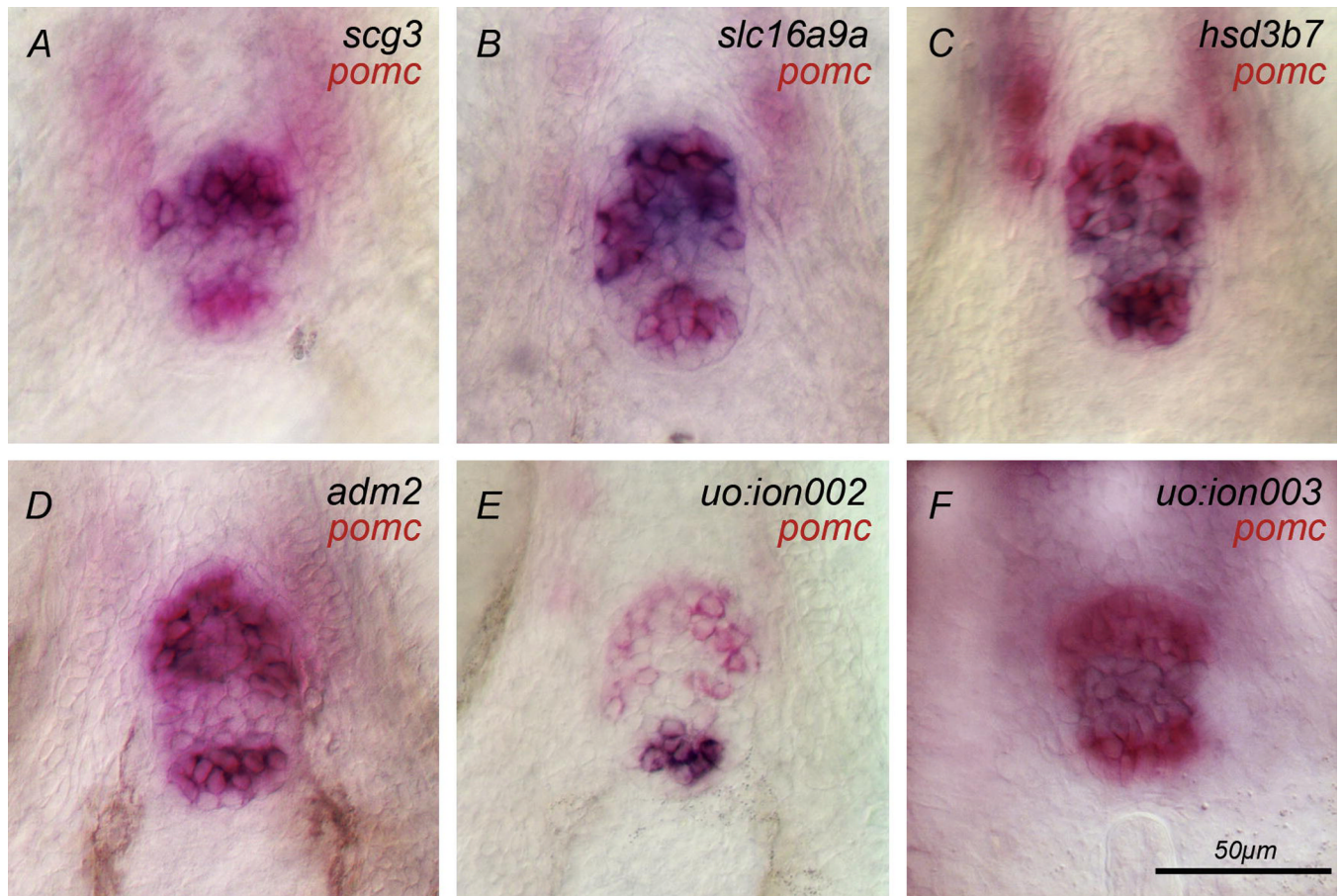


Fig. 1. Genes expressed in the adenohipophysis

(A) *scg3*, (B) *slc16a9a*, (C) *hsd3b7*, (D) *adm2*, (E) *uo:ion002* and (F) *uo:ion003* are expressed (blue) in the adenohipophysis that is delimited by *pomc* expression (red). *scg3* (A) and *slc16a9a* (B) are expressed in the rostral pars distalis (RPD). (C) *hsd3b7* is expressed in the entire gland. (D) The EST *adm2* is expressed in the proximal pars distalis (PPD) and pars intermedia (PI). (E) The novel gene *uo:ion002* is expressed in the PI. (F) The novel gene *uo:ion003* is expressed in the rostral pars distalis (RPD). Ventral views of adenohipophysis at 72h, anterior to the top; scale bar 50 μ m.

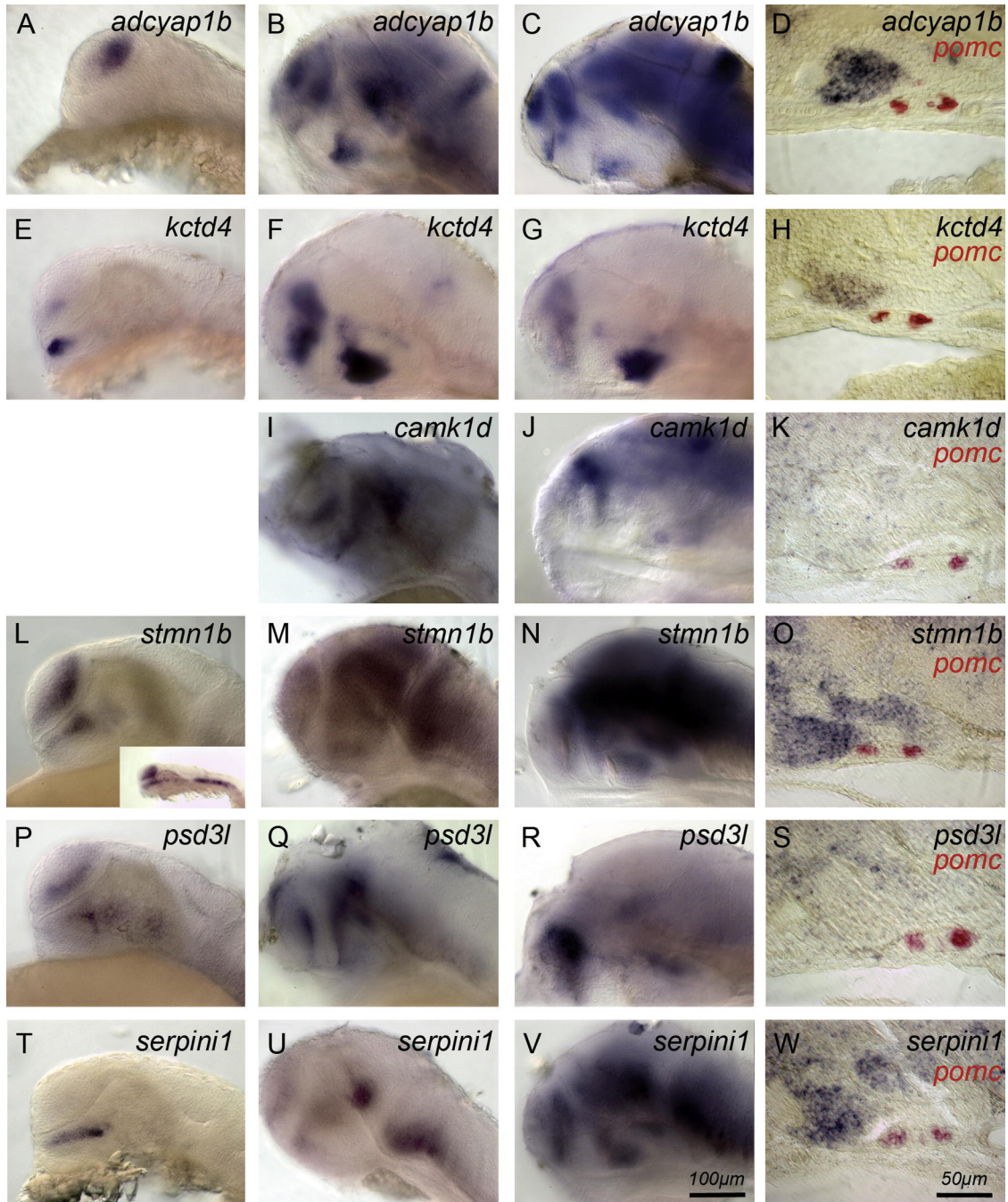


Fig. 2. Genes expressed in the hypothalamus

(A–D) *adcyap1b* and *kctd4* (E–H) are expressed in the hypothalamus. *adcyap1b* is expressed in the telencephalon from 24h to 72h (A–C) and in the hypothalamus at 48h and 72h (B–C). (D) *adcyap1b* and *kctd4* (H) are expressed by hypothalamic cells (black) close to the adenohypophysis (demarcated by *pomc*, red) but not in neurohypophysial cells. (I–K) *camk1d* is expressed in the dorsal hypothalamus, telencephalon, and midbrain at 48h and 72h. (L–N) *stmn1b* is expressed in telencephalic, mesencephalic, and hypothalamic cells between 24–72h. (O) The section at 72h suggests that ventral hypothalamic cells and some, but not all neurohypophysial cells express the gene. (P–R) *psd3l* is also expressed in telencephalon and hypothalamus between 24h–72h. (S) Hypothalamic cells express *psd3l*. (T–V) *serpini1* is

expressed in the dorsal hypothalamus at 24h (T), at 48h in telencephalon, diencephalon, and mesencephalon. (W) *serpini1* is expressed by hypothalamic cells (blue) close to the adenohypophysis (demarcated by *pomc*, red) but not in neurohypophysial cells. Side views, anterior to the left. (A,E,L,P,T) 24h; (B,F,I,M,Q,U) 48h; (C,G,J,N,R,V) 72h; Scale bar 100 μm . (D,H,K,O,S,W) 15 μm sagittal sections through the pituitary and adjacent hypothalamus at 72h, scale bar 50 μm .

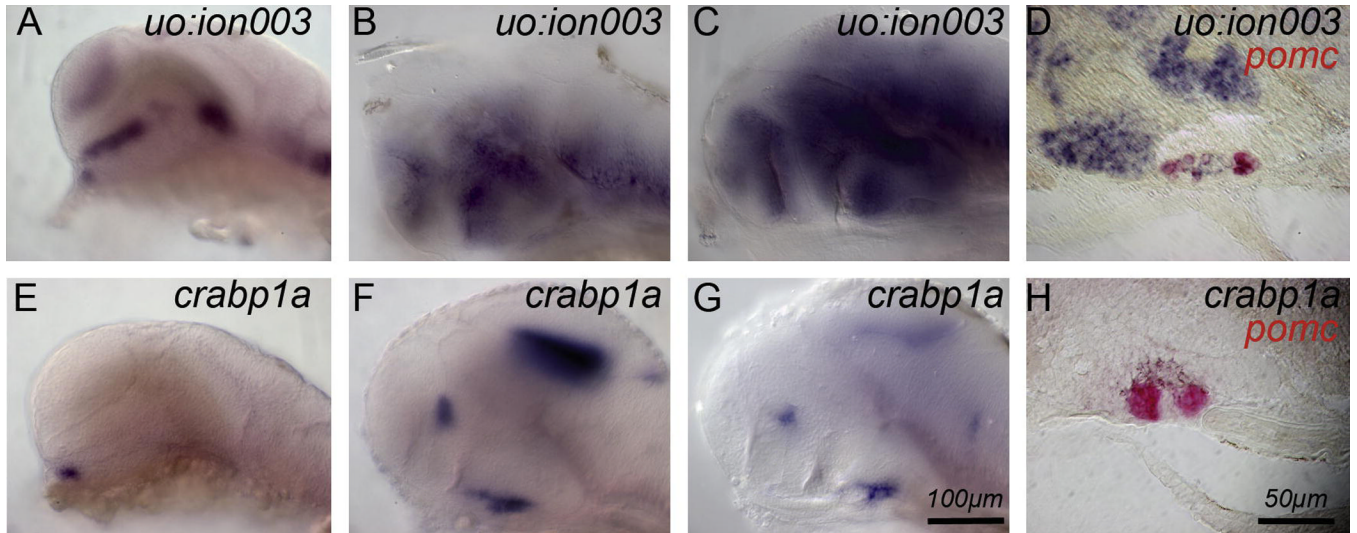


Fig. 3. Genes expressed in hypothalamus and adenohypophysis and in neurohypophysis
 (A–D) *uo:ion003* is expressed in telencephalon, diencephalon, and mesencephalon between 24h–72h. (D) The section shows that hypothalamic cells and cells in the posterior pars distalis (APD and PI are demarcated by *pomc*, red) express *uo:ion003*. (E–H) *crabp1a* is expressed in the presumptive neurohypophysis. *crabp1a* is expressed close to the adenohypophysis from 24h to 72h (E–H) and in the telencephalon and tectum at the later stages. Sagittal sections through the pituitary (*pomc*, red) show *crabp1a* expression dorsal to the adenohypophysis in neurohypophysial cells (L). Side views, anterior to the left. (A,E) 24h; (B,F) 48h; (C,G) 72h; scale bar is 100 μm. (D,H) 15 μm sagittal sections, anterior to the left, dorsal up, through the pituitary and adjacent hypothalamus at 72h; scale bar: 50 μm.

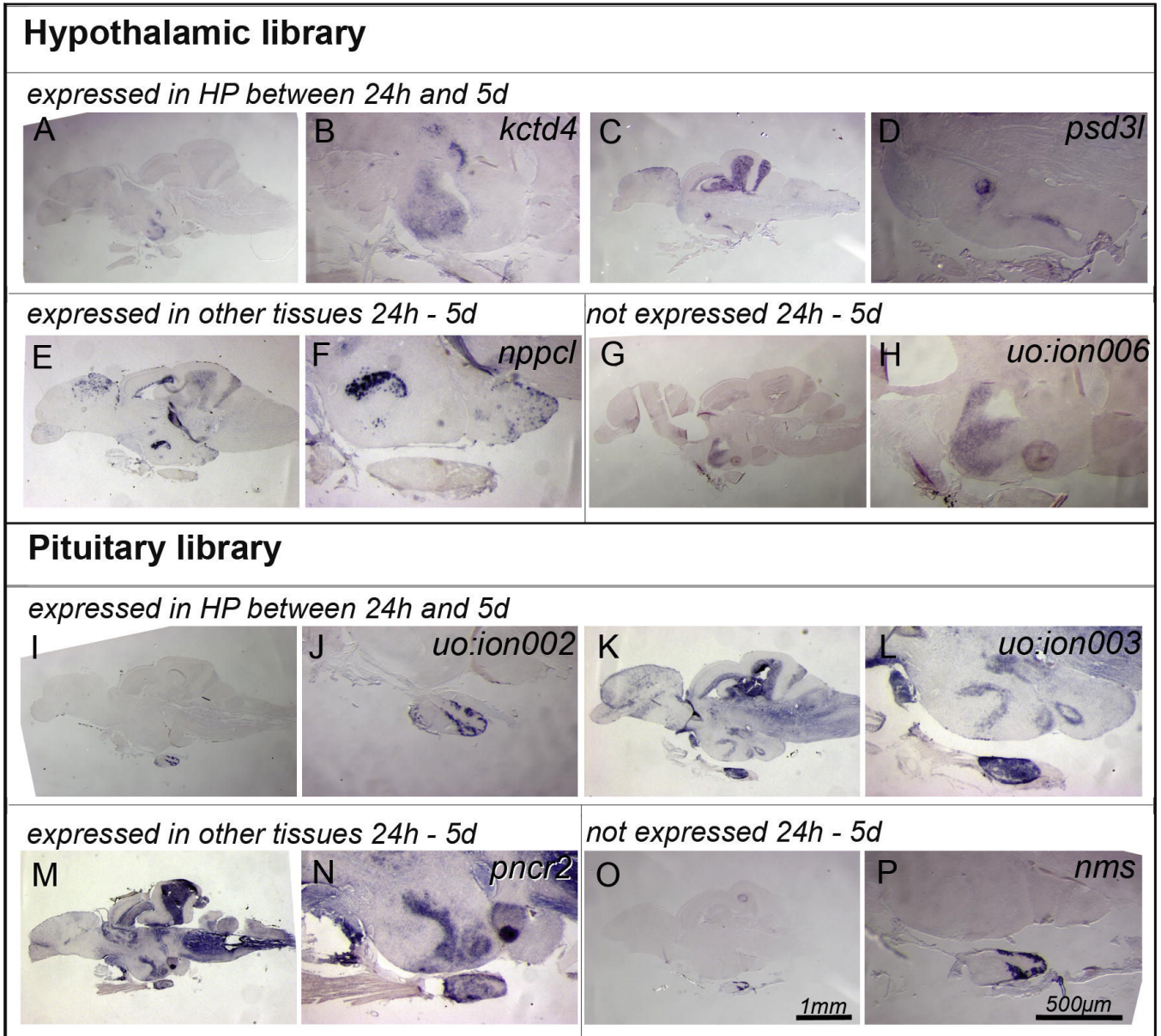


Fig. 4. Genes expressed in adult hypothalamic-pituitary axis

(A–H) Genes isolated in the Hypothalamic library expressed in the adult. (A–D) *kctd4* and *psd3l* were expressed in adult telencephalon and hypothalamus corresponding to the expression domains we found in 1–5 day old larvae (Fig. 2 E–H, 2 P–S). (E,F) In contrast, *nppcl* was not expressed in the hypothalamus between 1d and 5d; however, we found expression in a distinct set of cells in the adult hypothalamus. (G,H) The novel gene *uo:ion006* was not expressed in embryonic and larval tissues at all, however, we detected hypothalamic expression in the adult. (I–P) We made similar observations for the pituitary library: (I–L) genes such as *uo:ion002* and *uo:ion003* that were expressed in the embryonic larval HP were also found in the adult pituitary (I,J) or hypothalamus and pituitary (K,L). (M–P) *pncr2* that was not detected in embryonic and larval HP, or *nms* that was not detected in embryos and larvae at all, were both detected in adult HP (M,N) and adult pituitary (O,P). (A–P) 15 µm, sagittal sections of the adult brain, anterior left, dorsal up. (A,C,E,G,I,K,M,O,) scale bar 1 mm; (B,D,F,H,J,L,N,P) scale bar 500 µm.

Table 1

Hypothalamus and pituitary library construction.

	hypothalamic library		pituitary library	
	#	% of total	#	% of total
	sequences (5)	genes or genomic sequences (6)	sequences (5)	genes or genomic sequences (6)
# total clones (1)		448		558
total unusable clones (2)		114 (25.4%)		78 (14.0%)
total analyzed clones (3)		334 (74.6%)		480 (86.0%)
total identified sequences (4)		348		492
> mitochondrial sequences	21	5 (6.0%)	3	2 (0.6%)
> ribosomal sequences	4	2 (1.1%)	7	6 (1.4%)
> known genes	199	22 (57.2%)	438	54 (89.0%)
> ESTs and new genes	83	17 (23.9%)	34	23 (6.9%)
> genomic DNA sequences	41	12 (11.8%)	10	8 (2.0%)

(1) Represents the number of clones isolated after subtraction and stored as glycerol stocks. (2) From these clones, some did not survive, did not contain an insert, or were resistant to sequencing because of their base composition. The remaining clones were analyzed by sequencing. Some clones contained more than one insert. Therefore, the total number of identified sequences in row (4) is larger than the number of analyzed clones in row (3). Identified sequences were grouped into categories: mitochondrial sequences, ribosomal sequences, sequences of known genes, published ESTs, predicted genes or genomic DNA sequences. In each category, column (5) lists the number of sequences found, (6) indicates the number of unique genes or unique genomic sequences represented and in (7) the percentage of sequences is listed compared to the total number of identified sequences in column (4).

Table 2

List of genes expressed in the HP during the first 5d of development.

Gene	NCBI accession#	expression in HP (1)	library
known genes			
<i>cellular retinoic acid binding protein 1a</i>	NM 182858	neuro	pituitary
<i>follicle stimulating hormone beta-subunit</i>	NM 205624	adeno	pituitary
<i>glycoprotein hormones, alpha polypeptide</i>	NM 205687	adeno	pituitary
<i>growth hormone</i>	AJ937858	adeno	pituitary
<i>hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase</i>	NM-199809	adeno	pituitary
<i>luteinizing hormone beta-subunit</i>	AY714132	adeno	pituitary
<i>POU domain, class 1, transcription factor 1</i>	NM 212851	adeno	pituitary
<i>prolactin</i>	NM 181437	adeno	pituitary
<i>proopiomelanocortin</i>	NM 181438	adeno	pituitary
<i>secretogranin III</i>	NM 200757	adeno	pituitary
<i>solute carrier family 16 (monocarboxylic acid transporters), member 9a</i>	NM 200410	adeno	pituitary
<i>somatolactin beta</i>	NM 001037674	adeno	pituitary
<i>thyroid stimulating hormone, beta subunit</i>	NM 181494	adeno	pituitary
<i>adenylate cyclase activating polypeptide 1b</i>	NM 214715	hypo	hypothalamus
<i>cytochrome P450, family 19, subfamily A, polypeptide 1b</i>	NM 131642	hypo	hypothalamus
<i>pro-melanin concentrating hormone-like</i>	FJ392645	hypo	hypothalamus
ESTs and new genes			
<i>adrenomedullin 2</i>	FJ392613	adeno	pituitary
<i>uo:ion002</i>	FJ392616	adeno	pituitary

Gene	NCBI accession#	expression in HP (1)	library
<i>uo:ion003</i>	<u>FJ392618</u>	adeno+hypo	pituitary
<i>calcium/calmodulin-dependent protein kinase 1D</i>	<u>NM 001080658</u>	hypo	hypothalamus
<i>pleckstrin and Sec7 domain containing 3 like</i>	<u>FJ392621</u>	hypo	hypothalamus
<i>potassium channel tetramerization domain containing 4</i>	<u>FJ392642</u>	hypo	hypothalamus
<i>serpin peptidase inhibitor, clade I (neuroserpin), member 1</i>	<u>FJ392619</u>	hypo	pituitary
<i>stathmin 1/oncoprotein 18 b</i>	<u>FJ392638</u>	hypo	hypothalamus

Column (1) indicates that the gene is expressed in the neurohypophysis (neuro), adenohypophysis (adeno), or hypothalamus (hypotha) during the first 5d of development.