Altered gene expression profile of the hypothalamic arcuate nucleus of male mice suggests profound developmental changes in peptidergic signaling

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Abstract

Neuropeptides of the hypothalamic arcuate nucleus (ARC) regulate important homeostatic and endocrine functions and also play critical roles in pubertal development. Altered peptidergic and amino acidergic neurotransmission accompanying pubertal maturation of the ARC are not fully understood.

Here we studied the developmental shift in the gene expression profile of the ARC of male mice. RNA samples for quantitative RT-PCR studies were isolated from the ARC of day-14 infantile and day-60 adult male mice with laser-capture microdissection. The expression of 18 neuropeptide-, 15 neuropeptide receptor-, 4 sex steroid receptor and 6 classic neurotransmitter marker mRNAs were compared between the two timepoints.

Adult animals showed increased mRNA levels encoding cocaine- and amphetamine-regulated transcript, galanin-like peptide, dynorphin, kisspeptin, proopiomelanocortin, proenkephalin and galanin and reduced expression of mRNAs for pituitary adenylate cyclase activating peptide, calcitonin gene-related peptide, neuropeptide Y, substance P, agouti-related protein, neurotensin and growth hormone-releasing hormone. From the neuropeptide receptors tested, melanocortin receptor-4 showed the most striking (5-fold) increase. Melanocortin receptor-3 and the Y1 and Y5 neuropeptide Y receptors increased 1.5-1.8-fold, whereas δ-opioid receptor and neurotensin receptor-1 transcripts were reduced by 27 and 21%, respectively. Androgen-, progesterone- and α-estrogen receptor transcripts increased by 54-72%. The mRNAs of glutamic acid decarboxylase 65, and 67, vesicular GABA transporter and choline acetyltransferase remained unchanged. Tyrosine hydroxylase mRNA increased by 44%, whereas type-2 vesicular glutamate transporter mRNA decreased by 43% by adulthood.

Many of the developmental changes we revealed in this study suggest reduced inhibitory and/or enhanced excitatory neuropeptidergic drive on fertility in adult animals.

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Introduction

Puberty in mammals is a complex process of sexual development which leads to complete gonadal maturation and the attainment of full reproductive capacity [1-4]. Puberty can take place gonad-independently [5] with the activation of hypothalamic gonadotropin-releasing hormone (GnRH) neurons [1-3], leading to the onset of pulsatile GnRH secretion into the hypophyseal portal circulation. Neuronal and glial signals that play either causal or permissive roles in puberty initiation are multiplex and include increased levels of peripheral leptin [6], enhanced central glia-to-neuron signaling [7], reduced central inhibitory NPY tone [8] and in particular, reduced hypothalamic GABA- [9, 10], followed by increased glutamate [9, 11-13] release. Peptide and amino acid neurotransmitters synthesized and/or acting in the hypothalamic arcuate nucleus (ARC) are critically involved in pubertal development. Recent human genetics provide evidence that kisspeptin (KP) and neurokinin B (NKB) are particularly important for the pubertal awakening of the 'GnRH pulse generator', via signaling through their specific G protein-coupled receptors Kiss1r and NK3, respectively [14-16]. KP neurons in the ARC of the sheep and rodents co-express NKB and dynorphin [17, 18] and hence, are commonly referred to as 'KNDy neurons'. KNDy neurons of the ARC seem to represent a long-sought key element of the GnRH pulse generator network [18, 19] and intact KP/Kiss1r signaling to GnRH neurons is a requirement for puberty to occur in both humans [14, 15] and laboratory rodent species [20]. The mechanisms initiating puberty upstream from KP neurons are still poorly understood and highly complex, involving both genetic and epigenetic [21] regulatory events [22].

In the present study, we hypothesized that pubertal transition is accompanied by profound developmental changes in peptidergic and amino acidergic neurotransmission of the mediobasal hypothalamus which are reflected in an altered gene expression profile of the ARC. We carried out qPCR experiments on ARC tissue samples collected with laser capture microdissection (LCM) to compare

between day-14 infantile and day-60 adult male mice the expression of 18 neuropeptide-, 15 neuropeptide receptor-, 6 classic neurotransmitter marker- and 4 nuclear sex steroid receptor mRNAs that have been chosen based on their presence in the ARC transcriptome and known reproductive significance.

Materials and methods

Experimental animals

Male C57/BL/6 mice (N=16) were obtained from the local breeding colony of the Medical Gene Technology Unit of the Institute of Experimental Medicine (IEM) and housed in light- (12:12 light-dark cycle, lights on at 06:00h) and temperature (22 ± 2°C) controlled environment. The mice were used at postnatal day 14 (infantile group; N=8) and at postnatal day 60 (adult group; N=8). Mothers of the infantile mice and the adult mice had free access to standard food and tap water. Ethical permission was obtained from the Animal Welfare Committee of the IEM (No.: A5769-01) and studies were carried out in accordance with legal requirements of the European Community (Decree 86/609/EEC).

Section collection for laser capture microdissection

To collect ARC tissues for the qPCR studies, the mice were anesthetized between 0900 and 1100 h with a cocktail of ketamine (25 mg/kg), xylavet (5 mg/kg) and pipolphen (2.5 mg/kg) in saline, and then, perfused transcardially with ice-cold phosphate buffered saline (PBS; pH 7.4) containing 10% RNA*later* reagent (QIAGEN, Hilden, Germany) [23]. The brains were removed, snap-frozen in -40 °C isopentane (precooled with a mixture of dry ice and ethanol) and stored permanently at -80 °C. Then, 20-µm-thick coronal sections were cut serially from the ARC with a Leica CM3050 S cryostat (Leica Microsystems Nussloch Gmbh, Nussloch, Germany). Sections between Bregma levels -1.46mm and -1.94mm (corresponding to Paxinos atlas plates 43-47)[24] were collected onto PEN slides (Membrane Slide 1.0 PEN, Carl Zeiss, Göttingen, Germany), stained with 0.5% cresyl violet, dehydrated, air-dried and processed for LCM using a PALM Microbeam system (Zeiss). Prior to LCM, the region of interest (ROI) was adjusted to the boundaries of the ARC that were visualized by bright-field illumination of the cresyl

violet counterstain. The isolated tissues were pressure-catapulted from the object plane into 0.2 ml tube caps (Adhesive Cap 200, Zeiss) with a single laser pulse using a 10x objective lens (Fig. 1). The ARC mRNA profile of each animal was characterized from a tissue pool collected from both sides of every 3rd section. This sampling approach ensured the equal representation of rostral, mid- and caudal levels of the ARC from both age groups.

RNA isolation from the ARC samples

Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA quality was assessed with capillary electrophoresis using Pico RNA Chips on a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) [25]. The RNA samples processed further for qPCR studies exhibited RNA integrity numbers (RIN) between 6.2 and 7.5.

Reverse transcription and preamplification

Reverse transcription of 10 ng total RNA was carried out using routine procedures and the ViLO SuperScript III cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA) [25]. The quantity of the targeted cDNAs was increased with the TaqMan PreAmp Master Mix Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Quantitative real-time PCR studies

Custom TaqMan microfluidic cards (Thermo Fisher Scientific) were preloaded by the manufacturer with inventoried assays for genes of our interest, encoding 18 neuropeptides, 15 neuropeptide receptors, 4 nuclear sex steroid receptors and 6 presynaptic markers for classic neurotransmitters (Figs. 2-5). Each assay consisted of a FAM dye-labeled TaqMan MGB probe and two PCR primers. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl-transferase (*Hprt*) were used as housekeeping genes [25]. The geometric mean Ct value of the two housekeeping genes was used for ΔCt calculation [25]. Relative quantification against the calibrator samples was carried out to determine ΔΔCt values with the ViiA 7 RUO software (Applied Biosystems). In view that the reliability of the assay decreases at high cycle numbers, genes with mean Ct values above 30 were not evaluated.

Statistical analysis

Data were analyzed and results compared from 6 infantile and 6 adult animals. Animals excluded showed low RIN numbers or poor amplification of the two house-keeping genes *Hprt* and *Gapdh*. Data from the adult group were related to the mean of the infantile group and they were expressed and illustrated graphically as Relative quantities ($RQ = 2^{-\Delta\Delta Ct}$)±standard error of the mean. Statistical significance was analyzed with Student's t test (Statistica software, version 11.0, StatSoft Inc., Tulsa, OK) and developmental changes were stated at $p<0.05$.

Results

Neuropeptides

qPCR studies revealed robust developmental changes in the expression of several neuropeptide transcripts. Seven neuropeptide mRNAs showed increased, and another seven showed decreased levels in adult male mice, compared with their expression level in 14-day-old infantile mice (Fig. 2). As ranked by highest fold-change (RQ), mRNAs with increased adult levels encoded for CART (RQ=6.3), GALP (RQ=4.6), dynorphin (RQ=3.9), KP (RQ=3.3), POMC (RQ=3.0), proenkephalin (RQ=2.9) and galanin (RQ=2.5), whereas mRNAs with reduced adult levels encoded for PACAP (RQ=0.4), CGRP (RQ=0.4), NPY (RQ=0.4), SP (RQ=0.6), AGRP (RQ=0.6), NT (RQ=0.6) and GHRH (RQ=0.8). The assays failed in case of *Tac2* and *Cck* mRNAs. A different inventoried assay from Applied Biosystems (Mm01160362_m1) was also purchased but showed similarly poor amplification (Ct>30) of *Tac2* cDNA.

Neuropeptide receptors

The majority of neuropeptide receptor-encoding transcripts remained unchanged or changed less dramatically than changing peptide transcripts, with the exception of *Mc4r* (encoding melanocortin receptor-4) which increased robustly (RQ=4.9) between day 14 and 60. *Mc3r* mRNA (the transcript of melanocortin receptor-3) level also increased, although to a lower extent (RQ=1.8), similarly to the two NPY receptors examined (*Npy5r* and *Npy1r*) which had RQ values of 1.6 and 1.8, respectively. *Oprd1* (encoding δ-opioid receptor) and *Ntsr1* (encoding neurotensin receptor-1) decreased slightly, though

significantly (RQ=0.7 and 0.8, respectively), whereas the levels of other neuropeptide receptor transcripts remained unaltered (Fig. 3). The PCR assays failed to efficiently amplify *Tacr2* and *Galr1*.

Nuclear sex steroid receptors

The level of mRNAs encoding sex steroid receptors for progestins (*Pgr*), androgens (*Ar*) and estrogens (*Esr1*) increased by 50-70% during puberty (Fig. 4). The qPCR assay failed in case of the β estrogen receptor isoform (*Esr2*).

Classic neurotransmitters

Gene expression studies revealed that the expression of the dopaminergic marker enzyme tyrosine hydroxylase increased (RQ=1.44), whereas *Slc17a6* mRNA encoding for vesicular glutamate transporter-2 decreased significantly (RQ=0.57). There was no change in the expression of the presynaptic GABA markers *Gad1*, *Gad2* and *Slc32a1* and the cholinergic marker *Chat* (Fig. 5).

Discussion

Altered neuronal and glial signaling within the mediobasal hypothalamus plays a critical role in the onset of pulsatile GnRH/LH secretion at puberty. Here we hypothesized that changes in neuropeptide signaling coincide with an altered neuropeptide and neuropeptide receptor gene expression profile of the ARC. We used a high-precision tissue isolation method to selectively collect RNA from the ARC of day-14 infantile and day-60 adult male mice with the aid of LCM. The comparative analysis of these samples with qPCR identified a series of transcripts encoding for neuropeptides, neuropeptide receptors, presynaptic markers for classic neurotransmitters and nuclear sex steroid receptors that show dramatically different expression levels between adult and infantile animals.

Neuropeptides

Neuropeptides constituted the primary targets of these studies in the light of an abundant literature suggesting the involvement of altered peptidergic signaling in pubertal development. A set of neuropeptide mRNAs exhibited higher expression levels in adult compared with infantile male mice, whereas others showed significant developmental decreases.

Developmental increases in 'KNDy' peptide mRNAs

Much attention has been paid recently to the involvement of 'KNDy' neuropeptides in the regulation of puberty onset. Our present study provided evidence that pubertal maturation of male mice is accompanied by a 3.3-fold increase in *Kiss1* expression of the ARC between postnatal day 14 and 60. This observation differs from the reported absence of pubertal changes in *Kiss1* mRNA expression in female mice [26]. Given that both studies used similar qPCR methodologies, the discrepant results may be due to the different sex (and sex steroid milieu) of the model animals and/or the different tissue sampling methods used. Of note, the authors of the female study proposed that the negative regulation of Kiss1 expression by gonadal sex steroids could mask the pubertal changes in gene expression. Indeed, they have provided evidence for this concept by showing that the sex steroid-deficient hypogonadal female mice exhibit robust developmental increases in ARC *Kiss1* mRNA levels [26]. Negative regulation of *Kiss1* mRNA by gonadal steroids also characterizes the male mouse model which we used in this study. Indeed, testosterone produces a massive suppression of *Kiss1* expression in the ARC of adult male mice [27]. Despite the masking effect of this negative regulation, we were able to distinguish a net 3.3-fold developmental increase between days 14 and 60, what we attribute to the accurate LCM sampling method we used.

Tac2 mRNA encoding for NKB showed a robust pubertal increase in earlier studies of female mice [26]. This change was proposed to represent an early sign, but not a cause, of pubertal transition [26]. Because the TaqMan PCR failed using two different inventoried assays for *Tac2*, we were not able to address whether males show a similar pubertal increase of this transcript.

While relatively little attention has been paid to the possible role of the third KNDy peptide dynorphin in rodent puberty, the encoding *Pdyn* gene was ranked third among the peptide transcripts which increased the most (3.9-times) between days 14 and 60. This increase is likely developmental and independent of testosterone production because testosterone treatment of adult orchidectomized male mice does not seem to markedly influence the expression of *Pdyn* mRNA in the ARC (unpublished observation of our laboratory). Of note, in a study of adult female mice estradiol regulated *Pdyn* negatively [18]. This observation makes it further unlikely that the presence of sex steroids accounted for the increased *Pdyn* expression we observed in adult male mice.

Enhanced *Cartpt* expression

The neuropeptide transcript with the highest fold-change between day 14 and day 60 encodes for CART. *In situ* hybridization studies have established that *Cartpt* is widely distributed in the rodent brain, including various hypothalamic sites [28]. In the rodent ARC, CART has been localized to the anorexigenic Pomc neurons [29]. In our present qPCR study, these POMC/CART cells also showed elevated *Pomc* expression following pubertal transition. Somewhat surprisingly and unlike in rodents, Cart in the mediobasal hypothalamus of the human colocalizes with the orexigenic peptides Npy and Agrp [30] and with KP [31] and NKB [31], but not with the Pomc-derived opioids [30]. Studies in rodents indicate that CART can influence the reproductive axis at multiple sites. Anatomical and functional experiments revealed that CART fibers arising from the ARC innervate both the preoptic GnRH neurons and the mediobasal hypothalamic KP neurons and CART can depolarize both target cell types in slice preparations [32]. *In vitro* studies of mediobasal hypothalamic explants provide evidence that CART is a potent stimulator of pulsatile GnRH release both in day 15 prepubertal and in day 50 adult rats [33]. The robust pubertal increase of *Cartpt*

expression that we report in this study is in accordance with an enhanced direct and/or indirect stimulatory action of CART on GnRH neurons in adult animals.

Enhanced *Galp* expression

The second largest fold-change (4.6) in the mRNA expression of ARC neuropeptides was in *Galp* transcript. The encoded galanin-like peptide (GALP) is a 60 amino acid neuropeptide isolated from porcine hypothalamus in the late 1990s. The sequence of GALP at positions 9-21 shows 100% identity with amino acids 1-13 of galanin. This segment is required for binding [34] and activation of the 3 types of galanin receptors (GalR1-GalR3) [34, 35], whereas the unique region between residues 38 and 54 suggests that a GALP-specific receptor might also exist [36].

Galanin is widely distributed in the brain, whereas *Galp* mRNA is detected only in the ARC, the median eminence, the infundibular stalk and the posterior pituitary of rats [37-40], mice [41] and primates [36]. GALP-containing cells are distinct from those synthesizing NPY [39], somatostatin [39] and KP [42]. The only colocalization reported so far was between GALP and α-MSH; in one immunohistochemical study, 3-12% of the α-MSH cell bodies were also GALP-immunoreactive [43]. Conflictingly, Takatsu et al. have not found evidence for this colocalization phenomenon [39]. GALP plays an important role in the central regulation of reproductive functions. Accordingly, intracerebroventricular administration of GALP to male rats markedly increased plasma levels of LH [44]. This effect was mediated by GnRH neurons and could be blocked with the GnRH-receptor antagonist Cetrorelix [44]. Furthermore, in the same study GALP also induced Fos immunoreactivity in GnRH neurons of the medial preoptic area [44]. Using *in situ* hybridization histochemistry, Kawagoe and colleagues observed a marked pubertal increase in *Galp* mRNA levels of the ARC in male and female rats [45]. These observations are in concordance with the 4.6-fold pubertal increase in *Galp* transcript we report here in male mice, and suggest that increased levels of GALP might have a role in the maturation of reproductive functions. In support of this concept,

GALP treatment of food-restricted rats of both sexes was capable of rescuing the timing of puberty onset to that seen in controls fed ad libitum [42]. Of note, this action was proposed not to be mediated by the KP system as GALP did not induce Fos expression in KP cells [42].

Enhanced *Pomc* mRNA

The observation of a pubertal increase in *Pomc* mRNA expression in the ARC is reminiscent of previous *in situ* hybridization data from male rats [46] and monkeys [47]. It remains to be determined whether, and to what extent, this change is consequent to the pubertal increase of KP signaling in view that KP is capable of exciting POMC neurons via KP receptor mediated postsynaptic actions [48]. Although mutations in genes encoding for POMC [49] or the Mc3/4 receptors for the POMC-derived anorexigenic neuropeptide α-MSH [50] do not affect sexual development, the overexpression of the Mc3/4r antagonist AGRP causes infertility [51], clearly indicating that the unopposed α-MSH/Mc3/4r signaling plays an important role in puberty.

GnRH neurons receive direct input from POMC neurons [52], express *Mc4r* [53] and respond with increased Fos expression and firing activity to Mc4r activation [53]. In a different study, the majority of GnRH neurons were similarly activated by α-MSH as a result of a direct postsynaptic Mc3r and Mc4r activation [54]. These data indicate that the pubertal increase in *Pomc* mRNA may increase an excitatory drive directly onto GnRH cells. In addition, α-MSH might also regulate GnRH neurons indirectly via networks impinging on GnRH neurons.

Enhanced expression of *Penk* and *Gal*

Penk mRNA increased 2.9-fold. Of note, in female rats the level of these mRNAs only increased in the paraventricular and medial preoptic nuclei, but not in the ARC, during puberty [55]. Cell types expressing *Penk* in the mouse ARC may include tuberoinfundibular dopaminergic neurons. In female rats, these neurons respond with enhanced *Penk* gene expression to hyperprolactinemia [56].

In addition, *Penk* is also coexpressed with neuronal nitric oxide synthase in the ARC of rats, whereas Penk-immunoreactive neurons in the infundibular nucleus of human males exhibit overlap with KP and NKB neurons [57]; of note, in mice KP/met-Enkephalin colocalization has only been observed in the rostral periventricular area of the third ventricle, but not in the ARC [58].

Similarly to the *Penk* transcript, *Gal* mRNA was not found to change in the ARC of female rats during puberty [55], in contrast with the 2.5-fold increase we observed here in male mice. Cell types characterized by increased coexpression of *Gal* in adult mice may include KNDy neurons [58, 59] and/or growth hormone-releasing hormone neurons [60], among other cell types of the ARC.

Reduced expression of *Agrp* and *Npy*

The orexigenic peptides AGRP and NPY are both potent inhibitors of pulsatile LH secretion [61, 62].

AGRP is an endogenous antagonist of the Mc3/4r. Transgenic overexpression of AGRP in mice leads to obesity and infertility [51]. AGRP can exert its inhibitory effects on fertility at multiple sites, including its direct actions on GnRH neurons. AGRP is present in synaptic afferents to GnRH neurons [63] which latter express the *Mc4r* [53] and respond to AGRP with either increased or decreased electrical activity [54]. Previous developmental studies showed a gradual increase in *Agrp* mRNA levels of the ARC during P5-P21, along with a parallel increase in AGRP fiber densities in several hypothalamic regions [64]. Our qPCR results revealed 40% lower *Agrp* mRNA levels in adult *vs.* 14 day old mice, indicating that *Agrp* mRNA likely declines between P21 and P60.

NPY has long been known as an important developmental brake on puberty onset [65]. Accordingly, the postnatal pattern of GnRH pulse generator activity is inversely related to NPY mRNA and protein levels in the mediobasal hypothalamus of the male rhesus monkey, and central

administration of NPY Y1 receptor antagonist to infantile animals elicits precocious GnRH release [65]. Furthermore, intracerebroventricular administration of NPY to adult male [65] and female [66] monkeys inhibits pulsatile GnRH release.

The 60% reduction in *Npy* mRNA levels of the mouse ARC between P14 and P60 that we report in this study is in accordance with the concept that a pubertal decrease of an inhibitory NPY tone is also associated with pubertal development in male mice. In this species, NPY/AGRP neurons of the ARC provide direct synaptic input to the somatodendritic compartment of preoptic GnRH neurons and account for 50% of NPY inputs to GnRH cells, whereas an additional 25% originate from catecholaminergic cells of the brainstem [63]. Although we have to note that NPY can exert both stimulatory and inhibitory actions directly on GnRH neurons through the Y4 and Y1 receptors, respectively [54], there is abundant literature to suggest that the net effect of NPY on pubertal development is inhibitory [65]. The developmental decrease of ARC *Npy* expression we observed here is in accordance with a reduced inhibitory NPY tone on the reproductive axis in adult mice.

Reduced adult expression of *Ghrh*

The number of GHRH neurons in mice exhibited a complex developmental pattern in earlier studies. A decrease reported between day 5 and 20 was followed by an increase until day 40, and a decrease again by day 60 [31]. In our study, the slightly reduced levels of *Ghrh* mRNA in day-60 mice might be in accordance with a lower GHRH and growth hormone need in adult mice, compared with fast-growing infantile animals.

Reduced *Nts, Tac1, Calc*a and *Adcyap1* expression

Neurotensin in the ARC of the rat is colocalized with GHRH, TH, galanin and glutamic acid decarboxylase [67]. It remains to be determined which one of these cell populations shows reduced *Nts* mRNA levels in adult mice.

315 SP encoded by *Tac1* can influence reproduction via acting at the hypothalamic, pituitary and
316 gonadal levels of the reproductive axis. Both inhibitory and excitatory LH responses have been
317 reported, as reviewed recently [68]. For example, SP can inhibit the GnRH-stimulated LH secretion
318 from human pituitary cells *in vitro* [69]. On the contrary, intravenous, or intracerebroventricular SP
319 administration to ovariectomized estrogen primed rats stimulates, and conversely,
320 intracerebroventricular application of a SP antiserum to ovariectomized rats inhibits LH release
321 [70]. Our finding of reduced *Tac1* mRNA expression during development is in accordance with
322 reduced numbers of *Tac1* mRNA expressing neurons reported in several brain regions, including the
323 ARC, of day-60 adult mice [71].

324 *Calca* encoding for CGRP was expressed at low levels in the ARC of day-14 male mice and
325 expression levels were even lower by 60% in adults. CGRP immunoreactivity in the ARC is
326 sexually dimorphic, with higher cell numbers in males [72]. The functional significance of the
327 developmental decrease in ARC *Calca* expression, and the phenotype(s) of the neurons expressing
328 *Calca*, need to be determined.

329 The expression of the *Adcyap1* gene encoding for PACAP showed the most robust (61%)
330 reduction in this study. The ARC cell types expressing *Adcyap1* include *Pomc* and vesicular
331 acetylcholine transporter expressing neurons [73]. The inhibitory role of the PACAP peptide on
332 puberty has been demonstrated in several recent studies. Transgenic overexpression of pituitary
333 PACAP suppressed FSH, LH, and testosterone levels and delayed the timing of puberty in male
334 mice [74]. Furthermore, a single PACAP injection to neonatal female rats delayed vaginal opening
335 and decreased the intensity of GnRH immunostaining in the septo-preoptico-infundibular system.
336 Administration of PACAP antiserum had a reverse effect on GnRH immunoreactivity, suggesting
337 that neonatal PACAP administration delays puberty onset via influencing GnRH neurons [75]. The

reduced adult levels of ARC *Adcyap1* mRNA in our study suggest that PACAP of ARC origin significantly contributes to the regulation of puberty.

Transcriptional changes of neuropeptide receptor mRNAs

The most robust and intriguing change in neuropeptide receptor transcripts occurred in *Mc4r* expression which increased 4.9-fold between day 14 and day 60. The mRNA of the other melanocortin receptor *Mc3r* also increased, although by 77% only. The natural agonist of Mc4r (and Mc3r) is the *Pomc* gene-derived opioid peptide α-MSH, whereas AGRP antagonizes the action of α-MSH on these receptors. While mutations of *Pomc* and the melanocortin receptors Mc4r and Mc3r do not prevent sexual development, the transgenic overexpression of AGRP in mice leads to obesity and infertility [51]. Enhanced inhibitory AGRP tone acting via Mc4r has been strongly implicated in the infertility of the leptin receptor mutant *db/db* female mice. Accordingly, the absence of AGRP or haploinsufficiency of *Mc4r* can restore fertility in females, but not in males, of this animal model [53]. In wild-type female mice, AGRP deficiency results in advanced vaginal opening [53], whereas in *Lepr(db/db)* mice, it restores the normal timing of vaginal opening and estrous cyclicity [53]. The phenotype of ARC neurons in which *Mc4r* expression increases developmentally requires clarification. KNDy neurons are among the putative targets of melanocortin actions as they respond with FOS expression to the melanocortin analog MT-II [76]. Therefore, it seems likely that the inhibitory effect AGRP on puberty is mediated, at least partly, by *Mc4r* expressing KNDy neurons [76].

In addition to *Mc4r* and *Mc3r*, the other two receptors with increased levels of adult expression were also related to metabolic regulation. The developmental enhancements of *Npy5r* and *Npy1r* expression were moderate. The role of the slightly reduced *Ntsr1* (encoding for neurotensin receptor-1) and *Oprd1* (encoding δ-opioid receptor) expression is unclear. Notably, the majority of

the receptor transcripts analyzed, including *Kiss1r*, *Tacr3* and *Oprk1*, remained unchanged between day 14 and 60. The assays for *Tacr2* and *Galr1* failed.

Sex steroid receptors

Although this study focused on peptidergic neurotransmission, we also analyzed how the expression of classic sex steroid receptors changed between the infantile and adult periods. qPCR revealed a similar 50-70% upregulation of the *Ar* (encoding androgen receptor), *Esr1* (encoding estrogen receptor-α) and *Pr* (encoding progesterone receptor) transcripts, whereas the TaqMan assay failed in case of *Esr2* (encoding estrogen receptor-β). High expression levels of these receptors may account for enhanced sex steroid feedback signaling in postpubertal mice.

Presynaptic markers for classic neurotransmitters

In our experiments we also assessed pubertal changes of a few presynaptic markers for the classic neurotransmitters dopamine, acetylcholine, GABA and glutamate. The significant 44% increase we observed in ARC contrasts with observations in rats which latter showed increased *Th* expression and tyrosine hydroxylase immunoreactivity only in females, but not males, during this developmental period [77].

Expression of the cholinergic marker *Chat* did not change. Although pubertal development is preceded by reduced hypothalamic GABA release [9, 10, 78], the three GABAergic markers *Gad1*, *Gad2* and *Slc32a1* (encoding for vesicular inhibitory amino acid transporter) did not change. This finding is reminiscent to the lack of difference in ARC *Gad1* and *Gad2* transcript levels between the infantile and the pubertal stages of the male monkey [65].

Pubertal development of rhesus monkeys is associated with an enhanced hypothalamic glutamate release into the median eminence [78]. In rats, most of the glutamatergic input to the

median eminence originates outside the ARC [79], making it likely that *Slc17a6* mRNA expressing neurons of the mouse ARC are mostly non-hypophysiotropic. The marked developmental reduction (RQ=0.57) in *Slc17a6* mRNA expression we observed indicates that glutamatergic neurotransmission by the mouse ARC might be lower in adult than in infantile mice. In order to interpret these data, it will be important to identify the neuronal phenotype(s) showing the reduced levels of this glutamatergic marker in adult animals.

Methodological considerations

One important technical consideration is that in this qPCR study we measured relative and not absolute mRNA abundances due to normalization to housekeeping genes. Considering that the ARC volume is higher in adults, this implicates that the extent of an increase is likely more robust within the whole ARC than the actual RQs we determined. On the other hand, lack of change or decrease (RQ<1) also refer to relative mRNA abundances. This way, in cases with decreased relative mRNA representation in the adults (RQ<1), the total mRNA amounts may actually be increased within the total ARC volume. Finally, we note that the mRNAs we analyzed in the ARC are often expressed by heterogeneous cell populations. Differential regulation of such mRNAs could not be addressed in this study. For example, it remains possible that the GABA marker mRNAs (which showed no net change in the whole ARC) are regulated differentially among the distinct GABAergic cell populations. Similarly, the net developmental decrease in the expression of the glutamatergic marker VGLUT2 does not exclude that VGLUT2 is regulated in opposite or different manners in other cell types of this nucleus during development.

Gene expression changes related and unrelated to puberty

While many of the genes we selected for analysis are well established players of reproductive regulation, it remains unknown to what extent their developmental changes contribute to sexual

maturation. Of note, brain and body development and the underlying hormonal and nutritional changes temporally overlap with pubertal maturation in rodents. Therefore, it remains impossible to distinguish between transcriptional changes that are related to altered nutrition from those related to puberty. For example, levels of the adipocyte-derived leptin increase 5-10-fold in female mice during the second postnatal week [80] and this neonatal leptin surge not only alters permanently the projections of ARC neurons [81] but also affects the gene expression profile of this site to potentially modify reproduction and sexual maturation [82]. Blockage of the neonatal leptin surge in rats can decrease *Npy* and *Agrp* expression in males and *Kiss1r* expression in both sexes [82].

The activational effects of sex steroids in adult animals may complicate further the interpretation of some changes that we identified in this study. For example, the developmental increase in *kiss1* expression can be blunted by testosterone. Alternatively, some changes that appear to be developmental, may be entirely due to the presence of sex steroids in the adult animal group. Our preliminary data indicate that the activational effects of sex steroids contribute to the enhanced *mc4r* expression of adult mice.

Nutritional differences complicate further the comparison of gene expression levels in day 14 and day 60 mice.

In female mice, some of the early changes in genes regulating reproduction already occur at the end of the second postnatal week. Accordingly, the mRNA encoding *RF-amide related peptide* falls between day 15 and 20 in the dorsomedial nucleus [83] and Tac2 mRNA in the ARC rises between day 12 and 15 [26]. The choice of day 14 allowed us to study the earliest developmental changes. On the other hand, day 60 mice we chose for the adult group can be considered sexually fully mature as the production of motile sperm occurs between day 40 and 55 [4]. While with this research design we were able to study changes of a large number of target genes, the time when differences developed remained unknown. A recent *in situ* hybridization study of female mice by Semaan and Kaufmann [83] revealed initial drops in

Kiss1 mRNA/cell and total ARC *Kiss1* mRNA levels between day 15 and 20, followed by slight increases in the number of Kiss1 neurons and total ARC *Kiss1* mRNA levels by the time of vaginal opening. There was no overall increase in *Kiss1* cell numbers and ARC *Kiss1* mRNA levels between day 15 and 30, similarly to the lack of changes in *Kiss1* expression between day 10 and 60 in qRT-PCR studies by Gill and colleagues [26].

In summary, our study identified significant changes in the expression of 14 neuropeptide-, 6 neuropeptide receptor-, 3 nuclear sex steroid receptor and 2 classic neurotransmitter marker transcripts in the ARC of male mice in temporal association with pubertal maturation. While changes might be purely developmental and unrelated to the attainment of sexual function, several changes are well in agreement with a reduced inhibitory/enhanced excitatory peptidergic drive on the reproductive axis in adult animals. While a series of peptides and their receptors exert complex reproductive effects which largely depend on their site of action and the steroid hormone milieu, an overwhelming literature suggests that the enhanced expression of *Cartpt*, *Galp*, *Kiss1*, *Pomc*, *Gal*, *Mc4r* and *Mc3r* and reduced levels of *Agrp*, *Npy* and *Adcyap1* mRNAs tend to be associated with enhanced activity of the reproductive axis. Future studies will need to dissect the functional significance and the causal relationship to pubertal development of the individual changes in the gene expression profile of the ARC in male mice.

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453 **Legends**

454 **Figure 1. Laser-capture microdissection (LCM) allows the precise isolation of the ARC from cresyl**
455 **violet-stained sections. A:** Pre-LCM image of a cresyl violet-stained 20-µm section illustrates several
456 cell-rich hypothalamic areas, including the ventromedial (VMN), dorsomedial (DMN) and arcuate (ARC)
457 nuclei. **B:** The same section is shown following the isolation and collection of the ARC tissues from both
458 sides by means of LCM. Note the absence of sample contamination by the surrounding median eminence
459 (ME) and periventricular tanycytes (cell-rich layer lining the third ventricle). The ARC mRNA profile of
460 each animal was characterized from a tissue pool collected from both sides of every 3rd section. Scale
461 bar: 150µm.

462

463 **Figure 2. Pubertal transition is accompanied by robust changes in the expression profile of**
464 **neuropeptide genes.** Seven neuropeptide transcripts (shown in red) have been upregulated several-fold
465 in adult mice, compared with the mean of infantile mice. The highest relative quantities (RQs) can be
466 observed in *Cartpt* (6.3-fold) and *Galp* (4.6-fold) expression, but *Pdyn*, *Kiss1*, *Pomc*, *Penk* and *Gal*
467 mRNAs also increase 2.5-3.9-fold. Seven transcripts (shown in green) have been down-regulated by 20-
468 60%, whereas the assay failed in case of two transcripts (*Tac2* and *Cck*).

469

470 **Figure 3. A subset of the analyzed neuropeptide receptors also shows altered expression in the**
471 **ARC of male mice in adulthood.** Changes in receptor expression tend to be moderate, except for a
472 robust (five-fold) increase in *Mc4r* mRNA levels. Note that the four upregulated (red) G protein-coupled
473 receptors are involved in the regulation of metabolism and food intake. Seven receptor transcripts remain
474 unchanged (yellow), whereas two (*Ntsr1* and *Oprd1*) show mild down-regulation (green) by adulthood.

475

Figure 4. Nuclear sex steroid receptors for progestins (*Pgr*), androgens (*Ar*) and estrogens (*Esr1*) become upregulated by 50-70% during pubertal transition. The assay fails (mean Ct>30) in case of Esr2.

Figure 5. The altered expression profile of classic presynaptic neurotransmitter markers suggests increased dopaminergic (*Th*) and decreased glutamatergic (*Slc17a6*) neurotransmission, whereas the expression of GABAergic (*Gad1*, *Gad2*, *Slc32a1*) and cholinergic (*Chat*) presynaptic markers does not change in adult *vs.* infantile mice.

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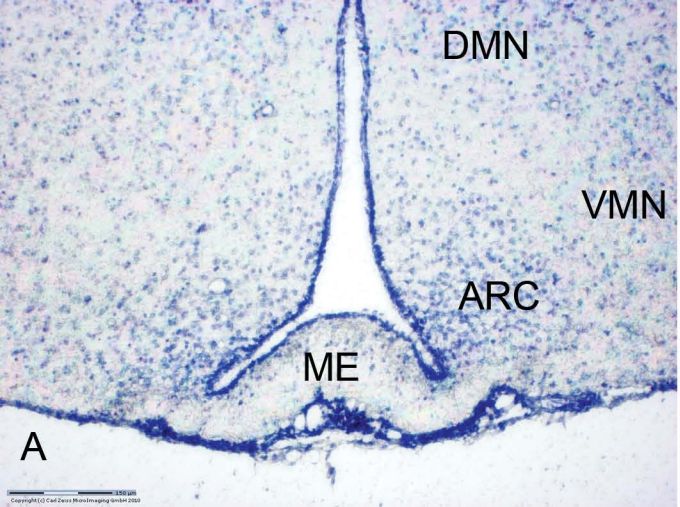
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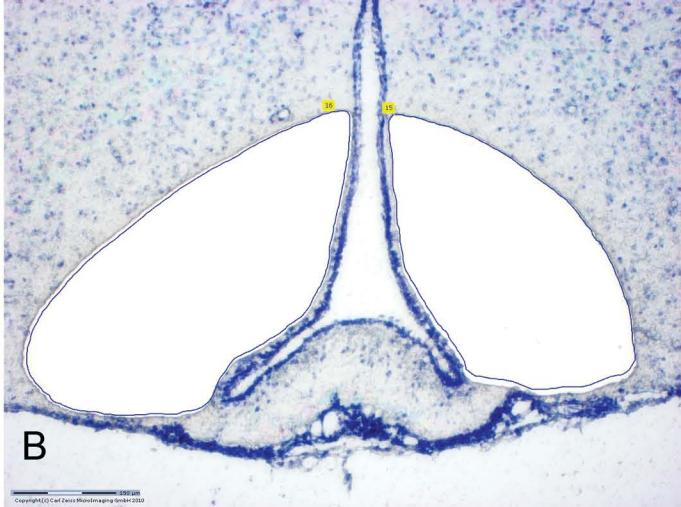
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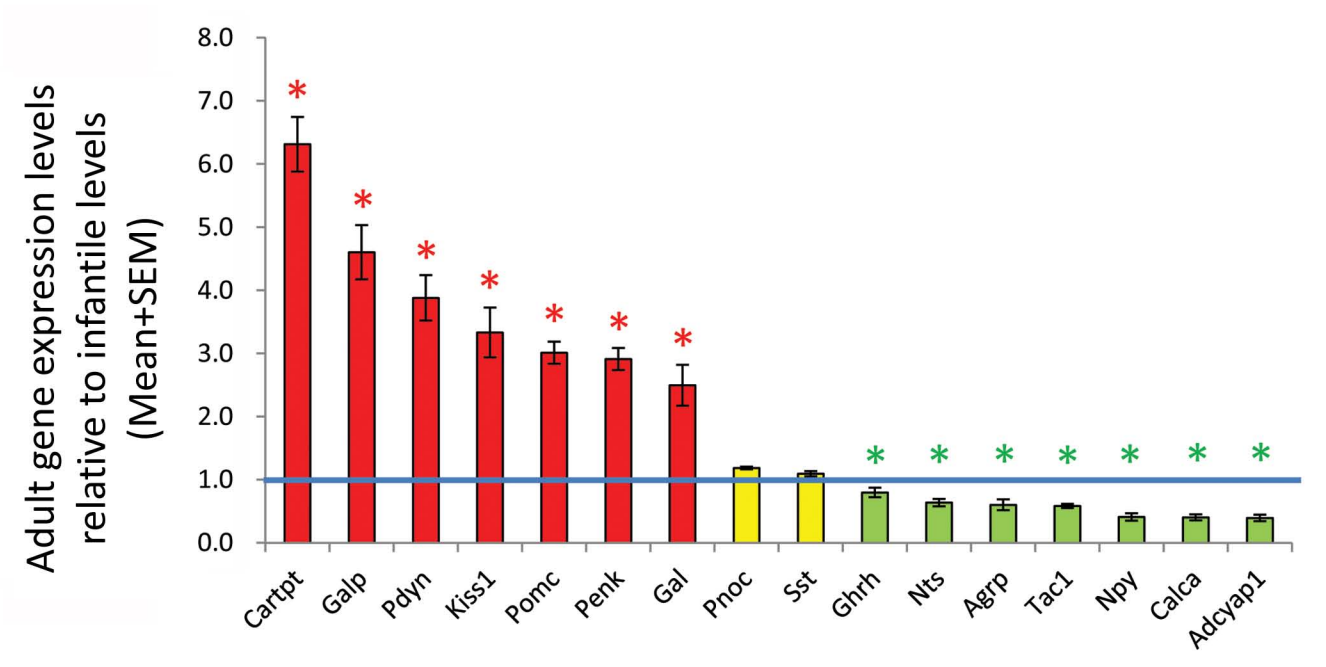
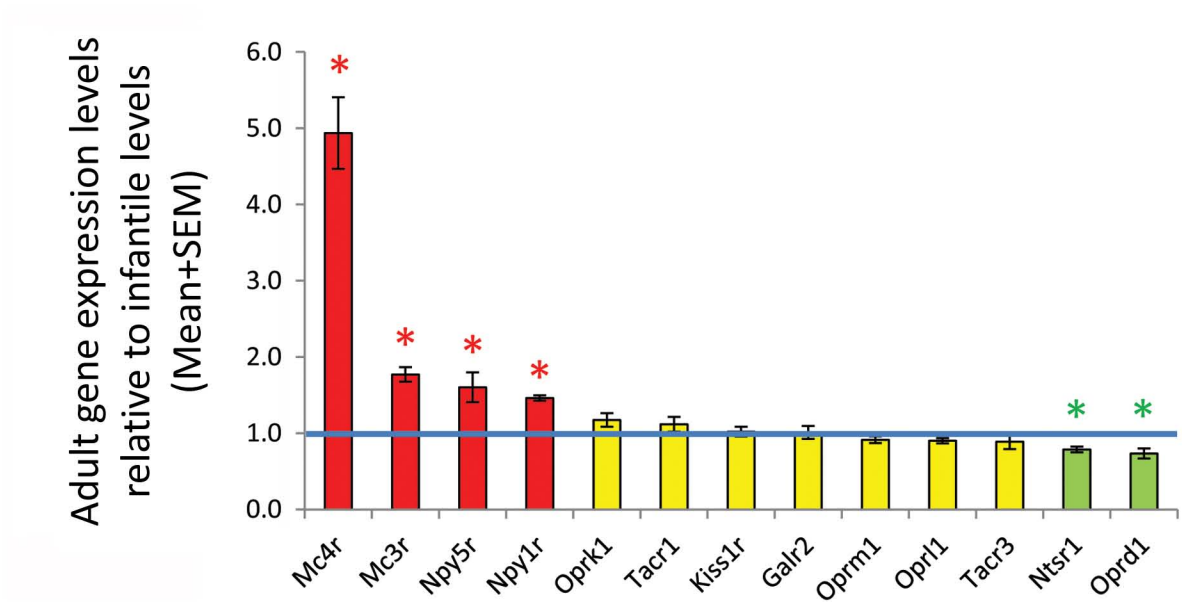
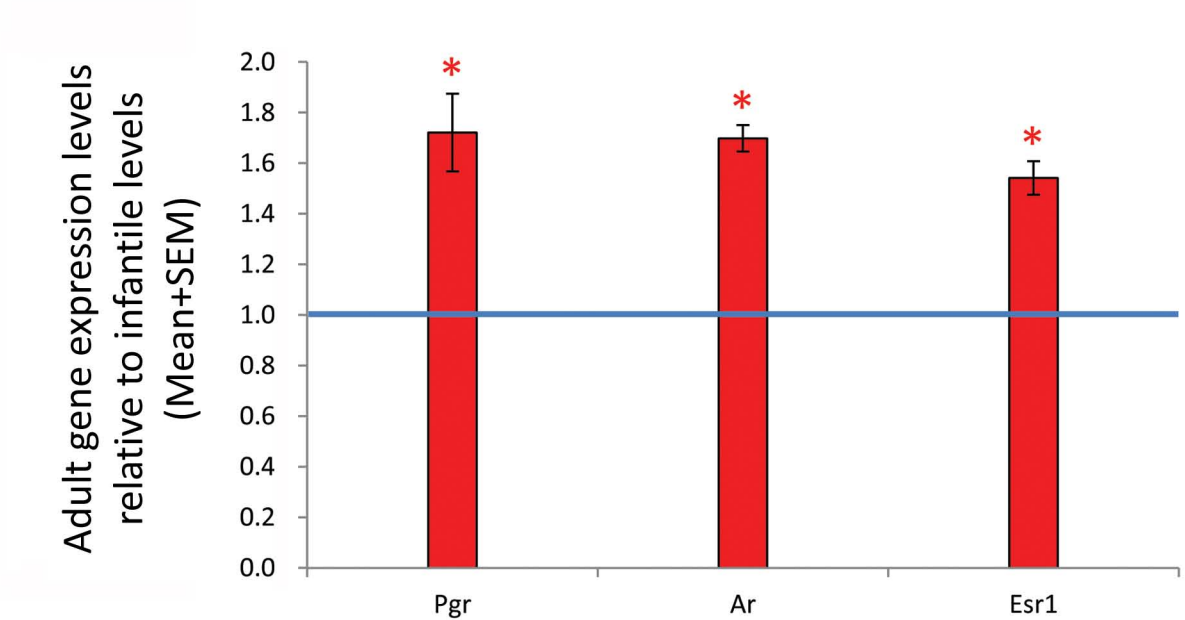
Gene/TaqMan ID	Neuropeptides	RQ	p	Change
Cartpt-Mm04210469_m1	**Cocaine- and amphetamine-regulated transcript (CART)**	**6.312**	**0.000**	**Increase (p<0.05)**
Galp-Mm00626135_m1	**Galanin-like peptide (GALP)**	**4.601**	**0.000**	
Pdyn-Mm00457573_m1	**Dynorphin**	**3.879**	**0.000**	
Kiss1-Mm03058560_m1	**Kisspeptin (KP)**	**3.331**	**0.001**	
Pomc-Mm00435874_m1	**Proopiomelanocortin (POMC)**	**3.011**	**0.000**	
Penk-Mm01212875_m1	**Proenkephalin**	**2.911**	**0.001**	
Gal-Mm00439056_m1	**Galanin**	**2.495**	**0.003**	
Pnoc-Mm00803087_m1	Prokineticin	1.185	0.099	No change
Sst-Mm00436671_m1	Somatostatin (SS)	1.094	0.169	
Ghrh-Mm00439100_m1	**Growth hormone-releasing hormone (GHRH)**	**0.797**	**0.043**	**Decrease (p<0.05)**
Nts-Mm00481140_m1	**Neurotensin (NT)**	**0.636**	**0.001**	
Agrp-Mm00475829_g1	**Agouti-related protein (AGRP)**	**0.601**	**0.003**	
Tac1-Mm01166996_m1	**Substance P (SP), Neurokinin A (NKA)**	**0.585**	**0.000**	
Npy-Mm03048253_m1	**Neuropeptide Y (NPY)**	**0.409**	**0.000**	
Calca-Mm00801463_g1	**Calcitonin gene-related peptide (CGRP)**	**0.402**	**0.000**	
Adcyap1-Mm00437433_m1	**Pituitary adenyl cyclase activating peptide (PACAP)**	**0.394**	**0.000**	
Tac2-Mm00436885_m1	Neurokinin B (NKB)	Not determined (Ct>30)		
Cck-Mm00446170_m1	Cholecystokinin (CCK)	Not determined (Ct>30)		



Gene/TaqMan ID	Neuropeptide receptors	RQ	p	Change
Mc4r-Mm00457483_s1	**Melanocortin receptor-4**	**4.935**	**0.000**	**Increase (p<0.05)**
Mc3r-Mm00434876_s1	**Melanocortin receptor-3**	**1.770**	**0.005**	
Npy5r-Mm02620267_s1	**Neuropeptide Y receptor-5**	**1.602**	**0.015**	
Npy1r-Mm00650798_g1	**Neuropeptide Y receptor-1**	**1.462**	**0.000**	
Oprk1-Mm01230885_m1	κ-opioid receptor	1.174	0.342	No change
Tacr1-Mm00436892_m1	Neurokinin receptor-1 (SP receptor)	1.118	0.683	
Kiss1r-Mm00475046_m1	Kisspeptin receptor	1.021	0.998	
Galr2-Mm00726392_s1	Galanin receptor-2	1.010	0.872	
Oprm1-Mm01188089_m1	μ-opioid receptor	0.915	0.220	
Oprl1-Mm00440563_m1	nociceptin/ orphaninFQ receptor	0.902	0.193	
Tacr3-Mm00445346_m1	Neurokinin receptor-3 (NKB receptor)	0.890	0.247	
Ntsr1-Mm00444459_m1	**Neurotensin receptor-1**	**0.786**	**0.032**	**Decrease (p<0.05)**
Oprd1-Mm00443063_m1	**δ-opioid receptor**	**0.734**	**0.029**	
Tacr2-Mm00436898_m1	Neurokinin receptor-2 (NKA receptor)	Not determined (Ct>30)		
Galr1-Mm00433515_m1	Galanin receptor-1	Not determined (Ct>30)		



Gene/TaqMan ID	Sex steroid receptors	RQ	p	Change
Pgr-Mm00435628_m1	**Progesterone receptor (PR)**	**1.721**	**0.004**	**Increase ($p<0.05$)**
Ar-Mm00442688_m1	**Androgen receptor (AR)**	**1.698**	**0.003**	
Esr1-Mm00433149_m1	**Estrogen receptor-α (ER-α)**	**1.541**	**0.003**	
Esr2-Mm00599821_m1	Estrogen receptor-β (ER-β)	Not determined (Ct>30)		



Gene/TaqMan ID	Markers for classic transmitters	RQ	p	Change
Th-Mm00447557_m1	**Tyrosine hydroxylase**	**1.442**	**0.005**	**Increase (p<0.05)**
Chat-Mm01221882_m1	Choline acetyltransferase (CHAT)	1.220	0.218	No change
Gad2-Mm00484623_m1	Glutamic acid decarboxylase-65 (GAD65)	1.179	0.366	
Slc32a1-Mm00494138_m1	Vesicular inhibitory amino acid transporter (VIAAT; VGAT)	1.164	0.311	
Gad1-Mm00725661_s1	Glutamic acid decarboxylase-67 (GAD67)	1.024	0.904	
Slc17a6-Mm00499876_m1	**Vesicular glutamate transporter-2 (VGLUT2)**	**0.570**	**0.000**	**Decrease (p<0.05)**