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Research article

The posterior pituitary expresses the serotonin receptor 2C

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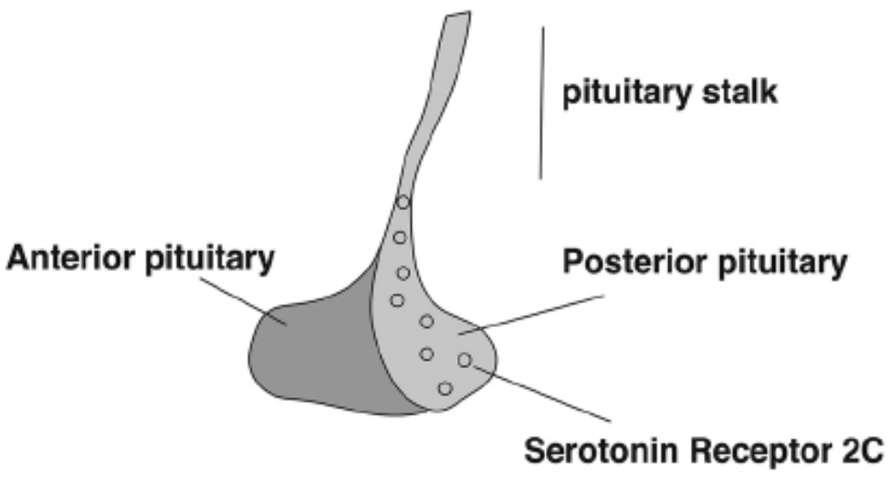
GRAPHICAL ABSTRACT



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ABSTRACT

The serotonin receptor 2C (5HT2C) is an important drug target to treat obesity and depression. Its pre-mRNA undergoes alternative splicing, encoding a short RNA1 isoform that is localized intracellularly and a full-length isoform (RNA2) that can reach the cell membrane. These splicing isoforms are deregulated in Prader-Willi syndrome (PWS), due to the loss of a trans-acting regulatory RNA, SNORD115. Here we show that the 5HT2C mRNA is expressed in the posterior pituitary, suggesting that 5HT2C mRNA is generated in the hypothalamus and subsequently conveyed by axonal transport. In the pituitary, the ratio of 5HT2C isoforms is regulated by feeding, and can be manipulated using a splice-site changing oligonucleotide injected into the blood. The pituitary expression of the 5HT2C mRNA may constitute a previously unknown mechanism whereby serotonin in the circulation or drugs targeting the 5HT2C might induce side-effects. Finally, the deregulation of 5HT2C splicing isoforms in PWS could contribute to the known hormonal imbalances.

1. Introduction

1.1. The serotonin receptor is targeted by a large number of drugs

The serotonin receptor 2C (5HT2C) is a seven transmembrane receptor regulating mood and appetite through its actions in the central nervous system [21,42]. Its deregulation is involved in depression [30], suicidal behavior [11], and spasticity after spinal cord injury [33] and 5HT2C knock-down induces obesity, making 5HT2C the target for antipsychotic drugs and appetite-controlling substances [30]. Several 5HT2C binding drugs are used clinically: The 5HT2C agonist lorcaserin exhibits high selectivity for 5HT2C and is approved as a drug for the short-term treatment of obesity, due to its enhancement of 5HT2C activity in POMC (pro-opiomelanocortin) neurons that results in an

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anorexic response [19]. In addition, a large number of drugs used to treat depression, anxiety, and schizophrenic disorders interact with the 5HT2C and with other serotonin receptor subtypes as well [8,30]. The change of the 5HT2C activity caused by these drugs contributes to their efficacy, but frequently causes side effects, most notably weight gain [2,37], as shown by the association between olanzapine-induced weight gain and 5HT2C polymorphisms [17,29].

1.2. 5HT2C gene structure

The human 5HT2C gene consists of six exons and hosts four miRNAs in intron II (Fig. 1A) [47] and two more miRNAs in intron IV [9]. In addition, intron II hosts SNORA35 [7]. The first three exons encompassing the 5' UTR are ubiquitously expressed, but are terminated through usage of an early polyadenylation site in intron III. Since neurons do not use this polyadenylation site, they express the remaining exons IV-VI [47], generating 5HT2C protein isoforms from a start codon in exon III. The pre-mRNA undergoes alternative splicing of exonVb creating two 5HT2C isoforms: RNA1 that encodes a truncated receptor and RNA2 that encodes a full-length receptor. Due to additional editing of RNA2, the 5HT2C gene generates a total of 33 mRNAs encoding 25 proteins: one protein encoded by RNA1 [48] and 32 RNA2 editing-isoforms encoding 24 proteins [5].

ExonVb inclusion is promoted by a snoRNA, SNORD115 [26,28], likely by changing the pre-mRNA structure around exonVb [38]. SNORD115 binds directly to exonVb via an 18 nucleotide base complementarity [26,28]. SNORD115 is not expressed in subjects with Prader-Willi syndrome (**PWS**), a genetic cause for obesity and intellectual disability [12]. Mouse models suggest that the loss of SNORD115 leads to an increase of the RNA1/RNA2 ratio in the hypothalamus, showing the in vivo relevance of SNORD115-dependent 5HT2C isoform regulation [16]. The truncated receptor encoded by RNA1 heterodimerizes with the full-length receptor protein, encoded by RNA2, leading to an internalization of the full-length receptor. This internalization stops 5HT2C signaling [31]. Since 5HT2C heterodimerizes with other receptors, such as the ghrelin receptor [36], the 5HT2C isoform ratio possibly influences a number of receptor systems.

1.3. Expression of the serotonin receptor 2C splice variants

So far, the expression of 5HT2C-RNA1 and 5HT2C-RNA2 has only been reported in the brain, with the highest abundance of RNA2 in the ventromedial hypothalamus, cerebral cortex, amygdala, and basal ganglia [34]. The choroid plexus almost exclusively expresses RNA1 [6,45], which is a minor form in all other brain regions [28]. Since the 5HT2C-Ser23 polymorphism leads to higher cortisol levels that are associated with anger, depression and type 2 diabetes [4] and PWS is associated with altered levels of growth hormone (GH) and oxytocin, we hypothesized that 5HT2C is expressed in the pituitary gland and that the ratio of 5HT2C-RNA splice variants is altered in PWS. In support of our hypothesis, we detected 5HT2C mRNA in pituitary tissue from mice, rats, and humans. Pituitary tissue from a PWS mouse model expresses an altered 5HT2C isoform ratio, suggesting that a pituitary deregulation of 5HT2C isoforms could contribute to hormonal imbalances characteristic for PWS. The 5HT2C splicing isoforms are influenced by food intake and are rapidly changed by splice-site changing oligonucleotides, suggesting that a pituitary expression of 5HT2C should be considered in evaluating drugs like olanzapine or lorcaserin that interact with the 5HT2C.

2. Material and methods

2.1. RT-PCR was performed using gene-specific primers as indicated

m5HT2c ex6 rev1: TTCACGAACACTTTGCTTTCG
m5HT2c ex4 for2: GCTGATATGCTGGTGGGACT
h5HTex6_R2: CGCAGAACGTAGATGGTCAG
h5HTex4_F2: TGTCTCTCCTGGCAATCCTT

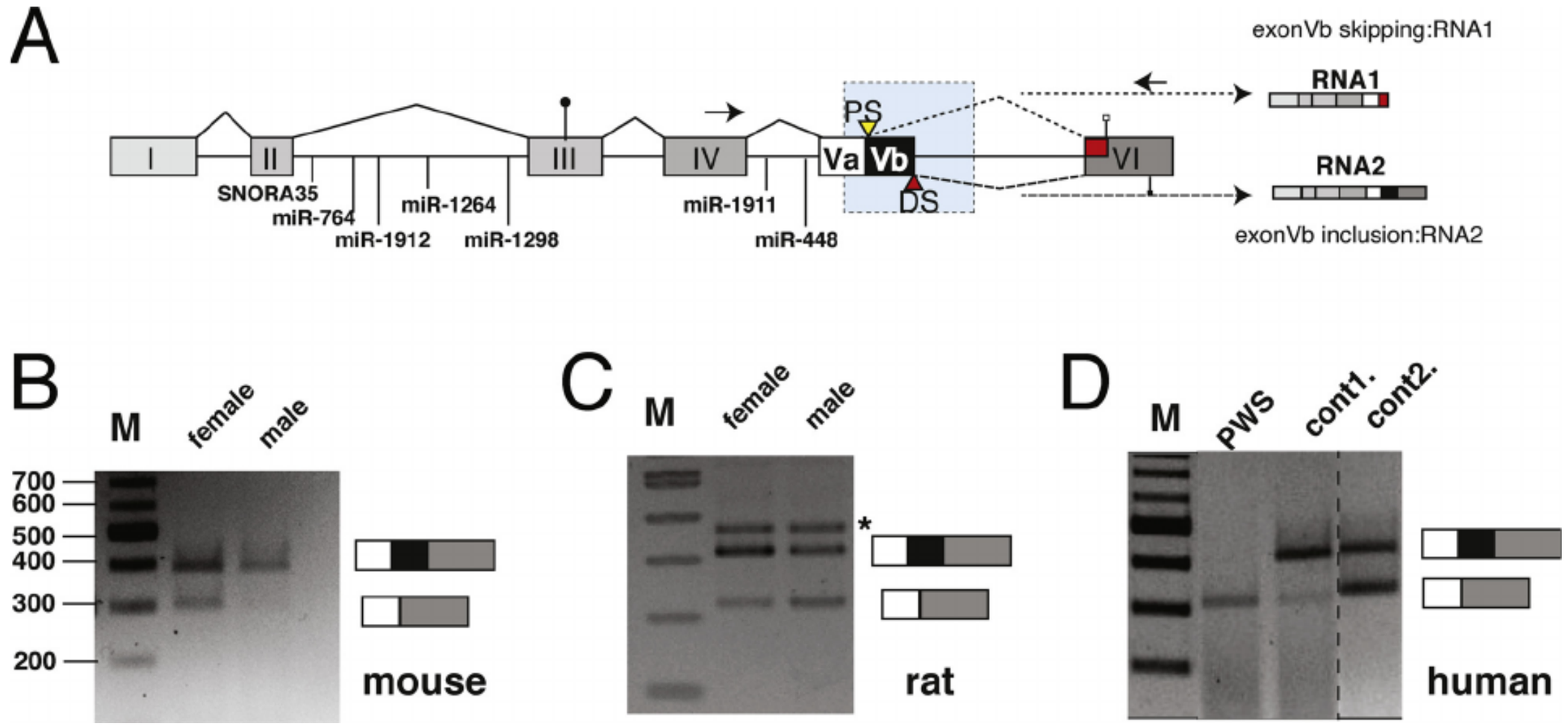

Fig. 1. 5HT2C isoforms are expressed in pituitary.
A) Gene structure of the 5HT2C receptor and location of the primers used for RT-PCR (arrows). PS, DS: proximal and distal splice sites. Roman numbers indicate the exons. The start codon in exon III is indicated by a circle, the two stop codons in exon VI by open and filled squares.
B) Expression of the 5HT2C isoforms in mouse pituitary. The upper band corresponds to RNA2, the lower one to RNA1.
C) Expression of the 5HT2C isoforms in rat pituitary. The star indicates a heterodimer.
D) Expression of the 5HT2C isoforms in human pituitary. PWS: sample from a PWS patient (NIH NeuroBioBank #5298). Controls: pituitaries from genetically normal

subjects, control #1: #5398, control 2: #5449; A third human control RNA located between cont1 and cont2 did not amplify and was omitted (dotted line).

ActinB Rev: GTACATGGCTGGGGTGTTGA
ActinB For: CCCCATTGAACACGGCATTG
Htr2cEx4 For1: ACTTGTCATGCCCCTGTCT
mHtr2cExVb Rev1: CCTTAGTCCGCGAATTGAAC
mHtr2cEx4 F: CCATTGCTGATATGCTGGTG
mRNA1junc R: ACTGAAACTCCCGGTCCAG
Rat 115 Rev: GCCTCAGCGTAATCCTATTGA
Rat 115 For: GGGTCAATGATGACAACATTAA

RNA was isolated using Purelink RNA mini kits from Ambion/Life Technology.

2.2. Animals

We used male and female Sprague-Dawley rats, 10–12 weeks old and male and female C57BL/6 mice, 6 months old. The animals were fed with standard rat and mouse chow and were exposed to a 14L:10D light dark cycle, with lights on at 6:00 AM EDT. Animals were deeply anesthetized with isofluorane between 2:40-4:50 PM EDT and euthanized by decapitation. Using a lighted magnifying glass, three tissue samples were dissected from each animal, i.e., the hypophyseal stalk and median eminence, the posterior pituitary gland, and the anterior pituitary, and were frozen until assays were conducted. During dissection of the rodent pituitary, the pars intermedia typically adheres to the posterior pituitary. The animal procedures, which were consistent with AAALAC guidelines, were designed to minimize pain and discomfort and were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

2.3. PWS-IC^{del} mice

The PWS-IC $^{+/}$ (referred to hereafter as PWS-IC ^{del}) and wild-type (WT) mice used in this study were bred under the authority of the Animals (scientific procedures) Act 1986 (UK), with all procedures conforming to the institutional and national guidelines, including those for genetically modified animals, and specifically approved by local ethical review. PWS-IC ^{del} mice and WT littermates were generated by crossing IC^{del}-positive males with WT females as previously described [18], briefly PWS-IC^{del} mice and WT littermates were generated by crossing IC ^{del}-positive males with WT females. Given the nature of the epigenetic regulation of imprinted genes, the paternally inherited IC deletion results in a lack of gene expression from the PWS interval. Since PWS-IC^{del} animals on a pure C57BL/6 J background suffer severe postnatal lethality, we crossed IC ^{del} positive males with CD1 females and selectively culled WT littermates (identified on the basis of their increased size 48 h after birth) leaving only 1 or 2 WT pups per litter. Animals were weaned at approximately 4 weeks of age and were single-sexed group housed with WT littermates (2–5 animals per cage). The mice were maintained in the JBIOS vivarium of Cardiff University on a 12 h light/dark cycle (lights on 07:00 h), with *ad libitum* access to water and standard laboratory chow (Rat and Mouse No. 3 Breeding Diet, Special Diet Services Ltd., Witham, Essex, UK).

2.4. Human tissue

Pituitaries were obtained from the NIH NeuroBioBank. Pituitaries were from #5298 (PWS, 31 years old, male, 10 h PMI (post mortem interval); #5398 unaffected control, 36 years old, male, 24 h PMI, #5449 unaffected control, 39 years old, male, 24 h PMI).

3. Results

pre-mRNA in mouse pituitary using RT-PCR. Using primers in exons Va and VI (Fig. 1A), we could detect RNA1 and RNA2 in pituitary tissue from both male and female mice, the upper band corresponding to RNA2, the lower one to RNA1 (Fig. 1B). The RNA2/RNA1 ratio is variable among individual mice (see also Fig. 4). We next investigated male and female rat pituitaries and found similar expression (Fig. 1C). Finally, we determined the expression in human pituitaries, both from genetically normal subjects and one available PWS subject. Again, the 5HT2C isoforms were readily detectable. RNA2 is not detectable in the pituitary of the one available PWS subject (Fig. 1D). Due to the nature and availability of human post-mortem tissue, it remains to be determined whether this is a general feature.

3.2. 5HT2C is expressed in the posterior pituitary

Based on its embryological origins, the pituitary gland is anatomically subdivided into two major parts, the adenohypophysis and neurohyphophysis. The adenohypophysis includes the pars distalis (anterior lobe), which is the main part of the gland, as well as the pars tuberalis surrounding the infundibular stalk, and a rudimentary pars intermedia. The pars intermedia is a small region that is vestigial in adult humans but functional in rodents. The anterior lobe contains two types of secretory cells, the acidophils which secrete growth hormone and prolactin, and the basophils, which secrete adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), and luteinizing hormone (LH). The neurohypophysis has two subdivisions, the pars nervosa (neural or posterior lobe) and the infundibulum, which includes the median eminence and the infundibular stalk, consisting of axonal projections emanating from the magnocelluar neurosecretory cells located in the hypothalamic supraoptic (SON) and paraventricular nucleus (PVN). These magnocellular neurons synthesize vasopressin and oxytocin and release them from axon terminals in the neural lobe (Fig. 2A). In addition to unmyelinated axons, the neurohypophysis contains pituicytes, astrocyte-like glial cells whose processes surround the axons of the neuroendocrine neurons.

To investigate the localization of 5HT2C mRNA in the pituitary, we dissected the anterior lobe, posterior lobe, and stalk/median eminence from rat pituitaries. To allow comparisons, the mRNA isolated from each part was dissolved in an equal volume of water. RT-PCR analysis showed that the anterior pituitary lacked 5HT2C mRNA expression, which was detectable in posterior pituitary and most abundant in pituitary stalk (Fig. 2B). This relative abundance is in contrast to the total RNA concentration, which was highest in anterior pituitary and lowest in pituitary stalk, which was also reflected by the abundance of beta-actin mRNA (Fig. 2D) and could be validated by qPCR (Fig. 2E). Since 5HT2C mRNA with the exception of the choroid plexus is neuron-specific, its most likely source are thus magnocelluar neurosecretory cells in the paraventricular and supraoptic nucleus that generate the axonal projections forming the pituitary stalk and posterior pituitary.

3.3. Pituitary expresses SNORD115 that regulates 5HT2C isoforms

The inclusion of 5HT2C's exon Vb is promoted by SNORD115, a neuron-specific C/D box snoRNA that is not expressed in subjects with PWS [7,27,42]. Using RNase protection analysis [26] and RNAseq shorter fragments of SNORD115 have been detected [23].

To test whether SNORD115 is also expressed in pituitary, we first used RT-PCR and detected SNORD115 in both the anterior and posterior pituitary as well as in the pituitary stalk (Fig. 2C). To test

3.1. 5HT2C is expressed in pituitary from mouse, rat and human

We hypothesized that 5HT2C expression outside the brain could contribute to off-target effects of antidepressent drugs and possibly to hormonal imbalances in PWS. We thus tested the expression of 5HT2C

SNORD115 expression with a different method that will not detect possible DNA contaminants, we used RNase protection analysis and human samples, employing a probe against human SNORD115 [14,15]. We used frontal cortex from PWS subjects and human controls for comparison. SNORD115 is processed into smaller RNAs, which is reflected by the occurrence of bands smaller than the full-length 89 nt

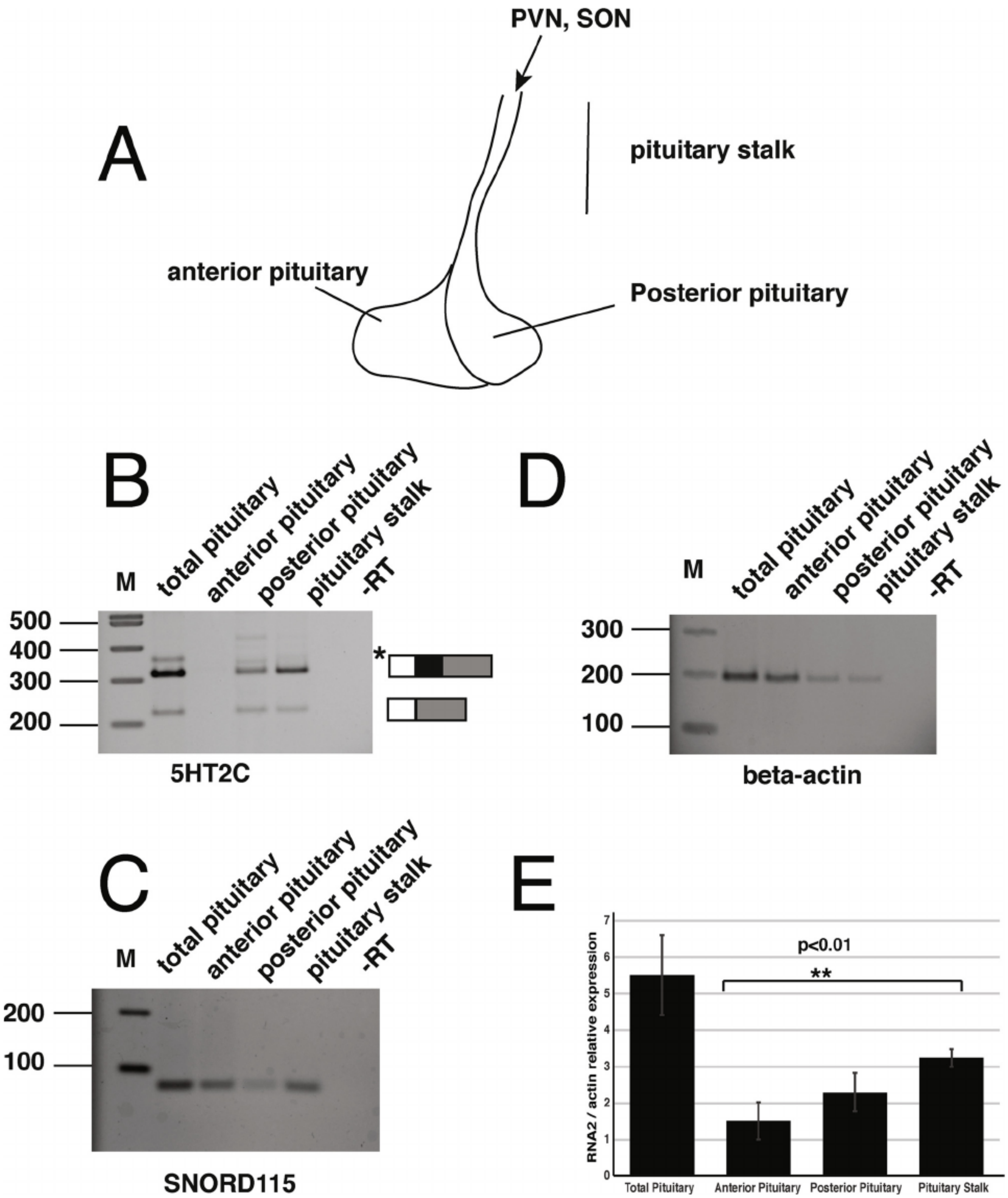

Fig. 2. Expression of 5HT2C in anterior and posterior pituitary.

A) Schematic representation of rat pituitary. PVN: Paraventricular nucleus; SON: supraoptic nucleus

A) Schematic representation of rat pituitary. PVN: Paraventricular nucleus; SON: supraoptic nucleus.
B) Rat pituitaries were dissected into anterior and posterior pituitary and stalk. The RNA corresponding to each part was dissolved in an equal volume of water and equal volumes were used in the RT-PCR reaction to detect 5HT2C mRNA. The star indicates a heterodimer.
C) RT-PCR of the same RNA aliquots using primers against SNORD115.
D) RT-PCR of the same RNA aliquots using primers against beta-actin.
E) qPCR analysis of RNA2 expression in dissected pituitary, normalized to beta-actin, n = 4.

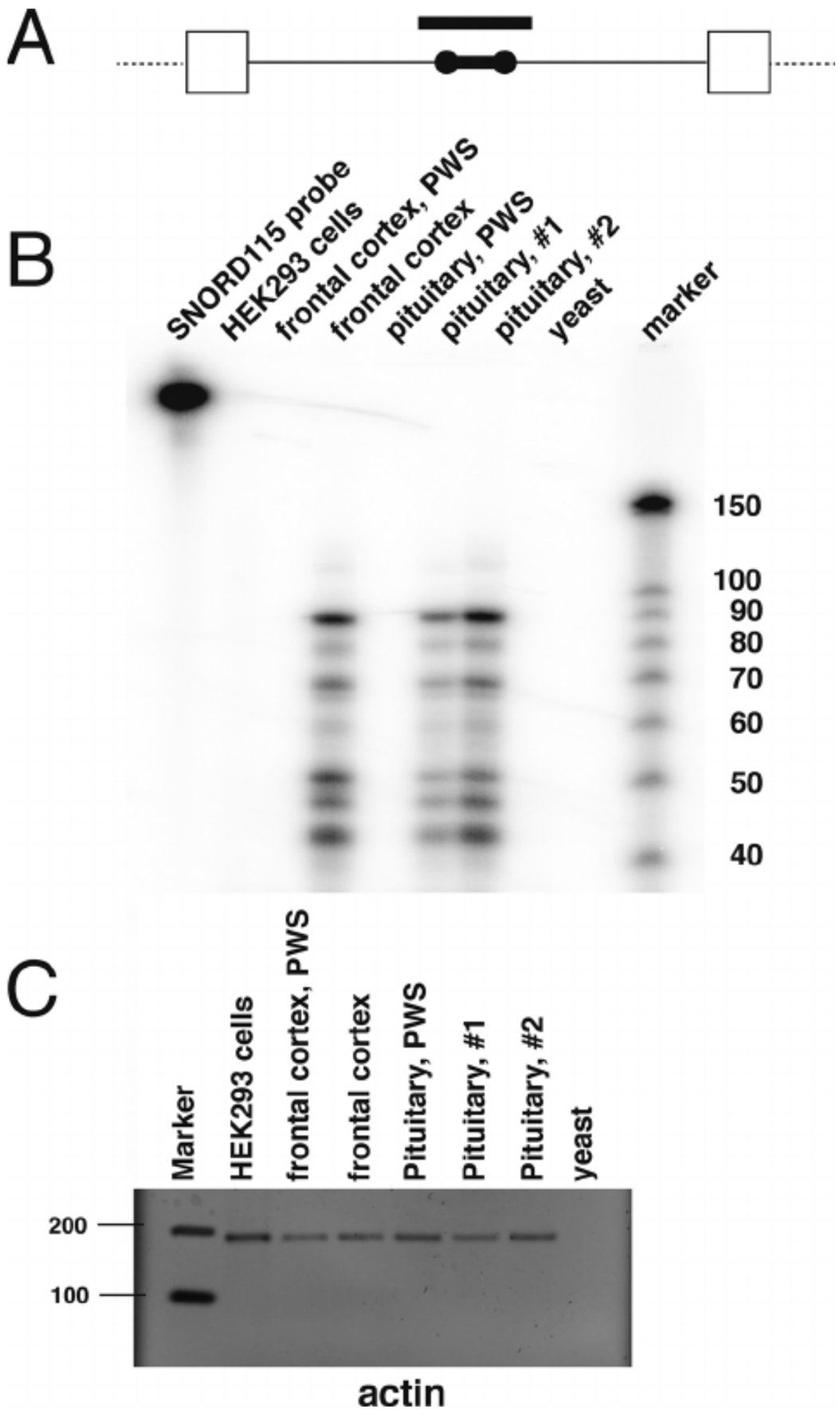

Fig. 3. Human pituitary expresses SNORD115.
A) Schematic representation of the RNase protection probe (thick black line) that hybridizes to SNORD115 (thick line between two dots), which is located in an intron between two non-coding exons (boxes).
B) 10 g of brain (frontal cortex) or pituitary total RNA was analyzed with a uniformly labeled antisense RNA probe that exhibit sequence complementarity to SNORD115; yeast: yeast RNA as a control, pituitary#1 and #2 were from subjects #5398, #5449, respectively.
C) RT-PCR of the same samples using primers against beta-actin.

fragment. As expected, SNORD115 is absent in subjects with PWS, both in their frontal cortex and pituitary. However, SNORD115 can be detected in pituitaries from non-PWS subjects. The pattern of protected bands is similar to frontal cortex, strongly suggesting similar processing

compared with wild-type littermates. Compared with the wild-type, the PWS-IC^{del} mice show a statistically significant ($p < 0.01$, $n = 12$) increase of RNA1 in hypothalamus, when measured by end-point PCR (Fig. 4A–C). This increase likely reflects the missing SNORD115 that promotes exon Vb inclusion. The data were confirmed by qPCR, where we observed a decrease in RNA2 levels in PWS mice when we compared the RNA2 expression relative to beta actin (Fig. 4D).

3.5. Ratios of 5HT2C isoforms change in feeding

Based on the inter-individual fluctuations in the pituitary 5HT2C isoform ratios (Fig. 1B–D), we considered the possibility that physiological stimuli influence the RNA1/RNA2 ratios. Given the role of 5HT2C in food uptake, we tested the influence of food withdrawal as a physiological condition on the isoform ratio. Mice underwent food withdrawal for either 14 or 38 h, but had free access to water. The 5HT2C isoforms were measured by RT-PCR. In male mice, food withdrawal strongly increased the RNA2/RNA1 ratio (Fig. 5A, B). In contrast, there was no statistical significant change in female mice (data not shown).

3.6. RNA2/RNA1 ratios can be modulated by splice site-changing oligonucleotides

We recently developed an oligonucleotide, oligo#5, that causes exon Vb inclusion and reduces food intake in mice upon either intracerebroventricular (ICV) or systemic injection [48]. Oligo#5 binds to an intronic region of intron V that is part of the dsRNA structure regulating exon Vb inclusion. Oligo#5 is a 2′-*O*-methyl- phosphothioate that accumulates in the brain after systemic injections [48]. Since systemic injections of oligo#5 causes a decrease in food intake [48], lasting for two days, we tested whether oligo#5 also affects splicing isoforms in pituitary. Male C57BL/6 mice were injected in the tail vein with 10 g oligo#5 (dissolved in 10 microliters of PBS) and sacrificed after 2–6 hrs. We used an oligo with cy3 attached, allowing its detection in tissue. The cy3 labeled oligo accumulates in pituitary and can be detected six hours after injection, indicating that the pituitary is accessible to oligonucleotides in the blood (Fig. 5C, D). When measured by RT-PCR, we saw a marked increase of exonVb inclusion, as early as 2 h after injection, indicating that 5HT2C isoforms can be rapidly regulated in pituitary.

4. Discussion

4.1. The serotonin receptor 2C mRNA is expressed in pituitary

We report for the first time that 5HT2C mRNA is expressed in pituitary. To pinpoint the anatomical site of expression, we dissected rat pituitaries and located the expression to the posterior pituitary and the pituitary stalk; both structures are formed by axons from magnocelluar neurosecretory cells in the paraventricular and supraoptic nuclei. 5HT2C expression has been detected in the paraventricular nucleus [20]. The most likely explanation of our findings is that neuron-specific

in pituitary and other parts of the brain (Fig. 3A, B). RT-PCR analysis shows the presence of RNA in all samples (Fig. 3C). Thus, the pituitary also expresses the splicing regulator for 5HT2C.

3.4. The RNA2/RNA1 ratio is decreased in pituitaries of a mouse model for PWS

We next asked whether there is a deregulation of 5HT2C isoforms in the pituitaries of a mouse model of PWS. We used PWS-IC ^{del} mice [46] that have a deletion in the imprinting center, which contains the promoter of the SNRPN gene. PWS-IC ^{del} mice thus lack expression from the Prader-Willi critical region, including the expression of mouse SNORD115 [10]. The ratio of RNA2/RNA1 in PWS-IC ^{del} mice was

5HT2C mRNA is made in the cell bodies of magnocellular neurosecretory cells and then transported to the posterior pituitary through axonal transport. At this point, it is unclear whether 5HT2C protein is also present in the posterior pituitary. We consider this possibility very likely, as the magnocellular neurosecretory cells transport peptides like oxytocin and vasopressin to the posterior pituitary. Given its neuron-specific expression it is unlikely that 5HT2C RNA is synthesized in pituicytes, capillary endothelial cells or in endocrine cells in the pars intermedia adhering to the posterior pituitary.

4.2. 5HT2C isoforms are deregulated in the pituitary of a PWS mouse model

Prader-Willi syndrome is a genetic disorder characterized by

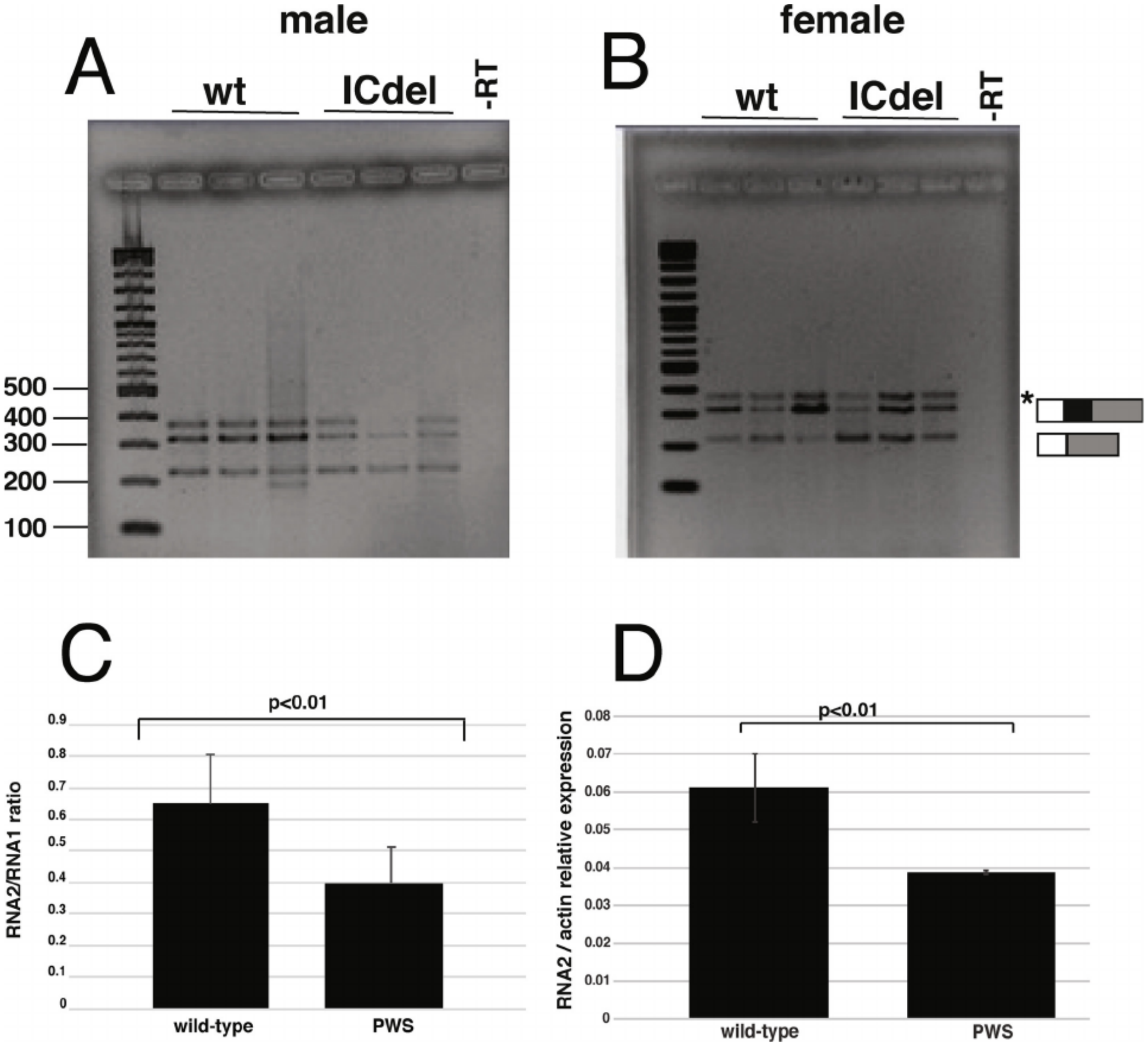

Fig. 4. Change of RNA2/RNA1 ratio in ICdel mice.
A) RT-PCR of 10 g total RNA from male wild-type and IC^{del} mice; -RT control without reverse transcription.
B) RT-PCR of 10 g total RNA from female wild-type and IC^{del} mice.
C) Quantification of the end-point PCR form panels A–B, $p < 0.01$, $n = 12$.
D) qPCR detecting RNA2 normalized to beta-actin, $n = 12$, $p < 0.01$.

hypothalamic defects and hormonal imbalances. The gene region lost in PWS contains SNORD115 that unlike canonical SNORDs does not ex-

4.3. The 5HT2C isoforms in the pituitary are rapidly changed after stimulation

hibit sequence complementarity towards rRNAs, but shows complementarity towards 5HT2C exon Vb [7]. A fraction of SNORD115 is not associated with the RNA methylase fibrillarin [39], suggesting that similar to other SNORDs [13], SNORD115 regulates alternative splicing of 5HT2C without fibrillarin in a 'non-methylating' complex.

We thus analyzed PWS-IC mice that do not express SNORD115. We found that PWS-IC mice have a lower RNA2/RNA1 ratio than wild type mice likely reflecting the lower RNA2/RNA1 ratio observed in their hypothalamus [16]. In PWS patients, oxytocin production/release is diminished and intranasal oxytocin substitution is tested in adolescent PWS and shows promise in decreasing appetite [32]. Delivery of oxytocin improves postnatal feeding in PWS, both in human subjects and mouse models [35,44]. The secretion of both vasopressin and oxytocin is stimulated by serotonin receptors, including 5HT2C [22] and thus a down-regulation of the full-length 5HT2C isoforms, encoded by RNA2 could contribute to low oxytocin levels seen in PWS.

Often, alternative splice site selection is controlled by physiological stimuli, usually through signaling pathways that regulate the phosphorylation of splicing factors [24,40,41]. We tested food withdrawal as a stimulus and observed a loss of the RNA1 isoform and a subsequent increase of the RNA2 isoform in male mice, demonstrating that 5HT2C splicing is regulated by feeding status. The changes in 5HT2C isoforms were not significant in females, suggesting additional regulatory mechanism, for example changes due to the estrus of the animals.

To test the possible effects of drugs on 5HT2C isoforms, we systemically injected mice with a 5HT2C splice site changing oligo. We previously found that unexpectedly, this oligo accumulates in the brain after systemic injection of more than 50 g in mice [48]. Here we found rapid accumulation of the oligo in pituitary and a change of 5HT2C splice sites within six hours. The change in splice site selection likely occurs in the hypothalamic magnocelluar neurosecretory cells and it

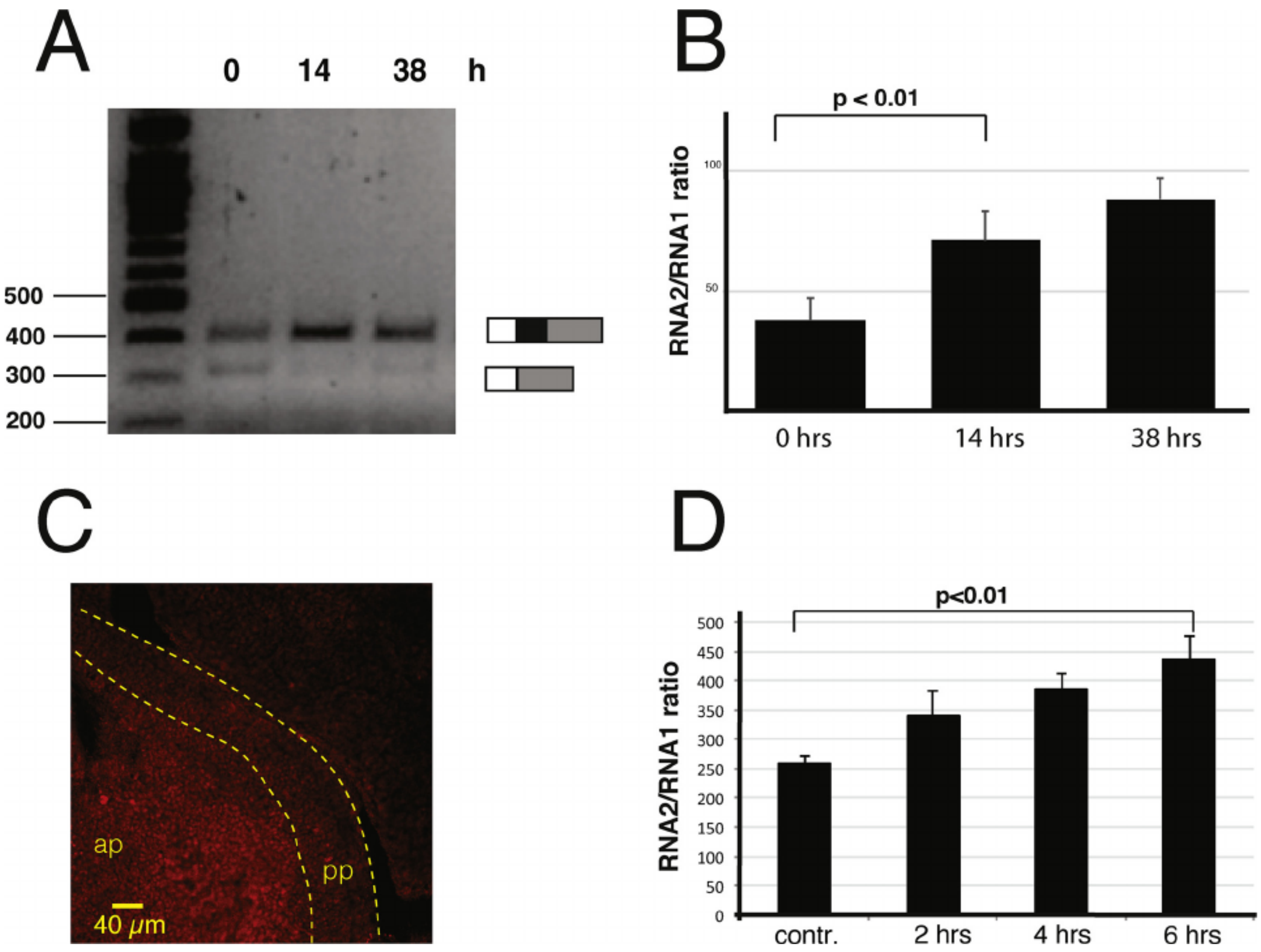

Fig. 5. The 5HT2C in pituitary isoforms are regulated by feeding and splice-site changing oligonucleotides.
A) Change of 5HT2C pituitaries in mouse isoforms after 14 and 38 h of food withdrawal. RNA was isolated from complete (anterior and posterior) mouse (male) pituitaries and analyzed by RT-PCR. Male C57BL/6 mice were used for all experiments.
B) Quantification of the RNA2/RNA1 ratio from male mice after food withdrawal. n = 4 per group.
C) Staining of cy3-oligo#5 in mouse pituitary, 30 min post injection of 10 g cy3-oligo#5, ap: anterior pituitary, pp: posterior pituitary
D) Change of RNA2 to RNA1 ratios in mouse pituitary after oligo#5 injection. The ratio of RNA2/RNA1 was measured using real-time PCR. Contr. Injection of an oligo against SMN2 [48], 2–6 h, injection of oligo#5 n = 4, $p < 0.01$.

remains to be determined whether there is a retrograde transport of the oligo from the pituitary into these cells.

Several drugs targeting serotonin receptors in the CNS are in use, among them the 5HT2C agonist lorcaserin and the antidepressant

such as vasopressin and oxytocin, which have been implicated in obesity and metabolic disorders. In addition, the deregulation of the 5HT2C isoforms could contribute to hormonal imbalances seen in PWS.

mirtazapine that binds to the 5HT2C [1]. It is possible that these drugs also act on the posterior pituitary, which could contribute to their side effects.

4.4. *Does the 5HT2C connect the gut with the pituitary?*

An estimated 90% of the body's serotonin is generated from enterochromaffin cells in the gut from where it reaches the blood and is stored in platelets [3]. Diets influence serum serotonin concentrations, but diet composition and caloric intake seem to have diverse effects, for example high fat diet increases serum serotonin concentration [25], which has also been reported to be caused by fasting [43]. It is thus possible that diet information is relayed to the pituitary via plasma serotonin levels that activate 5HT2C receptors in the pituitary. The resulting 5HT2C signaling, likely via PLC, could then cause nutrition-dependent changes in hormone release.

In summary, the posterior pituitary expresses 5HT2C RNA and the ratio of its isoforms is sensitive to feeding/fasting status. Drugs that act on 5HT2C may influence secretion of posterior pituitary hormones,

Acknowledgments

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