

1 **Altered gene expression profile of the hypothalamic arcuate nucleus of male mice suggests**
2 **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.

52

53 **Introduction**

54 Puberty in mammals is a complex process of sexual development which leads to complete gonadal
55 maturation and the attainment of full reproductive capacity [1-4]. Puberty can take place gonad-
56 independently [5] with the activation of hypothalamic gonadotropin-releasing hormone (GnRH) neurons
57 [1-3], leading to the onset of pulsatile GnRH secretion into the hypophyseal portal circulation. Neuronal
58 and glial signals that play either causal or permissive roles in puberty initiation are multiplex and include
59 increased levels of peripheral leptin [6], enhanced central glia-to-neuron signaling [7], reduced central
60 inhibitory NPY tone [8] and in particular, reduced hypothalamic GABA- [9, 10], followed by increased
61 glutamate [9, 11-13] release. Peptide and amino acid neurotransmitters synthesized and/or acting in the
62 hypothalamic arcuate nucleus (ARC) are critically involved in pubertal development. Recent human
63 genetics provide evidence that kisspeptin (KP) and neurokinin B (NKB) are particularly important for the
64 pubertal awakening of the 'GnRH pulse generator', via signaling through their specific G protein-coupled
65 receptors Kiss1r and NK3, respectively [14-16]. KP neurons in the ARC of the sheep and rodents co-
66 express NKB and dynorphin [17, 18] and hence, are commonly referred to as 'KNDy neurons'. KNDy
67 neurons of the ARC seem to represent a long-sought key element of the GnRH pulse generator network
68 [18, 19] and intact KP/Kiss1r signaling to GnRH neurons is a requirement for puberty to occur in both
69 humans [14, 15] and laboratory rodent species [20]. The mechanisms initiating puberty upstream from
70 KP neurons are still poorly understood and highly complex, involving both genetic and epigenetic [21]
71 regulatory events [22].

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

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

80 **Materials and methods**

81 **Experimental animals**

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

89 **Section collection for laser capture microdissection**

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

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

106 ***RNA isolation from the ARC samples***

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

111 ***Reverse transcription and preamplification***

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

116 **Quantitative real-time PCR studies**

117 Custom TaqMan microfluidic cards (Thermo Fisher Scientific) were preloaded by the manufacturer
118 with inventoried assays for genes of our interest, encoding 18 neuropeptides, 15 neuropeptide receptors,
119 4 nuclear sex steroid receptors and 6 presynaptic markers for classic neurotransmitters (Figs. 2-5). Each
120 assay consisted of a FAM dye-labeled TaqMan MGB probe and two PCR primers. Glyceraldehyde-3-
121 phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl-transferase (*Hprt*) were
122 used as housekeeping genes [25]. The geometric mean Ct value of the two housekeeping genes was used
123 for Δ Ct calculation [25]. Relative quantification against the calibrator samples was carried out to
124 determine $\Delta\Delta$ Ct values with the ViiA 7 RUO software (Applied Biosystems). In view that the reliability
125 of the assay decreases at high cycle numbers, genes with mean Ct values above 30 were not evaluated.

126 *Statistical analysis*

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

133 **Results**

134 **Neuropeptides**

135 qPCR studies revealed robust developmental changes in the expression of several neuropeptide
136 transcripts. Seven neuropeptide mRNAs showed increased, and another seven showed decreased levels in
137 adult male mice, compared with their expression level in 14-day-old infantile mice (Fig. 2). As ranked by
138 highest fold-change (RQ), mRNAs with increased adult levels encoded for CART (RQ=6.3), GALP
139 (RQ=4.6), dynorphin (RQ=3.9), KP (RQ=3.3), POMC (RQ=3.0), proenkephalin (RQ=2.9) and galanin
140 (RQ=2.5), whereas mRNAs with reduced adult levels encoded for PACAP (RQ=0.4), CGRP (RQ=0.4),
141 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
142 case of *Tac2* and *Cck* mRNAs. A different inventoried assay from Applied Biosystems
143 (Mm01160362_m1) was also purchased but showed similarly poor amplification ($Ct > 30$) of *Tac2* cDNA.

144 **Neuropeptide receptors**

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

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

153 **Nuclear sex steroid receptors**

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

157 **Classic neurotransmitters**

158 Gene expression studies revealed that the expression of the dopaminergic marker enzyme tyrosine
159 hydroxylase increased (RQ=1.44), whereas *Slc17a6* mRNA encoding for vesicular glutamate transporter-
160 2 decreased significantly (RQ=0.57). There was no change in the expression of the presynaptic GABA
161 markers *Gad1*, *Gad2* and *Slc32a1* and the cholinergic marker *Chat* (Fig. 5).

162

163 **Discussion**

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

173 **Neuropeptides**

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

178 **Developmental increases in ‘KNDy’ peptide mRNAs**

179 Much attention has been paid recently to the involvement of ‘KNDy’ neuropeptides in the
180 regulation of puberty onset. Our present study provided evidence that pubertal maturation of male
181 mice is accompanied by a 3.3-fold increase in *Kiss1* expression of the ARC between postnatal day
182 14 and 60. This observation differs from the reported absence of pubertal changes in *Kiss1* mRNA
183 expression in female mice [26]. Given that both studies used similar qPCR methodologies, the
184 discrepant results may be due to the different sex (and sex steroid milieu) of the model animals
185 and/or the different tissue sampling methods used. Of note, the authors of the female study
186 proposed that the negative regulation of *Kiss1* expression by gonadal sex steroids could mask the
187 pubertal changes in gene expression. Indeed, they have provided evidence for this concept by
188 showing that the sex steroid-deficient hypogonadal female mice exhibit robust developmental
189 increases in ARC *Kiss1* mRNA levels [26]. Negative regulation of *Kiss1* mRNA by gonadal steroids
190 also characterizes the male mouse model which we used in this study. Indeed, testosterone produces
191 a massive suppression of *Kiss1* expression in the ARC of adult male mice [27]. Despite the masking
192 effect of this negative regulation, we were able to distinguish a net 3.3-fold developmental increase
193 between days 14 and 60, what we attribute to the accurate LCM sampling method we used.

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

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

207 **Enhanced *Cartpt* expression**

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

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

223 **Enhanced *Galp* expression**

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

230 Galanin is widely distributed in the brain, whereas *Galp* mRNA is detected only in the ARC, the
231 median eminence, the infundibular stalk and the posterior pituitary of rats [37-40], mice [41] and
232 primates [36]. GALP-containing cells are distinct from those synthesizing NPY [39], somatostatin
233 [39] and KP [42]. The only colocalization reported so far was between GALP and α -MSH; in one
234 immunohistochemical study, 3-12% of the α -MSH cell bodies were also GALP-immunoreactive
235 [43]. Conflictingly, Takatsu et al. have not found evidence for this colocalization phenomenon [39].
236 GALP plays an important role in the central regulation of reproductive functions. Accordingly,
237 intracerebroventricular administration of GALP to male rats markedly increased plasma levels of
238 LH [44]. This effect was mediated by GnRH neurons and could be blocked with the GnRH-receptor
239 antagonist Cetrorelix [44]. Furthermore, in the same study GALP also induced Fos
240 immunoreactivity in GnRH neurons of the medial preoptic area [44]. Using *in situ* hybridization
241 histochemistry, Kawagoe and colleagues observed a marked pubertal increase in *Galp* mRNA levels
242 of the ARC in male and female rats [45]. These observations are in concordance with the 4.6-fold
243 pubertal increase in *Galp* transcript we report here in male mice, and suggest that increased levels
244 of GALP might have a role in the maturation of reproductive functions. In support of this concept,

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

248 **Enhanced *Pomc* mRNA**

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

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

263 **Enhanced expression of *Penk* and *Gal***

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

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

272 Similarly to the *Penk* transcript, *Gal* mRNA was not found to change in the ARC of female rats
273 during puberty [55], in contrast with the 2.5-fold increase we observed here in male mice. Cell
274 types characterized by increased coexpression of *Gal* in adult mice may include KNDy neurons [58,
275 59] and/or growth hormone-releasing hormone neurons [60], among other cell types of the ARC.

276 **Reduced expression of *Agrp* and *Npy***

277 The orexigenic peptides AGRP and NPY are both potent inhibitors of pulsatile LH secretion
278 [61, 62].

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

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

291 administration of NPY Y1 receptor antagonist to infantile animals elicits precocious GnRH release
292 [65]. Furthermore, intracerebroventricular administration of NPY to adult male [65] and female [66]
293 monkeys inhibits pulsatile GnRH release.

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

304

305 **Reduced adult expression of *Ghrh***

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

311 **Reduced *Nts*, *Tac1*, *Calca* and *Adcyap1* expression**

312 Neurotensin in the ARC of the rat is colocalized with GHRH, TH, galanin and glutamic acid
313 decarboxylase [67]. It remains to be determined which one of these cell populations shows reduced *Nts*
314 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

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

340 **Transcriptional changes of neuropeptide receptor mRNAs**

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

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

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

363 **Sex steroid receptors**

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

370 **Presynaptic markers for classic neurotransmitters**

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

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

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

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

389 **Methodological considerations**

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

403 **Gene expression changes related and unrelated to puberty**

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

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

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

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

422 In female mice, some of the early changes in genes regulating reproduction already occur at the end
423 of the second postnatal week. Accordingly, the mRNA encoding *RF-amide related peptide* falls between
424 day 15 and 20 in the dorsomedial nucleus [83] and *Tac2* mRNA in the ARC rises between day 12 and 15
425 [26]. The choice of day 14 allowed us to study the earliest developmental changes. On the other hand,
426 day 60 mice we chose for the adult group can be considered sexually fully mature as the production of
427 motile sperm occurs between day 40 and 55 [4]. While with this research design we were able to study
428 changes of a large number of target genes, the time when differences developed remained unknown. A
429 recent *in situ* hybridization study of female mice by Semaan and Kaufmann [83] revealed initial drops in

430 *Kiss1* mRNA/cell and total ARC *Kiss1* mRNA levels between day 15 and 20, followed by slight
431 increases in the number of *Kiss1* neurons and total ARC *Kiss1* mRNA levels by the time of vaginal
432 opening. There was no overall increase in *Kiss1* cell numbers and ARC *Kiss1* mRNA levels between day
433 15 and 30, similarly to the lack of changes in *Kiss1* expression between day 10 and 60 in qRT-PCR
434 studies by Gill and colleagues [26].

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

447

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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- μ m 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: 150 μ m.

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

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

479
480 **Figure 5. The altered expression profile of classic presynaptic neurotransmitter markers suggests**
481 **increased dopaminergic (*Th*) and decreased glutamatergic (*Slc17a6*) neurotransmission, whereas**
482 **the expression of GABAergic (*Gad1*, *Gad2*, *Slc32a1*) and cholinergic (*Chat*) presynaptic markers**
483 **does not change in adult vs. infantile mice.**

484

485

486 **References**

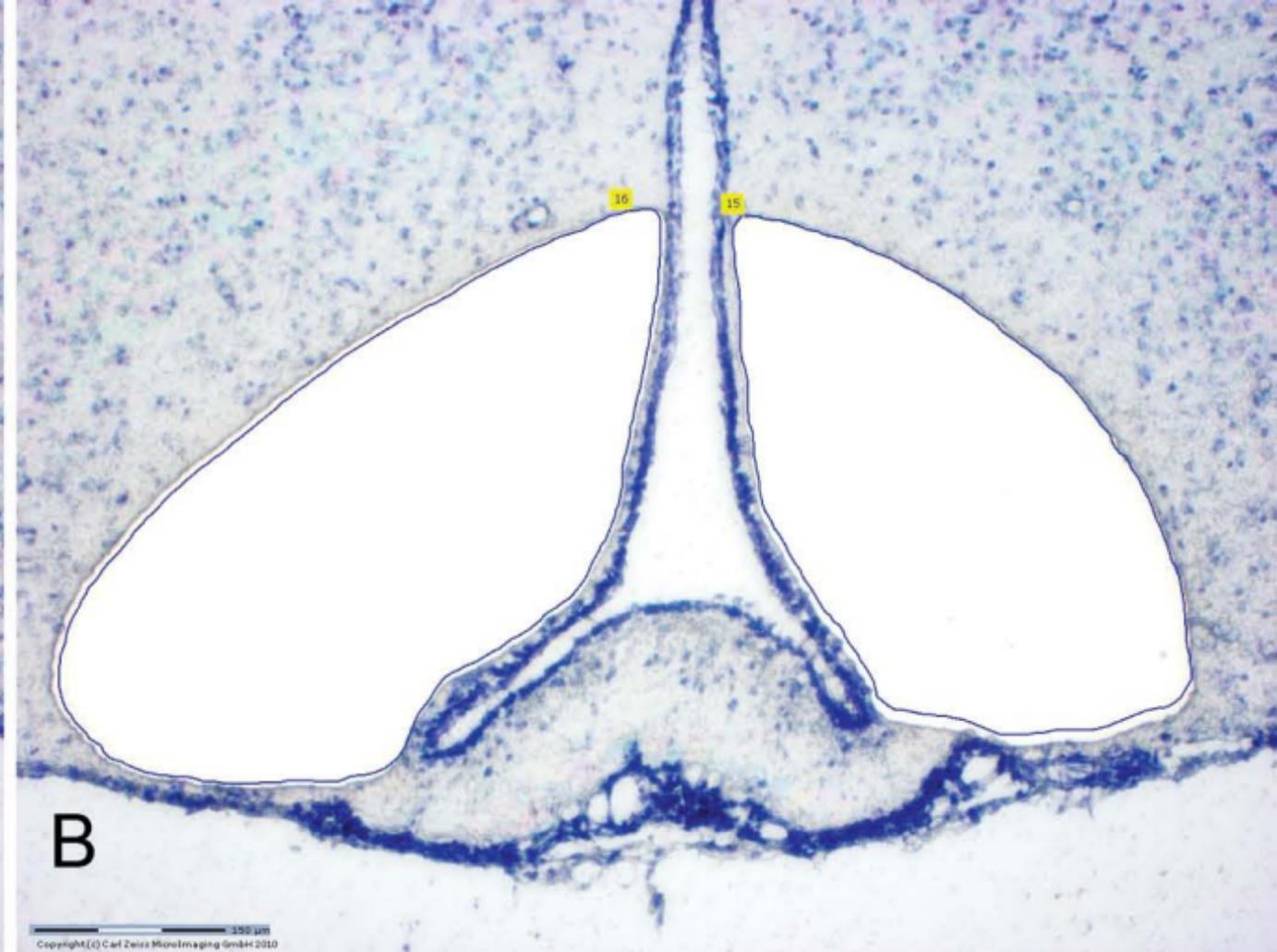
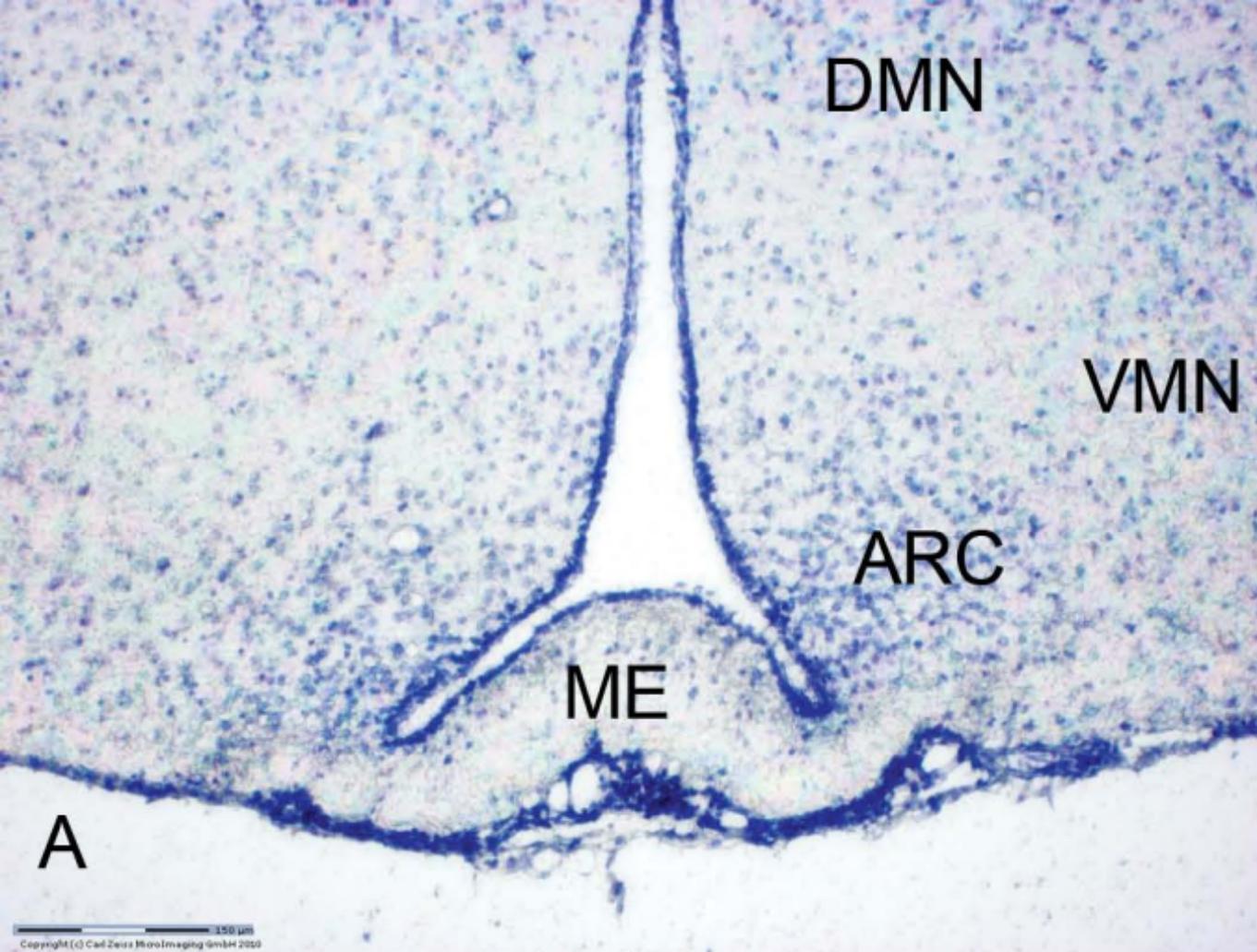
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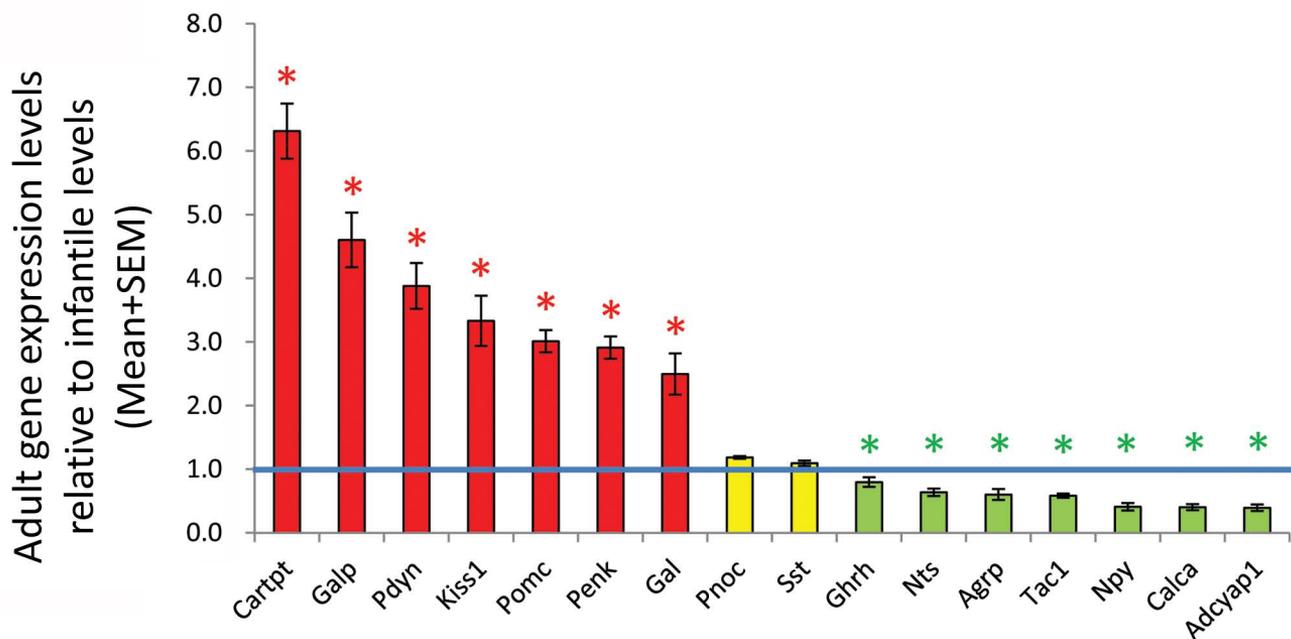
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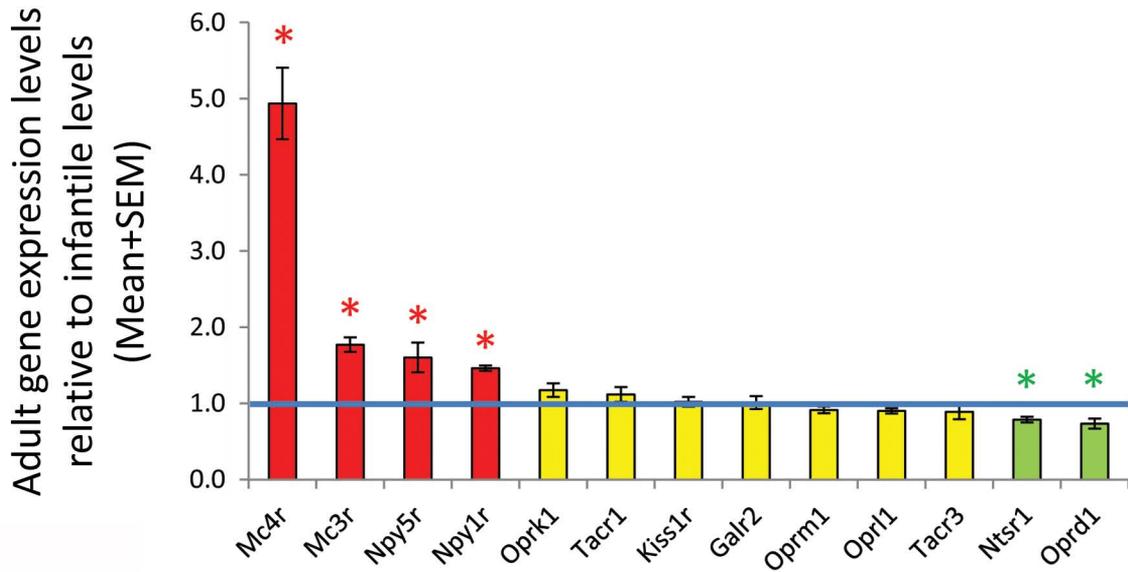
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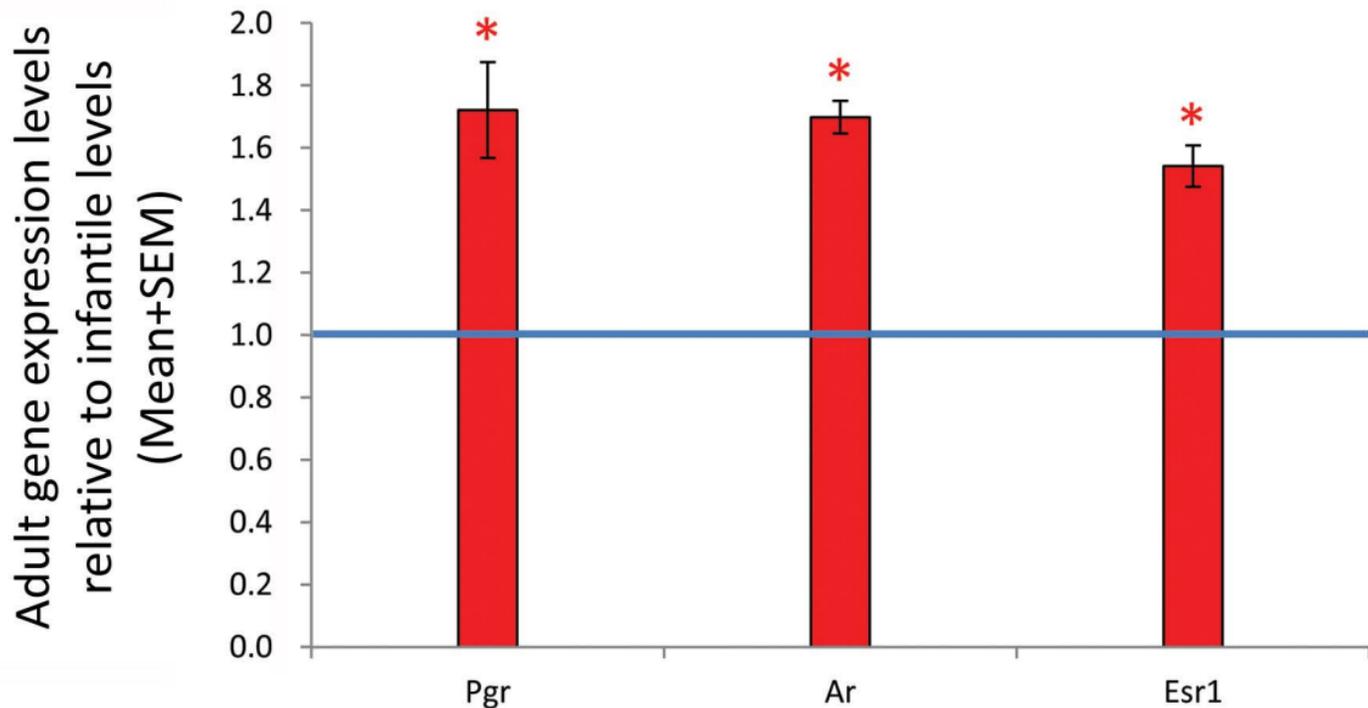
Gene/TaqMan ID	Neuropeptides	RQ	p	Change
Cartpt-Mm04210469_m1	Cocaine- and amphetamine-regulated transcript (CART)	6.312	0.000	Increase (p<0.05)
Galp-Mm00626135_m1	Galanin-like peptide (GALP)	4.601	0.000	
Pdyn-Mm00457573_m1	Dynorphin	3.879	0.000	
Kiss1-Mm03058560_m1	Kisspeptin (KP)	3.331	0.001	
Pomc-Mm00435874_m1	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 adenylyl 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)

