RESEARCH ARTICLE

Cocaine-and Amphetamine Regulated Transcript (CART) Peptide Is Expressed in Precursor Cells and Somatotropes of the Mouse Pituitary Gland

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Abstract

Cocaine-and Amphetamine Regulated Transcript (CART) peptide is expressed in the brain, endocrine and neuroendocrine systems and secreted into the serum. It is thought to play a role in regulation of hypothalamic pituitary functions. Here we report a spatial and temporal analysis of *Cart* expression in the pituitaries of adult and developing normal and mutant mice with hypopituitarism. We found that *Prop1* is not necessary for initiation of *Cart* expression in the fetal pituitary at e14.5, but it is required indirectly for maintenance of *Cart* expression in the postnatal anterior pituitary gland. *Pou1f1* deficiency has no effect on *Cart* expression before or after birth. There is no 1:1 correspondence between CART and any particular cell type. In neonates, CART is detected primarily in non-proliferating, POU1F1-positive cells. CART is also found in some cells that express TSH and GH suggesting a correspondence with committed progenitors of the POU1F1 lineage. In summary, we have characterized the normal temporal and cell specific expression of CART in mouse development and demonstrate that postnatal CART expression in the pituitary gland requires PROP1.

Citation: Mortensen AH, Camper SA (2016) Cocaine-and Amphetamine Regulated Transcript (CART) Peptide Is Expressed in Precursor Cells and Somatotropes of the Mouse Pituitary Gland. PLoS ONE 11(9): e0160068. doi:10.1371/journal.pone.0160068

Editor: Raul M. Luque, University of Cordoba, SPAIN

Received: May 3, 2016

Accepted: July 13, 2016

Published: September 29, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by NIH R01HD30428 (SAC).

Competing Interests: The authors have declared that no competing interests exist.

Introduction

CART is expressed in several organs of the neuroendocrine and endocrine system including the pituitary gland, brain, adrenal gland, and the somatostatin producing cells of the pancreatic islets [1–4]. CART is most abundant in the hypothalamus [5]. In rodents, two different splice variants of the *Cart* transcript result in the production of two pro-peptides of different lengths, called proCART 1–89 and proCART 1–102. The proCART peptides contain several cleavage sites that allow post-translational processing by prohormone convertases resulting in two biologically active forms: CART 55–102 and CART 62–102. CART 55–102 is the predominant form in the anterior pituitary gland [5–12]. CART peptides may have a hormonal role as they are found in the pituitary portal blood system and peripheral blood [13], in addition to the anterior and posterior pituitary lobes [1, 14].

CART is thought to function in inhibition of food intake, stimulation of energy expenditure, and regulation of the hypothalamic-pituitary axes [15–19]. In the hypothalamic-pituitary-thyroid (HPT) axis, functional studies in rats and cell lines demonstrate that CART peptide modulates TRH-induced prolactin secretion by influencing the stimulatory affect of TRH [18, 20–22]. There is also evidence that CART regulates the hypothlamic-pituitary-adrenal (HPA) axis at the level of the hypothalamus, where it is expressed together with corticotropin-releasing hormone (CRH) [23]. In vitro studies have shown that CART stimulates the release of CRH from hypothalamic explants [24]. These studies suggest that CART could regulate pituitary function both directly and indirectly.

Several genes have been identified that are required for pituitary development and function in humans and mice [25–27]. Among the best known are *PROP1* and *POU1F1*, which encode two pituitary-specific homeobox transcription factors [28–30]. Mutations in POU1F1 cause Combined Pituitary Hormone Deficiency (CPHD), which is typically characterized by lack of GH, TSH and PRL [30–34], while mutations in PROP1 cause progressive hormone deficiency that can include GH, TSH, PRL, gonadotropins and ACTH [29, 35–39].

Mice with mutations in *Prop1* and *Pou1f1* have been invaluable for revealing the genetic hierarchy of regulatory control and for understanding disease pathophysiology. PROP1 is expressed in Rathke's pouch, the rudiment of the anterior and intermediate lobes of the rodent pituitary gland, at e10.5 and it wanes by e14.5 [37]. The expression of *Pou1f11* is detectable at e14.5, and expression of *Tshb*, *Gh* and *Prl* are detectable a day later, e15.5 [40, 41]. Ames dwarf mice (*Prop1*$^{df/df}$) have an abnormally shaped Rathke's pouch and hypoplastic anterior lobe because proliferating progenitor cells are unable to migrate from the niche into the anterior lobe [42–44]. *Prop1*$^{df/df}$ mice also fail to activate *Pou1f1*, and although the Snell dwarf mice (*Pou1f1*$^{dw/dw}$) express *Prop1*, they fail to activate *Tshb*, *Gh* and *Prl* expression [32, 37, 39, 44]. These types of studies, together with lineage tracing experiments, established that *Prop1* is expressed in all pituitary progenitors, it binds the *Pou1f1* regulatory elements and activates its expression, and subsequently, POU1F1 directly activates the hormone genes that define somatotropes, thyrotropes and lactotropes [37, 45].

Identification of target genes is an important step in understanding the molecular mechanisms of transcription factor action. Many downstream targets of POU1F1 have been identified [32, 46, 47], but other than *Pou1f1* and *Hesx1*, few direct targets besides *Prop1* are known [37, 48]. We carried out gene expression profiling with RNA from neonatal pituitaries of normal, *Prop1*$^{df/df}$, and *Pou1f1*$^{dw/dw}$ mice to identify differentially expressed genes that were uniquely changed in *Prop1*$^{df/df}$ relative to the other samples, as these would be candidates for downstream targets of *Prop1*, specifically. The Cocaine- and amphetamine-regulated transcript (CART) was decreased 21 fold in the pituitaries of *Prop1*$^{df/df}$ newborns, but no change in *Cart* expression was detected between *Pou1f1*$^{dw/dw}$ and its wild type control [49]. This suggests that CART is a downstream target of *Prop1*, either directly or indirectly.

To determine whether CART is a direct downstream target of *Prop1* and epistatic to *Pou1f1*, we investigated the developmental regulation of CART expression in normal, *Prop1*$^{df/df}$ and *Pou1f1*$^{dw/dw}$ mutant mice. In addition, we investigated the cell specific expression of CART in mouse pituitary. Our results reveal that *Prop1* is not necessary for initiation of *Cart* expression during pituitary embryogenesis, but it is required indirectly for maintenance of *Cart* expression in the postnatal anterior pituitary gland.

Materials and Methods

Mice

All mice were housed in a 12-h light, 12-h dark cycle in ventilated cages with unlimited access to tap water and Purina 5020 chow. All procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and approved by the University of Michigan Committee for the Use and Care of Animals. Ames dwarf mice (*Prop1*$^{df/df}$) were originally obtained from Dr. A. Bartke (Southern Illinois University, Carbondale, IL) as a non-inbred stock (DF/B), and Snell dwarf mice (*Pou1f1*$^{dw/dw}$) were purchased from The Jackson Laboratory as an inbred stock (DW/J) (Bar Harbor, ME). Both strains have been maintained as colonies at the University of Michigan through heterozygous matings. The morning after conception is designated e0.5 and the day of birth is designated as P1. Mice were euthanized using 10 to 30% carbon dioxide inhalation followed by bilateral pneumothorax.

PCR genotyping

The genotypes of *Prop1*$^{df/df}$ (p.Ser83Pro) and *Pou1f1*$^{dw/dw}$ (p.Trp251Cys) mice [32, 37] were determined as described [38, 50].

Tissue Preparation and Histology

Embryos were fixed in 4% formaldehyde in PBS at 4°C for different times depending on the age of the embryo. Fixation was 2 hours for e14.5 and e16.5, overnight for P1 and P8, and 30 minutes for adult mouse pituitaries. The tissue was washed with PBS, dehydrated to 70% ethanol and embedded in a Tissue Tek VIP Paraffin tissue processing machine (Miles Scientific). 6 μm thick sagittal sections were prepared from embryos collected at e14.5 and e16.5, and coronal sections were prepared from postnatal pups and adult pituitaries.

Immunohistochemistry

Immunostaining for CART protein was done with the anti-CART (55–102) antibody (1:5000, Phoenix Pharmaceuticals). The specificity of the CART (55–102) antibody has been proven with immunohistochemistry experiments were the CART antisera was preabsorbed with the CART peptide [51]. Immunostaining for pituitary hormone markers was performed using anti-TSH, anti-ACTH, anti-LH, anti-FSH, anti-GH, and anti-PRL antibodies, (1:1000, National Hormone and Peptide Program, UCLA Medical Center, Torrance, CA). For double immunostaining of CART and pituitary hormones, the paraffin sections were hydrated and incubated for 20 minutes in an aqueous solution of 3%H_2O_2, 50% methanol to block endogenous peroxidases. All slides were placed in normal goat serum block (5% goat serum, 3% BSA, and 0.5% Tween-20 in PBS) for 10 minutes at room temperature. 100 μl of anti-CART antibody was placed on each slide on a wax ring and incubated over night at 4°C. A 100 μl aliquot of biotinylated anti-rabbit IgG secondary antibody (Jackson Immuno Research, West Grove, PA) was incubated on each slide for 30 minutes, and subsequently CART was detected using the tyramide signal amplification (TSA) and Fluorescein Isothiocynate (FITC) kit (according to protocol, Perkin-Elmer, Boston, MA). After washing slides in 1XPBS-T (phosphate buffered saline with 0.01% Tween-20), slides were boiled in 0.01 M citrate buffer to expose epitopes. Specifically, slides and citrate buffer were placed in the microwave for 5 minutes at 40% power. The slides were cooled for 30 minutes in the same citrate buffer [52] After washing the slides in 1X PBS, an Avidin/Biotin block was done for 15 minutes according to protocol (Vector Labs, Burlingame, CA). All hormone antibodies were diluted in this block and were incubated on the slides over night at 4°C, except for GH which was incubated for 1 hour at room temperature. The following secondary antibodies were used: biotinylated anti-guinea pig IgG (1:100,Jackson Immuno Research) for anti-TSHβ, ACTH, LHβ, and FSHβ, biotinylated anti-rabbit IgG (1:100, Jackson Immuno Research) antibody for anti-PRL, and anti-human biotin (1:200, ab97223, Abcam) for anti-GH. The biontinylated secondary antibodies were detected with streptavidin-conjugated Cy3 (Jackson Immuno Research) for 30 minutes at room temperature. Cell nuclei were stained with DAPI (1:200) for 5 minutes, washed with 1XPBS, and mounted with aqueous mounting media.

For double immunostaining with CART and antibodies against the nuclear factors anti-Ki67 (1:200, Novocastra, Newcastel, United Kingdom), anti-TPIT (1:200, from Dr. Jacques Drouin), anti-NR5A1 (1:100, from Ken-ichirou Morohashi), anti-POU1F1 (1:100, from Simon Rhodes), paraffin sections were boiled in 0.01M citrate for 10 minutes, or 15 minutes for anti-PROP1 antibody (1:100, from Aimee Ryan) [45]. These incubations were followed by a 20 minute incubation in an aqueous solution of 3% H_2O_2, 50% methanol, and a normal goat serum block (5% goat serum, 3% BSA, and 0.5% Tween-20 in PBS) for 10 minutes at room temperature. 100 μl of CART antibody was placed on each slide within a wax ring and incubated overnight at 4°C and the secondary and tertiary antibodies as described above were used. 100 μl of the nuclear antibodies were added to the slides and incubated over night at 4°C. We used biotinylated anti-rabbit IgG (Jackson Immuno Research) as a secondary antibody for anti-Ki67, anti-NR5A1, anti-TPIT, and anti-POU1F1, and biotinylated anti-guinea pig IgG (Jackson Immuno Resaerch) secondary antibody for anti-PROP1. Nuclear antibodies were detected using either the tyramide signal amplification (TSA) and fluorescein isothiocynate (FITC) kit (according to protocol, Perkin-Elmer, Boston, MA), or streptavidin-conjugated Alexa-fluor 488 (1:200, S11223, Invitrogen). Cell nuclei were stained with DAPI (1:200) for 5 minutes, washed with 1XPBS, and mounted with aqueous mounting media. See Table 1.

Pregnant mice were injected IP with 100mg BrdU per gram of body weight 2 hours prior to collecting embryos. After processing, tissue sections were boiled in 0.01M citrate for 10 minutes, followed by 20 minute incubation in an aqueous solution of 3% H_2O_2, 50% methanol. The slides were then incubated for 1 hr in a mouse IgG block. BrdU detection was performed as described by [43] with the anti-BrdU antibody (1:100, AbD Serotec). The secondary biotin anti-rat IgG (1:200, 711-066-152, Jackson Immunoresearch, Westgrove, PA) was used followed by detection with Alexa Flour 488. Microwave treatment, Avidin/Biotin Block, and CART immunostaining were all as described above.

All single and double- immunohistochemistry experiments were done with the appropriate negative controls (S1 Fig).

Cells were counted using ImageJ (1.51a) Software (National Institute of Health).

Results

CART is expressed in the developing anterior and posterior pituitary gland

If *Cart* were a direct target of *Prop1*, then we would expect *Cart* expression to be activated in a spatial and temporal manner coincident or overlapping with *Prop1* expression in normal mice and absent or reduced in *Prop1*$^{df/df}$ mutants. CART was not detected at e12.5 in the pituitary gland when PROP1 is expressed (Fig 1A and 1B). We detected the CART protein at e14.5 in the developing anterior lobe and infundibulum using immunohistochemistry. This pattern persists at e16.5 and the intensity of staining is increased, but there is no striking difference between the control and the *Prop1*$^{df/df}$ mutants at either of these time points (Fig 1C–1F). There are fewer cells that stain for CART in the anterior lobe of *Prop1*$^{df/df}$ at e16.5, but the anterior lobe is hypoplastic at that time. At P1 the CART immunostaining is increasingly prominent in the posterior lobes of both the normal and *Prop1*$^{df/df}$ pituitary glands (Fig 1G and 1H). By P8 CART is detected throughout the anterior and posterior lobe in the control pituitary gland, but it is almost completely absent from the anterior lobe of *Prop1*$^{df/df}$ mutants (Fig 1I and 1J). In contrast, there is no obvious difference between CART expression in control and

Table 1.

Immunohistochemistry experiments									
Experiment	**Citric acid boil**	**CH_3OH: H_2O_2**	**Block**	**Primary Antibody**	**Secondary antibody and detection**	**Avidin/ Biotin block[8]**	**5 min Microwave with 10 mM citric acid**	**Other primary antibody**	**Other secondary and detection**
CART	none	yes	NGS[1], TNB[2]	1:5000 anti-CART, P.P.[3]	anti-rabbit biotin[4], TSA FITC[9]	no	no		
CART and POU1F1	10 min	yes	NGS, TNB	1:5000 anti-CART, P.P.	anti-rabbit biotin, TSA TRITC[9]	yes	yes	1:500 anti-POU1F1, gift from S. Rhodes	Anti-rabbit biotin, SA Alexa Flour 488[10]
CART and NR5A1	10 min	yes	NGS, TNB	1:5000 anti-CART, P.P.	anti-rabbit biotin, TSA TRITC	yes	yes	1:1000 anti-NR5A, gift from K. I. Morohashi	Anti-rabbit biotin, SA Alexa Flour 488
ISL1 and TPIT	10 min	yes	NGS, TNB	1:5000 anti-CART, P.P.	anti-rabbit biotin, TSA TRITC	yes	yes	1:200 anti-TPIT, gift from J. Drouin	Anti-rabbit biotin, SA Alexa Flour 488
CART and ACTH	none	yes	NGS, TNB	1:5000 anti-CART, P.P.	anti-rabbit biotin, TSA FITC	yes	no	1:1000, anti-ACTH, NHPP[5]	Anti-guinea pig biotin[4], SA Cy3[4]
CART and TSHb	none	yes	NGS, TNB	1:5000 anti-CART, P.P.	anti-rabbit biotin, TSA FITC	yes	no	1:1000 anti-TSHb, NHPP	Anti-guinea pig biotin, SA SA Cy3
CART and LHb	none	yes	NGS, TNB	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA FITC	yes	no	1:600 anti-ISL1, DSHB	Anti-guinea pig biotin, SA SA Cy3
CART and GH	none	yes	NGS, TNB	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA FITC	yes	no	1:1000 anti-GH[6], NHPP	anti-human biotin[7], SA SA Cy3
CART and FSHb	none	Yes	NGS, TNB	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA FITC	yes	no	1:1000 anti-FSHb, NHPP	Anti-guinea pig biotin, SA SA Cy3
CART and Ki67	10 min	Yes	NGS, TNB	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA TRITC	yes	yes	1:200, anti-Ki67, Novocastra	Anti-rabbit biotin, SA Alexa Flour 488
CART and BrdU	10 min	Yes	TNB, M. O.M.[8]	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA TRITC	yes	yes	1:100, anti-BrDu, AbD Serotec	Anti-rat biotin[4], SA Alexa Flour 488
PROP1 and CART	15 min	yes[11]	NGS, TNB	1:100 anti-PROP1, gift from A. Ryan	Anti-guinea pig biotin, TSA FITC	yes	yes	1:5000 anti-CART, P.P	anti-rabbit biotin, TSA TRITC

[1] 5% Normal Goat Serum, 3%BSA in 1XPBS

[2] Block from Perkin-Elmer TSA FITC kit

[3] Phoenix Pharmaceuticals

[4] Jackson Immuno Research

[5] National Hormone and Pituitary Program

[6] anti-GH antibody left on for 1 hour

[7] Abcam

[8] Vector Labs

[9] Perkin-Elmer

[10] Invitrogen

[11] Second CH_3OH:H_2O_2 block done before second primary antibody

doi:10.1371/journal.pone.0160068.t001

Pou1f1$^{dw/dw}$ mutant pituitary glands at P8 (Fig 1K and 1L). Thus, activation of CART expression is *Prop1*-independent, but *Prop1* is clearly essential for sustaining CART expression in the

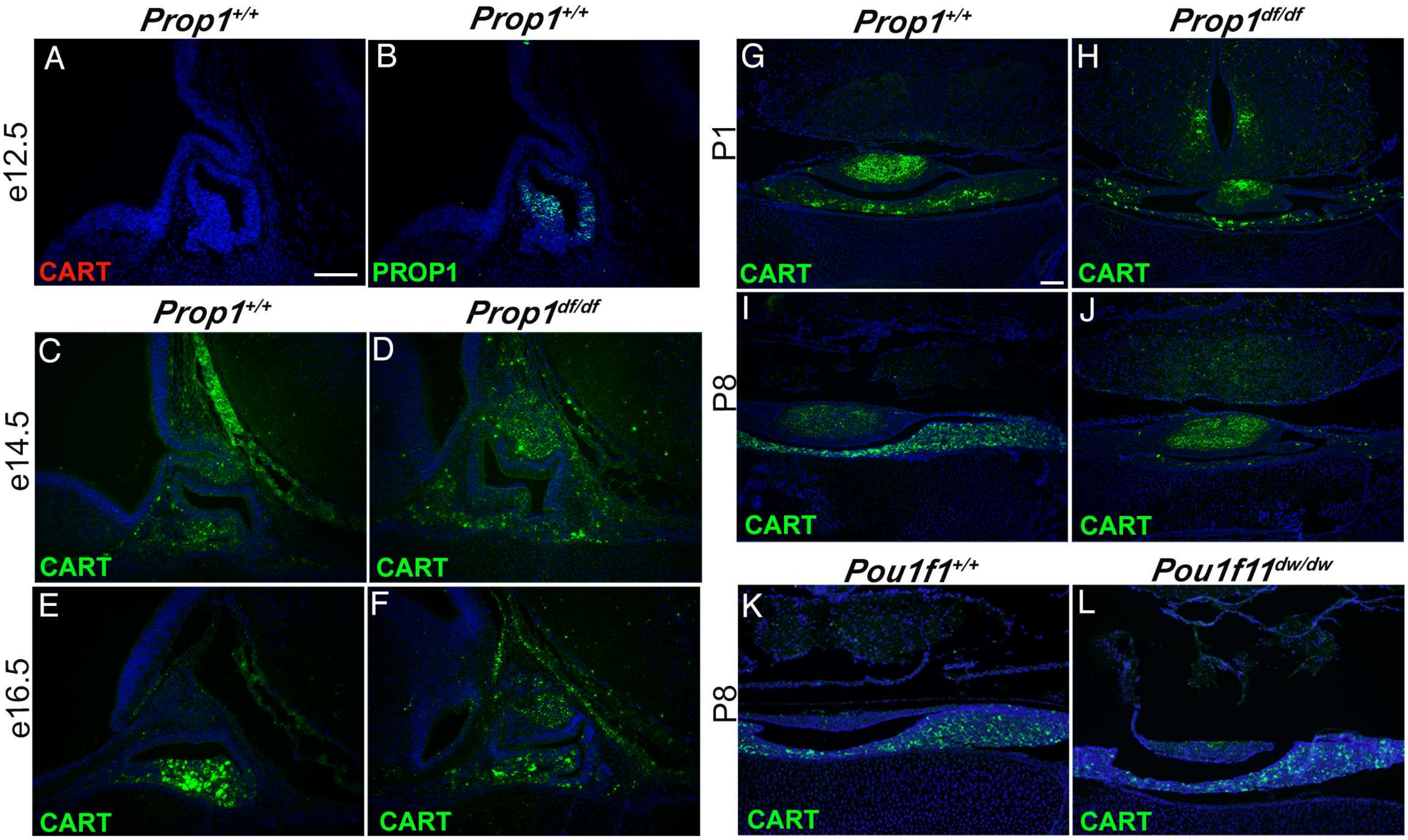

Fig 1. CART is expressed throughout normal pituitary development. CART protein is not detected in e12.5 pituitary gland at e12.5 (A). PROP1 protein is detected in Rathke's pouch of the e12.5 pituitary gland (B). CART protein was detected in paraffin sections of pituitary glands with a specific antibody (green fluorophor) in *Prop1*$^{+/+}$ (C, E, G) and *Prop1*$^{df/df}$ (D, F, H) anterior pituitary lobes at e14.5 (C, D), e16.5 (E, F), and P1 (G, H). At P8 CART is expressed abundantly in the anterior pituitary lobe (I), but it is absent in the *Prop1*$^{df/df}$ anterior pituitary lobe (J). CART protein is present in the *Pou1f1*$^{+/+}$ (normal) (K) and Pou1f1$^{dw/dw}$ anterior lobe of the pituitary gland (L). Images A-F were taken at 200X and scale bar is 200 μm. Images G-L were taken at 100X and scale bar is 200 μm.

doi:10.1371/journal.pone.0160068.g001

anterior lobe postnatally. In contrast, *Pou1f1* is not essential for either activation or maintenance of *Cart* expression.

CART expression predominates in somatotropes of the postnatal pituitary gland

We determined the cell specific expression of CART in the pituitary gland at P8 using immunohistochemistry to co-stain for CART and each of the pituitary hormones individually. TSHβwas expressed in 16 to 21% of the CART positive cells in the pituitary gland at P8 (n = 3, Fig 2A, yellow arrow). This is a large portion of the TSHβpopulation as 62 to 79% (n = 3) of the TSHβcells were CART positive. More than half of the CART expressing cells were also positive for GH (57 to 68%, n = 3). (Fig 2B, yellow arrow). There were many GH only cells (31 to 40%, n = 3, red arrow) and CART only cells (green arrow). We did not detect any co-localization of CART with ACTH, LHβ, FSHβ, or PRL at P8 (Fig 2C–2F). The *Pou1f1*$^{dw/dw}$ and *Prop1*$^{df/df}$ mutant pituitaries are nearly devoid of GH, TSHβ, and PRL [41, 44]. It is intriguing that despite identical deficits in *Pou1f1* lineage determination, CART protein expression is normal in *Pou1f1*$^{dw/dw}$ but not detectable in *Prop1*$^{df/df}$ mutants (Fig 1).

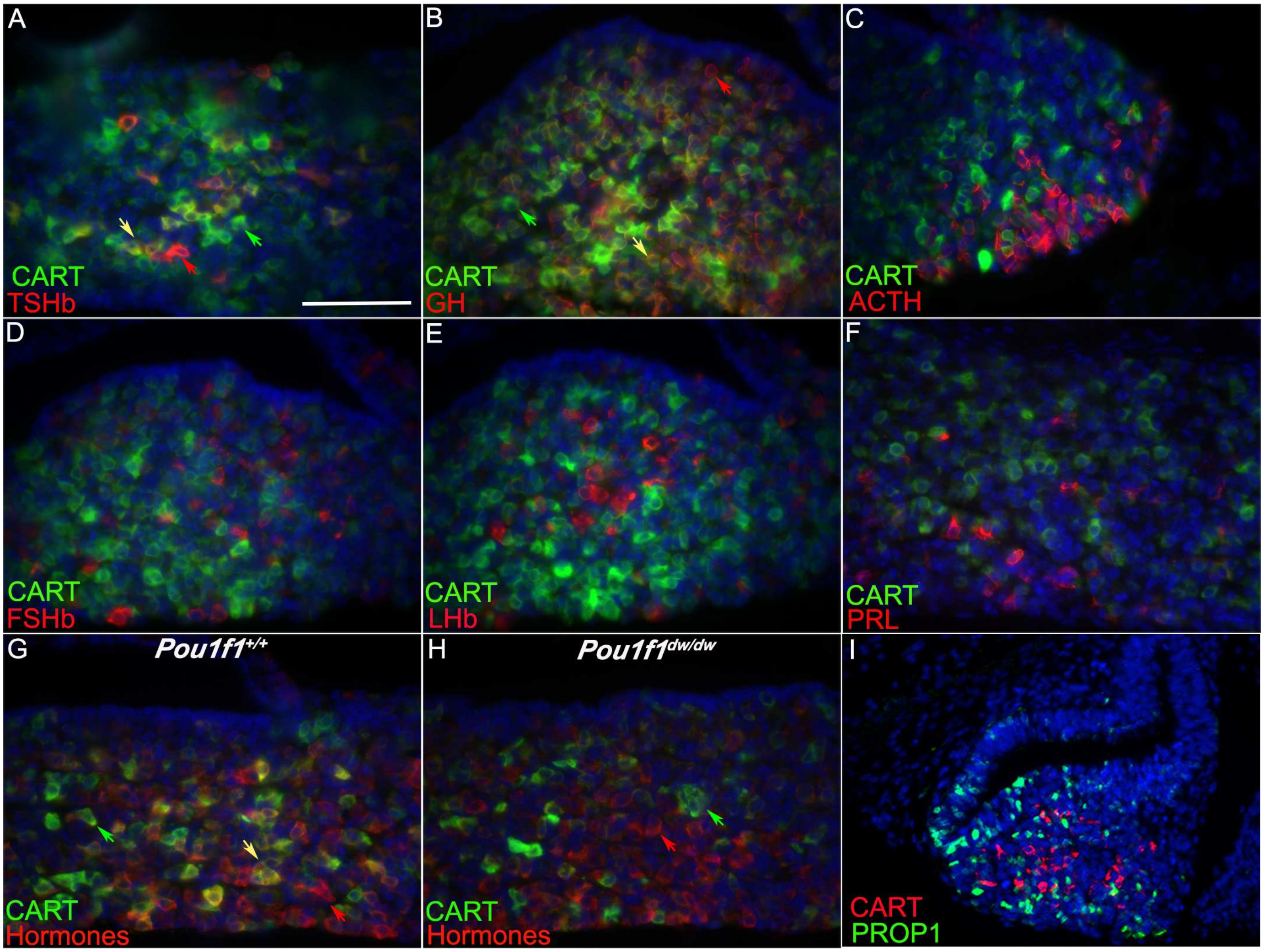

Fig 2. CART expression the pituitary hormones. Co-localization of CART with specific pituitary hormones was determined with a CART-specific antibody (green) and hormone specific antibodies (red) in sections from pituitary glands collected at P8. The hormone specific antibodies were TSHb (A), GH (B), ACTH (C), FSHb (D), LHb (E), and PRL (F). Co-localization is indicated with yellow arrows (A, B). In one CART immunostaining experiment all hormone antibodies were applied together and detected with the same fluorophore (G, H). A few CART positive cells do not express a detectable level of hormone (green arrow) in the *Pou1f1*$^{+/+}$ pituitary (G), but almost all CART positive cells do not co-localize with any hormones in the somatotrope deficient *Pou1f1*$^{dw/dw}$ anterior pituitary (H). The CART protein (red) does not co-localize with PROP1 (green) in the developing pituitary gland at e14.5 (I). Images A-I were taken at 400X and scale bar is 100 μm.

doi:10.1371/journal.pone.0160068.g002

To determine whether any of the CART positive cells in the *Pou1f1*$^{dw/dw}$ mutant were producing hormones, we carried co-staining with CART the antibody and an "all hormone" immunostain, by combining antibodies specific for TSHβ, GH, ACTH, LHβ, FSHβ, and PRL and using a common tertiary streptavidin-Cy3 antibody. In the control, 59 to 65% (n = 3) of the CART positive cells co-localized with a hormone producing cell (yellow arrow) (Fig 2G), but all of the CART positive cells were hormone negative in the *Pou1f1*$^{dw/dw}$ pituitaries (Fig 2H). The nature of the CART positive, hormone negative cells is unknown, but the cell morphology resembles that of hormone producing cells.

PROP1 is detectable in progenitors that reside in the marginal zone of the anterior lobe and in scattered cells in the parenchyma of the anterior lobe [53]. Lineage tracing experiments show that all hormone-producing cells of the anterior pituitary gland pass through a *Prop1* expressing progenitor [45]. At e14.5, PROP1 immunostaining is detectable in cells of the most caudal end of the marginal zone and the developing anterior lobe, but expression does not overlap with CART at this time (Fig 2I). This suggests that CART expression may be associated with a progenitor that has initiated commitment to hormone production, but has accumulated low levels of hormone, if any.

CART co-localizes with multiple cell specific transcription factors, but primarily with POU1F1

Some lineage specific transcription factors are detectable prior to the hormone. For example, *Pou1f1* [54] and *Nr5a1* [*55*, *56*] expression precede the detection of GH and LH respectively [38, 57]. To test whether CART is expressed in committed progenitors, we carried out additional co-immunostaining experiments. At P8 the majority of PROP1 immunostaining is detected in the cytoplasm of cells that have migrated away from the marginal zone, and there is little or no co-localization with CART (data not shown). At P8 CART does co-localize with a large portion of the POU1F1 positive cells in the control pituitary gland (74 to 82%, n = 3, Fig 3A), which supports the idea of a CART expressing progenitor. The CART immunostaining is similar in wild type and *Pou1f1*$^{dw/dw}$ mutant pituitaries, despite the fact that POU1F1 is not functional and not detectable in the mutants (Fig 3B). TPIT (TBX19) is a positive regulator for late POMC cell differentiation and POMC expression, although it is not required for lineage commitment [58, 59]. POMC is processed to produce ACTH in the anterior lobe. We did not detect any co-localization of CART with ACTH at P8 (Fig 2), but we did detect a few TPIT and CART double positive cells in both the control and *Pou1f1*$^{dw/dw}$ pituitary glands (Fig 3C and 3D, yellow arrows). The transcription factor NR5A1 (SF1) is specific to the gonadotropes of the pituitary gland [60]. Although we did not see any co-localization of LHβ or FSHβ with CART, we detected a few CART and NR5A1 positive cells in the control pituitaries (12 to 13% of CART cells, n = 3). We saw slightly more CART and NR5A1 co-localized cells in *Pou1f1*$^{dw/dw}$ mutants (41 to 47% of CART cells, n = 3) compared to the control pituitaries (Fig 3E and 3F, yellow arrow), which may be due to the increased number of cells committed to the gonadotrope fate in these mutants [61]. Taken together these results support the idea that CART is expressed in some committed progenitors that have not yet accumulated hormones. Maintenance of CART expression is indirectly dependent upon *Prop1* and independent of *Pou1f1*, placing it between these two transcription factors in a transcriptional hierarchy.

CART is expressed primarily in non-proliferating cells of the postnatal pituitary gland

We hypothesized that the CART expressing cells that have no detectable hormone expression could be committed progenitors that are still in a proliferative state. To test this idea we carried out co-immunostaining with antibodies specific for Ki67 and CART at P8. Ki67 marks all active phases of the cell cycle (G_1, S, G_2, and M), but it is absent from resting cells (G_0). Only a small fraction of the CART positive cells were also positive for Ki67 in both the control (8 to 15%, n = 3) and *Pou1f1*$^{dw/dw}$ (12 to 15%, n = 3) pituitary gland (white arrow) (Fig 4A and 4B). We also carried out bromodeoxyuridine (BrdU) labeling at P8 to identify cells in S phase of the cell cycle. Only a small portion of the CART expressing cells also immunostained with BrdU in the control (10–11%, n = 3) and *Pou1f1*$^{dw/dw}$ (9 to 13%, n = 3) (Fig 4C and 4D, white arrow), confirming that the majority of CART expressing cells are non-proliferative. The few CART expressing cells that are proliferating cells may be among the subset of proliferating, hormone negative, POU1F1 positive cells in the P8 pituitary gland [62].

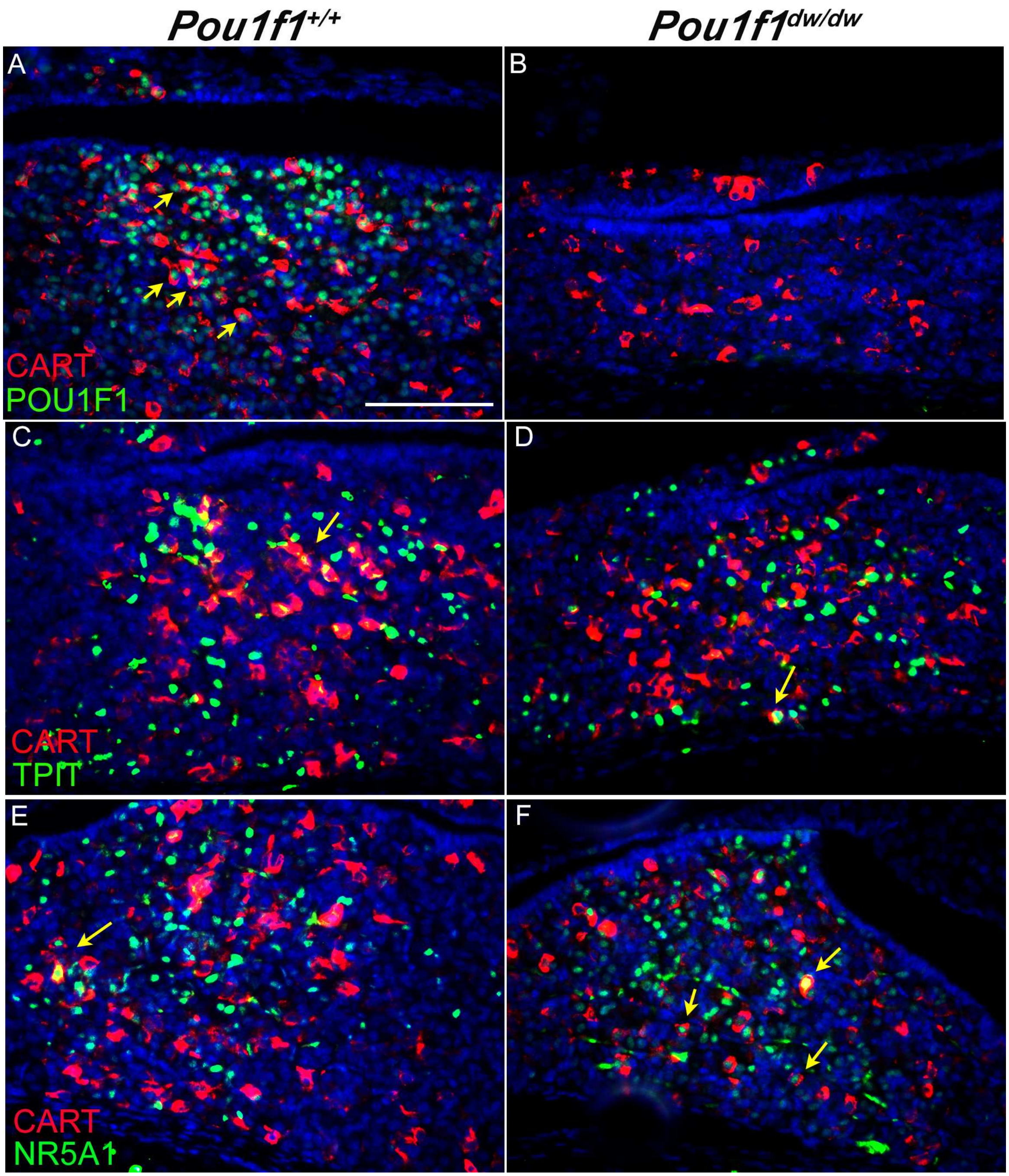

Fig 3. CART is co-expressed with several cell specific transcription factors. Immunostaining with antibodies specific for CART (red, cytoplasmic) and lineage specific transcription factors (green, nuclear) was carried out on paraffin sections of pituitaries collected at P8 from *Pou1f1*$^{+/+}$ mice (A, C, E) and *Pou1f1*$^{dw/dw}$ mutants (B, D, F). The lineage specific transcription factors were POU1F1 (A, B), TPIT (C, D), and NR5A1 (E, F). All images were taken at 400X and scale bar is 100 μm.

doi:10.1371/journal.pone.0160068.g003

CART co-localizes with TSH cells in the adult pituitary

We did not detect any co-localization of CART and PRL immunostaining in the pituitary glands of 12 week old adult mice (Fig 5A). In the adult pituitaries there was little to no co-staining of CART and GH (Fig 5B), which contrasts with the abundance co-localization at P8.

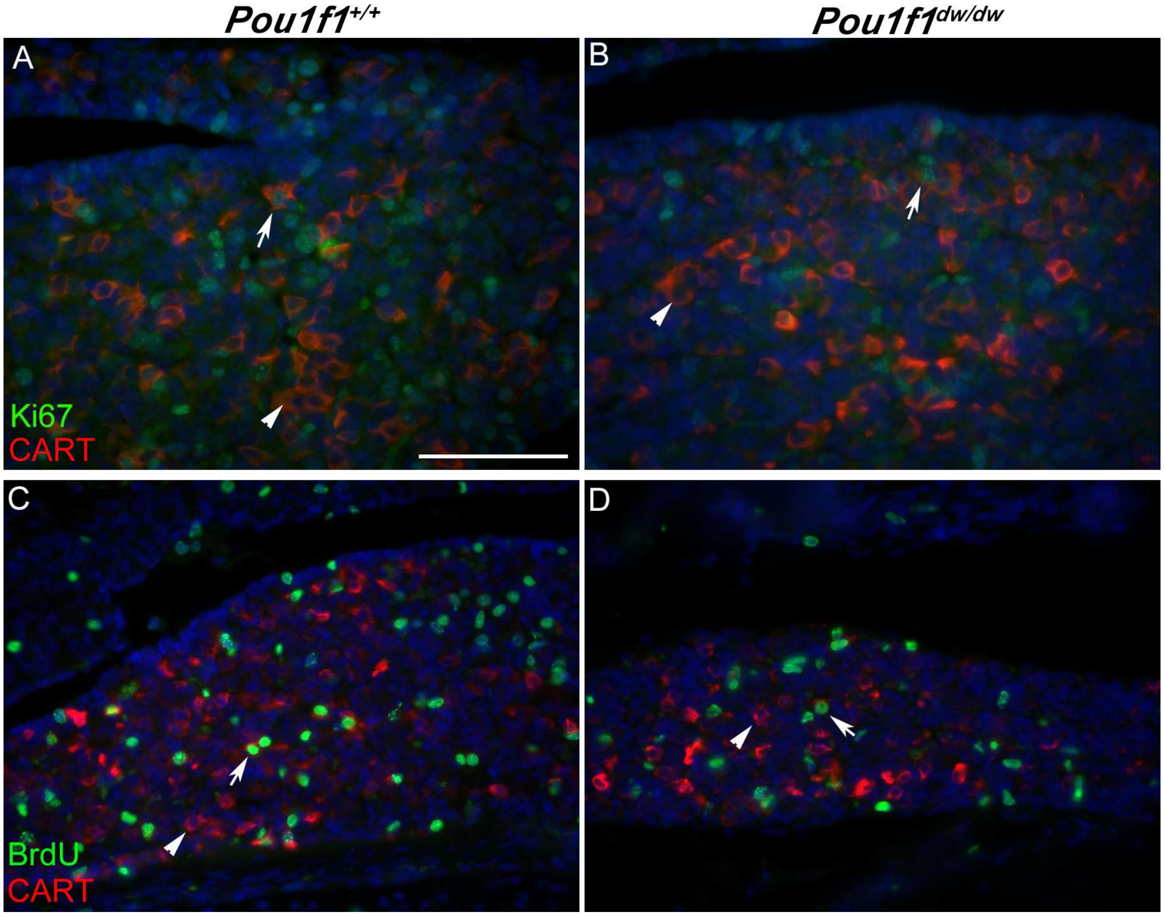

Fig 4. CART is expressed in the proliferating cells of the pituitary gland. Immunohistochemical staining was used to detect CART (red) and the proliferative markers Ki67 (green) or BrdU (green) in sections of pituitaries collect from *Pou1f1*$^{+/+}$ and *Pou1f1*$^{dw/dw}$ mice at P8. Co-localization is indicated by white arrows, and CART immunofluorescence in Ki67 or BrdU negative cells is indicated with arrow heads. There are many CART cells in both pituitary genotypes that do not co-localize with any proliferating cells (arrow heads). All images were taken at 400X, and scale bar is 100 μm.

doi:10.1371/journal.pone.0160068.g004

Many of the CART cells co-localize with TSHβ (yellow arrow) in the adult pituitary (67–76%, n = 3), but CART only (green arrow) and TSHβ only (red arrow) cells are detectable (Fig 5C). We did not observe any co-localization of CART with LHβ, FSHβ, or ACTH in the adult pituitary (Fig 5D–5F).

Discussion

Cart expression is dependent on *Prop1* after birth, but not embryonically

We identified *Cart* in a screen for genes whose postnatal expression was specifically dependent upon *Prop1* but not *Pou1f1* [49]. Here we report that the initiation and embryonic stages of *Cart* expression are independent of *Prop1*, and the spatial and temporal pattern of *Prop1* and *Cart* expression are inconsistent with *Cart* being a direct target of *Prop1*-mediated transactivation during pituitary embryogenesis. *Prop1* expression is transient in the fetus, diminishing to very low levels by e14.5, but expression is reactivated briefly after birth during a period of rapid pituitary growth and cell proliferation [37, 48, 53]. The postnatal expression of *Cart* is *Prop1* dependent, and we expect that it is indirect because they are not expressed in the same cells

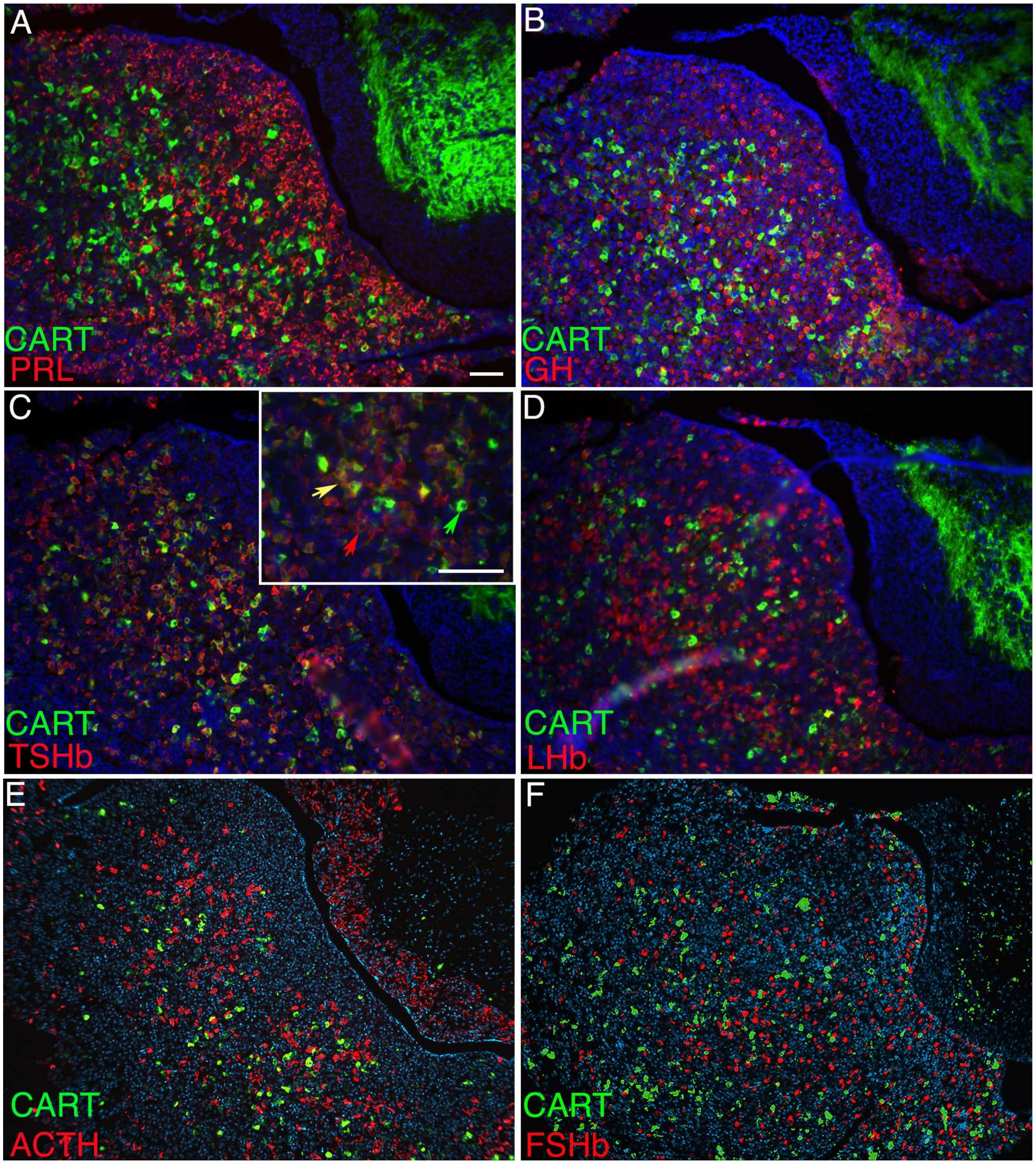

Fig 5. CART co-localizes with thyrotropes in the adult pituitary. Immunohistochemical staining was used to detect co-localization of CART (green) and hormones (red) in sections prepared from adult pituitary glands. The hormone specific antibodies were GH (A), PRL (B), TSHβ (C), LHβ (D), ACTH (E), and FSHβ (F). Representative cells (Inset panel C) exhibited co-localization of CART with TSHβ (yellow arrow), or lacked TSH expression (green arrow), and some TSHβ positive cells lacked CART staining (red arrow). All images were taken at 200X and scale bar is 100 μm. Inset of image C was taken at 400X and scale bar is 100 μm.

doi:10.1371/journal.pone.0160068.g005

embryonically. We expect that the failure to maintain *Cart* expression after birth in *Prop1*$^{df/df}$, but not *Pou1f1*$^{dw/dw}$ mutant mice, is due to the arrest in progenitor development at different stages in these two mutants. *Cart* expression may mark cells that have transitioned out of *Prop1* expression and begun commitment to produce hormones, but do not require *Pou1f1* function to maintain *Cart* expression. It is possible that initial activation of *Cart* expression involves some of the early acting transcription factors like *Pitx2*, *Lhx3*, or *Lhx4*, [63–65] and that other *Prop1* dependent transcription factors are necessary to maintain its expression postnatally. Candidates for regulating postnatal expression could include *Otx1*, *Notch*, *Math3*, *Zfhx3*, and *Zeb1* [66–70].

Cart expression predominates in the POU1F1 lineage but is not uniquely cell-type specific

Cart expression is dynamic and not uniquely cell type specific. *Cart* is first detected at e14.5 in the developing pituitary gland and then persists through adulthood. It does not exhibit exclusive cell specificity at any stage that we examined. We found predominant co-localization in cells expressing *Pou1f1* and GH in neonates, but less co-localization in adult pituitaries. A few cells were detected that express CART and other lineage specific transcription factors like NR5A1 and TPIT, but no co-localization was detected with LH, ACTH or PRL. This developmental shift in co-localization is consistent with the idea that *Cart* marks a *Prop1* dependent progenitor that can give rise to all major hormone producing cell types of the pituitary gland [45]. After *Prop1* activation, mutually antagonistic interactions between POU1F1, NR5A1, GATA2, and probably TPIT (TBX19) may ultimately drive cell specification to different lineages [58, 61]. If this is the case, it could explain why *Cart* expression is detected in a variety of hormone negative committed cells and ultimately becomes more enriched in somatotropes and thyrotropes. It is important to acknowledge that the lack of 1:1 correspondence with CART expression and any particular hormone producing cell type is not unique. The transcriptomes of gonadotropes vary, for example [71].

Given the dynamic *Cart* expression we observed in mice, it is perhaps not surprising that the expression and function reported for CART in the rat anterior pituitary is controversial and differs from the mouse. We did not observe *Cart* expression in mouse lactotropes. However, it was detected in the majority of adult rat lactotropes, expression and secretion of CART 62–102 increased in lactating animals, and CART inhibits TRH-induced prolactin release [20, 21]. *Cart* expression is repressed by thyroid hormone; levels increase in hypothyroid animals and decrease in hyperthyroid animals [20]. Although another group concurs that CART suppresses prolactin release, they detected CART co-localization only with gonadotropes using an antibody specific to CART 55–102, which is the same antibody that we used [22]. Finally, *Cart* transcripts reportedly to co-localize with ACTH cells in the rat, and CART secretion is regulated by CRH and glucocorticoids [72]. There may be species differences in CART expression and function, but the basis for the conflicting co-localization studies in the rat are unclear.

In conclusion, we demonstrate that PROP1 is necessary for maintenance, but not initiation, of CART expression in the pituitary gland. The postnatal maintenance of CART expression by PROP1 is likely indirect. We also show CART co-localization with somatotropes in the mouse pituitary gland, along with non-hormone producing cells that could be progenitors. CART expression could be a useful tool for sorting cells in an intermediate differentiation state.

Supporting Information

S1 Fig. Negative Controls for Co-Immunohistochemistry Experiments. Single antibody immunostaining were conducted as controls in all co-immunohistochemistry experiments. There was no cross-reactivity detected between the CART (55–102) antibody and the other hormone, transcription factor, and proliferation marker antibodies. A, A', B, B', C, C', D, D', H, H', I, I', J, J', L, and L' were taken at 400X, scale bar 100 μm. E, E', F, F' G, G' K, K' were take at

630X, scale bar 100 μm.
(TIF)

Acknowledgments

We thank Aimee Ryan, Simon Rhodes, Jacques Drouin, and Ken Morohashi for generously providing antibodies and the NIH R01HD30428 (SAC) for funding.

Author Contributions

Conceived and designed the experiments: AHM SAC.

Performed the experiments: AHM.

Analyzed the data: AHM SAC.

Contributed reagents/materials/analysis tools: AHM.

Wrote the paper: AHM SAC.

References

1. Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ. Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. J Neuroendocrinol. 1997; 9(11):823–33. Epub 1998/01/07. PMID: 9419833.
2. Koylu EO, Couceyro PR, Lambert PD, Kuhar MJ. Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. J Comp Neurol. 1998; 391(1):115–32. Epub 1998/04/04. PMID: 9527537.
3. Jensen PB, Kristensen P, Clausen JT, Judge ME, Hastrup S, Thim L, et al. The hypothalamic satiety peptide CART is expressed in anorectic and non-anorectic pancreatic islet tumors and in the normal islet of Langerhans. FEBS Lett. 1999; 447(2–3):139–43. Epub 1999/04/24. PMID: 10214934.
4. Couceyro PR, Koylu EO, Kuhar MJ. Further studies on the anatomical distribution of CART by in situ hybridization. J Chem Neuroanat. 1997; 12(4):229–41. Epub 1997/05/01. PMID: 9243343.
5. Douglass J, McKinzie AA, Couceyro P. PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. The Journal of neuroscience: the official journal of the Society for Neuroscience. 1995; 15(3 Pt 2):2471–81. Epub 1995/03/01. PMID: 7891182.
6. Kuhar MJ, Adams S, Dominguez G, Jaworski J, Balkan B. CART peptides. Neuropeptides. 2002; 36 (1):1–8. PMID: 12147208.
7. Kuhar MJ, Adams LD, Hunter RG, Vechia SD, Smith Y. CART peptides. Regulatory peptides. 2000; 89 (1–3):1–6. PMID: 10771306.
8. Adams LD, Gong W, Vechia SD, Hunter RG, Kuhar MJ. CART: from gene to function. Brain research. 1999; 848(1–2):137–40. PMID: 10612705.
9. Thim L, Kristensen P, Nielsen PF, Wulff BS, Clausen JT. Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. Proc Natl Acad Sci U S A. 1999; 96(6):2722–7. PMID: 10077578.
10. Ludvigsen S, Thim L, Blom AM, Wulff BS. Solution structure of the satiety factor, CART, reveals new functionality of a well-known fold. Biochemistry. 2001; 40(31):9082–8. PMID: 11478874.
11. Dey A, Xhu X, Carroll R, Turck CW, Stein J, Steiner DF. Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3. The Journal of biological chemistry. 2003; 278(17):15007–14. doi: 10.1074/jbc.M212128200 PMID: 12584191.
12. Stein J, Steiner DF, Dey A. Processing of cocaine- and amphetamine-regulated transcript (CART) precursor proteins by prohormone convertases (PCs) and its implications. Peptides. 2006; 27(8):1919–25. doi: 10.1016/j.peptides.2005.10.028 PMID: 16784796.
13. Larsen PJ, Seier V, Fink-Jensen A, Holst JJ, Warberg J, Vrang N. Cocaine- and amphetamine-regulated transcript is present in hypothalamic neuroendocrine neurones and is released to the hypothalamic-pituitary portal circuit. J Neuroendocrinol. 2003; 15(3):219–26. Epub 2003/02/18. PMID: 12588509.

14. Koylu R, Tozkoparan E, Pabuscu Y, Ciftci F, Bilgic H, Seber O. Unusual miliary tuberculosis presenting with generalized lymphadenopathy and abdominal involvement. Int J Tuberc Lung Dis. 1997; 1 (5):474–6. Epub 1998/01/24. PMID: 9441104.
15. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, et al. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature. 1998; 393(6680):72–6. doi: 10.1038/29993 PMID: 9590691.
16. Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ. CART peptides in the central control of feeding and interactions with neuropeptide Y. Synapse. 1998; 29(4):293–8. Epub 1998/07/14. doi: 10.1002/(SICI)1098-2396(199808)29:4<293::AID-SYN1>3.0.CO;2–0 PMID: 9661247.
17. Rohner-Jeanrenaud F, Craft LS, Bridwell J, Suter TM, Tinsley FC, Smiley DL, et al. Chronic central infusion of cocaine- and amphetamine-regulated transcript (CART 55–102): effects on body weight homeostasis in lean and high-fat-fed obese rats. Int J Obes Relat Metab Disord. 2002; 26(2):143–9. Epub 2002/02/19. doi: 10.1038/sj.ijo.0801863 PMID: 11850744.
18. Fekete C, Mihaly E, Luo LG, Kelly J, Clausen JT, Mao Q, et al. Association of cocaine- and amphetamine-regulated transcript-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic-pituitary-thyroid axis during fasting. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2000; 20(24):9224–34. Epub 2000/01/11. PMID: 11125000.
19. Vrang N, Larsen PJ, Kristensen P, Tang-Christensen M. Central administration of cocaine-amphetamine-regulated transcript activates hypothalamic neuroendocrine neurons in the rat. Endocrinology. 2000; 141(2):794–801. Epub 2000/01/29. doi: 10.1210/endo.141.2.7295 PMID: 10650962.
20. Raptis S, Fekete C, Sarkar S, Rand WM, Emerson CH, Nagy GM, et al. Cocaine- and amphetamine-regulated transcript co-contained in thyrotropin-releasing hormone (TRH) neurons of the hypothalamic paraventricular nucleus modulates TRH-induced prolactin secretion. Endocrinology. 2004; 145 (4):1695–9. Epub 2003/12/24. doi: 10.1210/en.2003-1576 PMID: 14691017.
21. Smith SM, Vaughan JM, Donaldson CJ, Fernandez RE, Li C, Chen A, et al. Cocaine- and amphetamine-regulated transcript is localized in pituitary lactotropes and is regulated during lactation. Endocrinology. 2006; 147(3):1213–23. doi: 10.1210/en.2005-1392 PMID: 16339196.
22. Kuriyama G, Takekoshi S, Tojo K, Nakai Y, Kuhar MJ, Osamura RY. Cocaine- and amphetamine-regulated transcript peptide in the rat anterior pituitary gland is localized in gonadotrophs and suppresses prolactin secretion. Endocrinology. 2004; 145(5):2542–50. Epub 2004/02/07. doi: 10.1210/en.2003-0845 PMID: 14764627.
23. Ceccatelli S, Eriksson M, Hokfelt T. Distribution and coexistence of corticotropin-releasing factor-, neurotensin-, enkephalin-, cholecystokinin-, galanin- and vasoactive intestinal polypeptide/peptide histidine isoleucine-like peptides in the parvocellular part of the paraventricular nucleus. Neuroendocrinology. 1989; 49(3):309–23. PMID: 2469987.
24. Stanley SA, Small CJ, Murphy KG, Rayes E, Abbott CR, Seal LJ, et al. Actions of cocaine- and amphetamine-regulated transcript (CART) peptide on regulation of appetite and hypothalamo-pituitary axes in vitro and in vivo in male rats. Brain research. 2001; 893(1–2):186–94. PMID: 11223006.
25. Castinetti F, Reynaud R, Saveanu A, Jullien N, Quentien MH, Rochette C, et al. Mechanisms in Endocrinology: An Update in the Genetic Aetiologies of Combined Pituitary Hormone Deficiency. Eur J Endocrinol. 2016. doi: 10.1530/EJE-15-1095 PMID: 26733480.
26. Bancalari RE, Gregory LC, McCabe MJ, Dattani MT. Pituitary gland development: an update. Endocrine development. 2012; 23:1–15. doi: 10.1159/000341733 PMID: 23182816.
27. Davis SW, Ellsworth BS, Perez Millan MI, Gergics P, Schade V, Foyouzi N, et al. Pituitary gland development and disease: from stem cell to hormone production. Current topics in developmental biology. 2013; 106:1–47. doi: 10.1016/B978-0-12-416021-7.00001–8 PMID: 24290346; PubMed Central PMCID: PMCPMC4039019.
28. Cogan JD, Wu W, Phillips JA 3rd, Arnhold IJ, Agapito A, Fofanova OV, et al. The PROP1 2-base pair deletion is a common cause of combined pituitary hormone deficiency. The Journal of clinical endocrinology and metabolism. 1998; 83(9):3346–9. doi: 10.1210/jcem.83.9.5142 PMID: 9745452.
29. Wu W, Cogan JD, Pfaffle RW, Dasen JS, Frisch H, O'Connell SM, et al. Mutations in PROP1 cause familial combined pituitary hormone deficiency. Nature genetics. 1998; 18(2):147–9. doi: 10.1038/ng0298-147 PMID: 9462743.
30. Tatsumi K, Miyai K, Notomi T, Kaibe K, Amino N, Mizuno Y, et al. Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene. Nature genetics. 1992; 1(1):56–8. doi: 10.1038/ng0492-56 PMID: 1302000.
31. Camper SA, Saunders TL, Katz RW, Reeves RH. The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. Genomics. 1990; 8(3):586–90. PMID: 1981057.

32. Li S, Crenshaw EB 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature. 1990; 347(6293):528–33. doi: 10.1038/347528a0 PMID: 1977085.

33. Ohta K, Nobukuni Y, Mitsubuchi H, Ohta T, Tohma T, Jinno Y, et al. Characterization of the gene encoding human pituitary-specific transcription factor, Pit-1. Gene. 1992; 122(2):387–8. PMID: 1487156.

34. Ohta K, Nobukuni Y, Mitsubuchi H, Fujimoto S, Matsuo N, Inagaki H, et al. Mutations in the Pit-1 gene in children with combined pituitary hormone deficiency. Biochem Biophys Res Commun. 1992; 189 (2):851–5. PMID: 1472057.

35. Tang K, Bartke A, Gardiner CS, Wagner TE, Yun JS. Gonadotropin secretion, synthesis, and gene expression in human growth hormone transgenic mice and in Ames dwarf mice. Endocrinology. 1993; 132(6):2518–24. doi: 10.1210/endo.132.6.8504754 PMID: 8504754.

36. Bartke A, Goldman BD, Bex F, Dalterio S. Effects of prolactin (PRL) on pituitary and testicular function in mice with hereditary PRL deficiency. Endocrinology. 1977; 101(6):1760–6. doi: 10.1210/endo-101-6-1760 PMID: 590190.

37. Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, et al. Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. Nature. 1996; 384 (6607):327–33. doi: 10.1038/384327a0 PMID: 8934515.

38. Gage PJ, Brinkmeier ML, Scarlett LM, Knapp LT, Camper SA, Mahon KA. The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. Mol Endocrinol. 1996; 10(12):1570–81. doi: 10.1210/mend.10.12.8961267 PMID: 8961267.

39. Andersen B, Pearse RV 2nd, Jenne K, Sornson M, Lin SC, Bartke A, et al. The Ames dwarf gene is required for Pit-1 gene activation. Developmental biology. 1995; 172(2):495–503. PMID: 8612966.

40. Japon MA, Rubinstein M, Low MJ. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem. 1994; 42(8):1117–25. PMID: 8027530.

41. Lin SC, Li S, Drolet DW, Rosenfeld MG. Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. Development (Cambridge, England). 1994; 120 (3):515–22. PMID: 8162852.

42. Raetzman LT, Ward R, Camper SA. Lhx4 and Prop1 are required for cell survival and expansion of the pituitary primordia. Development (Cambridge, England). 2002; 129(18):4229–39. PMID: 12183375.

43. Ward RD, Raetzman LT, Suh H, Stone BM, Nasonkin IO, Camper SA. Role of PROP1 in pituitary gland growth. Mol Endocrinol. 2005; 19(3):698–710. doi: 10.1210/me.2004-0341 PMID: 15591534.

44. Gage PJ, Roller ML, Saunders TL, Scarlett LM, Camper SA. Anterior pituitary cells defective in the cell-autonomous factor, df, undergo cell lineage specification but not expansion. Development (Cambridge, England). 1996; 122(1):151–60. PMID: 8565826.

45. Davis SW, Keisler JL, Perez-Millan MI, Schade V, Camper SA. All Hormone-Producing Cell Types of the Pituitary Intermediate and Anterior Lobes Derive From Prop1-Expressing Progenitors. Endocrinology. 2016; 157(4):1385–96. doi: 10.1210/en.2015-1862 PMID: 26812162; PubMed Central PMCID: PMC4816735.

46. Herman JP, Jullien N, Guillen S, Enjalbert A, Pellegrini I, Franc JL. Research resource: A genome-wide study identifies potential new target genes for POU1F1. Mol Endocrinol. 2012; 26(8):1455–63. doi: 10.1210/me.2011-1308 PMID: 22638072.

47. Skowronska-Krawczyk D, Ma Q, Schwartz M, Scully K, Li W, Liu Z, et al. Required enhancer-matrin-3 network interactions for a homeodomain transcription program. Nature. 2014; 514(7521):257–61. doi: 10.1038/nature13573 PMID: 25119036; PubMed Central PMCID: PMCPMC4358797.

48. Dasen JS, Barbera JP, Herman TS, Connell SO, Olson L, Ju B, et al. Temporal regulation of a paired-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis. Genes & development. 2001; 15(23):3193–207. doi: 10.1101/gad.932601 PMID: 11731482.

49. Mortensen AH, MacDonald JW, Ghosh D, Camper SA. Candidate genes for panhypopituitarism identified by gene expression profiling. Physiol Genomics. 2011; 43(19):1105–16. Epub 2011/08/11. doi: 10.1152/physiolgenomics.00080.2011 PMID: 21828248; PubMed Central PMCID: PMC3217323.

50. Douglas KR, Brinkmeier ML, Kennell JA, Eswara P, Harrison TA, Patrianakos AI, et al. Identification of members of the Wnt signaling pathway in the embryonic pituitary gland. Mamm Genome. 2001; 12 (11):843–51. PMID: 11845287.

51. Dun NJ, Dun SL, Wong PY, Yang J, Chang J. Cocaine- and amphetamine-regulated transcript peptide in the rat epididymis: an immunohistochemical and electrophysiological study. Biology of reproduction. 2000; 63(5):1518–24. PMID: 11058560.

52. Toth ZE, Mezey E. Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species. J Histochem Cytochem. 2007; 55(6):545–54. doi: 10.1369/jhc.6A7134.2007 PMID: 17242468.

53. Garcia-Lavandeira M, Quereda V, Flores I, Saez C, Diaz-Rodriguez E, Japon MA, et al. A GRFa2/Prop1/stem (GPS) cell niche in the pituitary. PLoS ONE. 2009; 4(3):e4815. doi: 10.1371/journal.pone.0004815 PMID: 19283075; PubMed Central PMCID: PMCPMC2654029.

54. Lin C, Lin SC, Chang CP, Rosenfeld MG. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. Nature. 1992; 360(6406):765–8. doi: 10.1038/360765a0 PMID: 1334535.

55. Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow AF, Camper SA, et al. Hypomorphic phenotype in mice with pituitary-specific knockout of steroidogenic factor 1. Genesis. 2001; 30(2):65–9. PMID: 11416865.

56. Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow AF, Camper SA, et al. Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. Development (Cambridge, England). 2001; 128 (2):147–54. PMID: 11124111.

57. Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, et al. The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. Genes & development. 1994; 8 (19):2302–12. PMID: 7958897.

58. Pulichino AM, Vallette-Kasic S, Tsai JP, Couture C, Gauthier Y, Drouin J. Tpit determines alternate fates during pituitary cell differentiation. Genes & development. 2003; 17(6):738–47. doi: 10.1101/gad.1065703 PMID: 12651892.

59. Lamolet B, Pulichino AM, Lamonerie T, Gauthier Y, Brue T, Enjalbert A, et al. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. Cell. 2001; 104(6):849–59. PMID: 11290323.

60. Zhao L, Bakke M, Parker KL. Pituitary-specific knockout of steroidogenic factor 1. Molecular and cellular endocrinology. 2001; 185(1–2):27–32. PMID: 11738791.

61. Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, et al. Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. Cell. 1999; 97(5):587–98. PMID: 10367888.

62. Ward RD, Stone BM, Raetzman LT, Camper SA. Cell proliferation and vascularization in mouse models of pituitary hormone deficiency. Mol Endocrinol. 2006; 20(6):1378–90. doi: 10.1210/me.2005-0409 PMID: 16556738.

63. Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. PITX genes are required for cell survival and Lhx3 activation. Mol Endocrinol. 2005; 19(7):1893–903. doi: 10.1210/me.2005-0052 PMID: 15761027.

64. Sheng HZ, Zhadanov AB, Mosinger B Jr., Fujii T, Bertuzzi S, Grinberg A, et al. Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. Science (New York, NY. 1996; 272(5264):1004–7. PMID: 8638120.

65. Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, et al. Multistep control of pituitary organogenesis. Science (New York, NY. 1997; 278(5344):1809–12. PMID: 9388186.

66. Acampora D, Mazan S, Tuorto F, Avantaggiato V, Tremblay JJ, Lazzaro D, et al. Transient dwarfism and hypogonadism in mice lacking Otx1 reveal prepubescent stage-specific control of pituitary levels of GH, FSH and LH. Development (Cambridge, England). 1998; 125(7):1229–39. PMID: 9477321.

67. Nantie LB, Himes AD, Getz DR, Raetzman LT. Notch signaling in postnatal pituitary expansion: proliferation, progenitors, and cell specification. Mol Endocrinol. 2014; 28(5):731–44. doi: 10.1210/me.2013-1425 PMID: 24673559; PubMed Central PMCID: PMCPMC4004773.

68. Zhu X, Zhang J, Tollkuhn J, Ohsawa R, Bresnick EH, Guillemot F, et al. Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. Genes & development. 2006; 20(19):2739–53. doi: 10.1101/gad.1444706 PMID: 17015435.

69. Qi Y, Ranish JA, Zhu X, Krones A, Zhang J, Aebersold R, et al. Atbf1 is required for the Pit1 gene early activation. Proc Natl Acad Sci U S A. 2008; 105(7):2481–6. doi: 10.1073/pnas.0712196105 PMID: 18272476; PubMed Central PMCID: PMCPMC2268162.

70. Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontaine GG, et al. Opposing LSD1 complexes function in developmental gene activation and repression programmes. Nature. 2007; 446(7138):882–7. doi: 10.1038/nature05671 PMID: 17392792.

71. Qiao S, Nordstrom K, Muijs L, Gasparoni G, Tierling S, Krause E, et al. Molecular Plasticity of Male and Female Murine Gonadotropes Revealed by mRNA Sequencing. Endocrinology. 2016; 157 (3):1082–93. doi: 10.1210/en.2015-1836 PMID: 26677881.

72. Stanley SA, Murphy KG, Bewick GA, Kong WM, Opacka-Juffry J, Gardiner JV, et al. Regulation of rat pituitary cocaine- and amphetamine-regulated transcript (CART) by CRH and glucocorticoids. Am J Physiol Endocrinol Metab. 2004; 287(3):E583–90. Epub 2004/05/13. doi: 10.1152/ajpendo.00576.2003 PMID: 15138156.