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Covell, Jeremy J

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Regional Expression of Apolipoprotein D

throughout Development of the Songbird Brain

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Science

by

Jeremy Covell

2018

ABSTRACT OF THE THESIS

Regional Expression of Apolipoprotein D

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by

Jeremy Covell

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor Barnett Schlinger, Chair

Apolipoprotein D (ApoD) is a prominent age related gene with an ancient genetic history with

numerous homologs. It has been shown to be expressed and functional in both the developing

and aging brain, maintaining neural health, and providing resistance to oxidative stress. Yet

despite both its ancient nature and seeming necessity, organisms have been found to display

varying expression patterns at different age points and among different organisms, including

sexually dimorphic expression. In this study, we investigated the expression of ApoD in zebra

finches (*Taeniopygia guttata*) across several regions of the brain. We found upregulation of

ApoD across the brain during early stages of development, the largest increase observed within

the cerebellum. Unlike what has been found in mammals, we observed no sex-differences in

expression. These results suggest that while ApoD expressed in the developmental brain, there

appear to be a variety of mechanisms that control ApoD production among vertebrates.

The thesis of Jeremy Covell is approved.

Scott H Chandler

David William Walker

Barnett Schlinger, Committee Chair

University of California, Los Angeles

2018

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Acknowledgements

Introduction

The understanding of the function of Apolipoprotein D (ApoD) has had a tumultuous history since its initial discovery. Originally identified as a secretion of breast cancer tumor cells (called GCDFP-24) (1), it was shown to have strong sensitivities to control by hormonal regulation. When the breast tumor cells were exposed to Testosterone (T) or Estrogen (E) ApoD expression and secretion increased and decreased respectively (1). It was later investigated in prostate cancer cells exhibiting similar behavior (2). It was under some interest then as a potential biochemical marker for these and other hormonally sensitive cancers.

As protein assays became more robust and sophisticated at the turn of the millenium, many began to wonder as to what potential cellular changes might behind the process of aging, particularly aging within the brain. Samples across multiple ages and individuals were assayed for a variety of proteins and the changes in productions of them measured and weighed against one another (3). No other protein showed a closer correlation to increased age than did ApoD (3), and at the time well-reasoned scientists thought they had found a potent protein marker for aging. They suspected that ApoD buildup may be the direct cause of the various age related changes, particularly those within the brain (4). This speculation was further strengthened by similar results in neurodegenerative diseases (NDDs) with those with more pronounced disease states, independent of age, showed increase ApoD production (4). It was at this point that it was thought that perhaps by lowering or even eliminating ApoD production it could be possible to immortalize cognitive functions of the brain and limit its aging and degradation with time.

Researchers then sought to eliminate ApoD via knockout or other blockade to view if this helped prolong life, neural function, or both. Initial results with the fly homologue for ApoD, Glial Lazarillo (GLaz), resulted in flies that had dramatically reduced lifespans (5) and dramatically reduced ability to handle starvation and oxidative stress (5). Further studies in rodents utilizing a ApoD KO failed to reveal the same decrease in longevity (6) as was seen in the flies, but showed significant decreases in cognition and brain function compared to controls (6,13) as well as reduced resistance to oxidative stress (13).

Under even normal mitochondrial function, approximately 0.1% of the reactions of the electron transport chain results in the formation of a reactive oxygen species (ROS) as opposed to the normal production of water (7). This ROS can wreak havoc around the cell, causing short term damage to protein, lipid, and RNA molecules, but can also at times reach the interior of the nucleus where its ability to strip electrons off of virtually any molecule allows it to irreversibly damage DNA (7). A cell is able to buffer against the average amount of ROS production with its own machinery, however when the production of ROS increases beyond this for any number of of reasons including stress, radiation, random chance, the cell is unable to adequately buffer against the ROS produced and it gains the permanent signature of oxidative stress as oxidative damage (7). This phenomenon led to the development of the oxidative damage and ROS theories of aging, with these ROS being what leads to the slow accumulation of damage and dysfunction in our somatic cells over time (7).

Returning then to the results of the GLaZ and ApoD KOs, this seemed to indicate then that ApoD was an incorrectly accused perpetrator of aging and instead ApoD offered resistance to the slow build up of oxidative damage with age. It has since then been reclassified as a neuroprotective protein, and its role in the response to NDDs and aging has been greatly sought out. All cells within the brain appear to be able express ApoD with glia expressing ApoD continuously and increasing expression with stress or injury, while neurons generally only producing it in response to stress or injury (12).

It had been long known that there are obvious sex differences in both aging and NDDs between men and women. Men are more likely to develop NDDs than are women, and generally seem to age faster than women (8). However, when women do develop NDDs, they proceed through the disease course faster and with a greater severity than do their male counterparts (8). Studies of human brain regions showed that ApoD increased for both age and disease severity, but also showed a relative increase in females among age matched and disease stage matched males (8). This suggests the ability for hormonal influences to play a role in the regulation of ApoD production, likely from the ApoD suppressive effects of E, as production of menopause goes does results in an increase in ApoD production which as speculated by Ordóñez et al. is to potentially make up for the decreased neuroprotective effects of E (8).

ApoD's role in the brain is not only in staving off the effects of age, but has also been shown to be prominent in development as well. In both rodents and humans increased production of ApoD compared to young adults has been seen in developing juveniles (8,9). There is an increase

through aging that corresponds to periods of dramatic brain growth, postnatal days in mice and adolescence in humans (9,10). Additionally it has been shown to facilitate neuronal growth and synaptogenesis (23). In line with ApoD's role in the mediation of oxidative stress, it suggests that ApoD increases to help compensate for the increased metabolic output, and therefore ROS production, during this period of development. Furthermore, overexpression of ApoD showed increased resistance to stress and increased lifespan, the complete opposite of what was expected following the original meta-analysis on aging (14).

Despite all the above that has been learned about ApoD, much about this enigmatic protein remains unknown, and the specific nature of both the hormonal and temporal controls on its expression require further investigation. Recently, a unique model organism, the zebra finch, has been used to investigate both of these factors. Expression of ApoD in brains of young and elderly adult zebra finches were analyzed using qPCR. Unlike their mammalian peers, the production of ApoD in the Hippocampus (HP) decreased with age (11). This suggested either the possibility of an increased resistance to oxidative stress in aged birds compared to mammals, or an alternate means of addressing the increased oxidative stress that comes with age that mammals lack. Likewise of interest, there were also no sex differences in ApoD expression, unlike what has been observed in both rodents and humans. This suggests taxa differences in the regulation of ApoD in the zebra finch, potentially via hormones (11).

We chose to investigate changes in ApoD expression throughout development. We hypothesized that similar to mammals, ApoD would initially increase as the birds developed from hatchlings

to fledglings then decline as growth diminished as the birds progressed into a juvenile phase. During an approximate one month period from hatchling to fledgling there is a dramatic increase in both brain size and mental capacity (15). This dramatic upsurge mirrors if not surpasses the dramatic growth seen in mammals, and thus would expectedly have an increase in ApoD to ameliorate oxidative stress that arises from significant development. Furthermore, this period also corresponds with the time when complex neural circuitry, the song system, develops only in males that allows them to learn, remember, and sing upon reaching sexual maturity. This has been shown to be a tightly regulated hormonal process that shows the ability of both systemic T and neuronally produced E to lead to dramatic changes in brain growth (15). Thus we further predicted that we would see a difference in expression of ApoD between the two sexes due to the sexual dimorphism present in both ApoD expression and in the development that occurs in the song nuclei.

To investigate this, we collected tissue samples from individuals within each of the three prominent developmental stages for zebra finch: hatchlings (~5 day old), fledglings (~25 day old) and juveniles (~75 day old). Tissue samples consisted of five brain regions, rostral telencephalon (RT), caudal telencephalon (CT), whole telencephalon (WT), hippocampus (HP), hypothalamus (Hy), and cerebellum (CB). Each of the samples then had their ApoD content expression measured by qPCR and compared across age, sex, and brain region.

Methods

Sample Collection

Individual birds were collected from 3 major time points of the young zebra finch life development stages: hatchlings (H) collected from 5-8 days old birds, fledglings (F) collected from 24-27 day old individuals, and juveniles (J) collected from 75-76 day old birds. The animals were euthanized using isoflurane then decapitated. Their brains were extracted by removing the top of the skull and loosening the brain from the bottom of the skull. The brain was placed on a glass petri dish. Due to the small size of the hatchlings (H) and the inability to precisely dissect individual brain regions, the telencephalon was taken whole (WT) by removing both the cerebellum (CB) and the brainstem and spinal cord. For the fledgling (F) and juvenile (J) birds, first the optic tecta (OT) and CB were removed from the ventral surface of the brain followed by the hypothalamus (Hy). The brain was turned to reveal the dorsal surface from which the HP was removed. The remaining telencephalon was bisected along the midsagittal line and rostral (RT) and caudal (CT) portions were removed.

Adult (A) HP samples were collected by first removing the front section of the telencephalon, them making two shallow cuts on the top of the remaining telencephalon and peeling off the HP revealing the top most ventricles beneath.

RNA Extraction

Tissue samples were homogenized and extracted utilizing a Trizol extraction procedure as outlined by the manufacturer (Ambion). Frozen brain samples were homogenized for 30-45 seconds in 1ml cold Trizol in glass test tubes placed in ice. Following homogenization samples were centrifuged as 12k*g* at 4°C for 10 minutes. The supernatant was decanted then incubated at room temperature for 5 minutes. 200 µl of chloroform were added and were shaken vigorously before incubating at room temperature for 3 minutes. The samples were then again centrifuged at the above settings for 15 minutes. Following then the removal and discard of the aqueous layer, 500 µl of isopropanol was added to each tube. In addition, any samples from the HP, Hy, or H CB had an additional 1 µl of glycoblue (Invitrogen) to assist in pellet binding and viability of small tissue samples. Samples were then vortexed and incubated at room temperature for 10 minutes, after which they were again centrifuged at above settings for 10 minutes. The supernatant was then discarded, leaving behind the RNA pellet which was further purified 1 ml of 75% ethanol was added to each tube, and then centrifuged at 7.5k*g* at 4°C for 5 minutes. The ethanol was then removed carefully from each tube via pipette and the pellets were allowed to try evaporating the remaining ethanol for 20 minutes. After this, samples were resuspended in 10-200 µl of sterilized water, with larger pellets being resuspended in greater volumes, and placed in a hot water bath at ~58°C for 10 minutes. RNA concentration and integrity was determined via nanodrop with concentration values ranging from 100 to 700 ng/µl, and A260/280 ratios between 1.8 and 2.10.

cDNA Reverse Transcription Synthesis

cDNA was reverse transcribed from 600 ng of RNA for each sample. 0.5 µl of DNAse (Promega) and 1.1 µl of DNAse buffer (Promega) were added to each sample which were incubated at 30 minutes at 37°C then incubated for 10 minutes at 65°C, killing the DNAse. Next, 1.5 µl of oligoDT (Sigma) and 0.5 µl dNTPs (Bioline) were added, and tubes were incubated for 10 min at 65°C. Finally, a mix of reverse transcriptase (Superscript II, Invitrogen; 1 µl/sample), RT buffer (4 µl/sample), DTT (1 µl/sample), and RNAse inhibitor (RNAsin, Promega; 1 µl/sample) was added, followed by incubation at 42°C for 50 min and 70°C for 15 min. Samples were then frozen at −20°C until qPCR analysis.

A confirmatory PCR was run using samples and blanks from each batch of cDNA to ensure both presence of product in the cDNA and lack of contamination in the samples utilizing commonly expressed mineralocorticoid receptor gene. The mineralocorticoid primer used was the same as described in Rensel et al. (22).

In addition, in the absence of juvenile ApoD and Peptidylprolyl isomerase A (PPIA) in Hy tissue, a PCR was conducted on all of the Hy samples for both sexes and across both ages utilizing again the mineralocorticoid receptor gene using the same primer as described above.

qPCR

Following numerous optimizations, best results for both our gene of interest, ApoD, and our reference gene, PPIA, occurred at a cDNA dilution of 1:30. The ApoD primer used was

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the same as in Kosarussavadi, S. et al. (11). A plate standard sample was used to be able to compare all plates with one another, the sample used was randomly chosen and included on every well and ran for both primers on each plate. The standard curves were constructed using a 1:1 pool of cDNA from all brain regions from both sexes across all ages. Standard curves were prepared for each gene and plate to confirm reaction efficiency (90–110%) and standard curve linearity ($\geq$98%)

We used an Applied Biosystems 7300 Real-Time PCR system to quantify gene expression relative to the PPIA in samples using SYBR Green. Samples were run at a 1:10 dilution and in duplicate wells. Reaction volume was 25 µl, and cycling conditions were as follows: (1) 2 min at 50°C, (2) 10 min at 95°C, (3) 15 s at 95°C, (4) 1 min at 60°C, repeat steps 3 and 4 40 times, (5) 15 s at 95°C, (6) 1 min at 60°C, (7) 15 s at 95°C, and (8) 15 s at 60°C.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics software version 24. Due to high variance, logarithmic transformations were applied to the data and so each of the following tests utilized the values of the natural log of the fold change as measured from the qPCR. Each of the samples were compared by sex, age, and region using univariate models with interactions between age and region. Interactions found to be nonsignificant were dropped from the model and the model run again. Due to a variable number of samples obtained from each bird, a mixed model analysis was conducted with an effect of Bird ID on the data. Bird ID had no significant

effect on the analyses. To compare hatchling whole telencephalons to those of the older birds, we pooled results of the RT, CT, and HP areas of the fledgling and juveniles.

Results

Interregional Comparisons

CB expressed significantly more ApoD than RT(p=.002), HP(p=.002), and CT(p=.050), with all the remaining regions statistically similar to one another: RT-CT(p=.180), RT-HP(p=.999),RT-WT(p=.109), CT-HP(p=.194), CT-WT(p=.708), HP-WT(p=.119), CB-WT(p=.154) on average across all age groups. (Fig 1)

WT

We observed a large difference between hatchling, juvenile and fledgling ApoD expression (p=.000), but no significant difference between the juveniles and fledglings (p>.874) and no significant sex differences (p=.263). (Fig. 2a).

CB

We found that ApoD expression increased significantly between hatchling and fledgling ages (p=.000) followed then by a significant decrease in expression between fledglings and juveniles (p=.036). There was no significant effect of sex for any ages for this region (p=.650). (Fig. 2b).

RT, CT

There was no significant effect of age (p=.215) or sex (p=.863) on ApoD expression in the

rostral telencephalon.(Fig. 2c). There were no significant effects of age (p=.248) or sex (p=.823)

on ApoD expression in the caudal telencephalon. (Fig. 2d).

HP

To compare results collected here with previously published levels of ApoD in adults (11), we

examined adult HP samples to samples of HP from our fledglings and juveniles. There was no

significant difference in ApoD expression between the age groups (p=.278) or between sexes

(p=.804). (Fig. 2e).

Hy

Interestingly, and unlike other brain regions, we were unable to compare expression changes due

to large differences in expression of both ApoD and our reference gene PPIA. ApoD was

detected in the Hy of fledglings, but neither gene was detectable in the Hy of juveniles. Using

PCR, Mineralocorticoid Receptor (MR) was detected, confirming the presence of useful mRNA

in the juvenile Hy samples. (Fig. 3).

Discussion

Our original hypothesis was that the dramatic rate of neural growth and metabolism during development would result in an upregulation of ApoD expression in the zebra finch brain. Following this increase, we proposed that ApoD expression would decline when upon completion of this maximal period of brain growth and organization, as has been reported for mammals. Furthermore, we predicted that ApoD would be expressed to a greater degree in males as compared to females given the sexually dimorphic growth of their song circuitry, on previous reports in other species for greater expression in males than in non-menopausal females and because sex steroids, particularly E2, has been shown to negatively influence ApoD expression. While our data point to increases in ApoD expression in two brain areas from the hatchling to fledgling phases (~7-25 days of age), a period a maximal brain growth with little change thereafter except in the cerebellum, we found no evidence for sex differences in neural ApoD expression. We discuss these findings below.

The two brain regions that we could accurately quantify ApoD expression in hatchlings, the whole telencephalon and the cerebellum, showed dramatic increases in expression at the fledgling stage. These results are consistent with previous observations in the mammalian brain that also showed increased ApoD expression during development. These data suggest that although birds and mammals may differ with respect to ApoD expression in the aging brain (11), they do appear to display utilize similar strategies early on life, particularly during periods of large-scale neural development.

With the exception of the cerebellum, other brain regions appear to retain levels of expression beyond the fledgling stage. Why expression in the cerebellum decreased precipitously after the fledgling stage is unknown. Zebra finches are altricial species, that is the young hatch at a very immature state of development and rely fully on their parents for their care (25). Nevertheless, up to the fledgling stage the young birds must perform a number of discrete motor patterns, such as opening their beaks and wagging the heads and tongues to attract parental attention and some ability to move about the nest to avoid injury from parents and other siblings. Thus, the cerebellum that guides some of these motor functions, may require rapid coordinated growth and function up to the fledgling stage, after which cerebellar function may become more quiescent. The fledgling stage is also the time when birds learn to fly, a complex motor task that may require extensive modification and development in the cerebellum. Given that in CB in mice, ApoD has been shown to increase in response to oxidative stress (21), these data are consistent with our hypothesis that increased oxidative stress during a period of extensive brain growth leads to increased expression of ApoD.

The results of ApoD in the hippocampus may be particularly instructive. ApoD expression appears to peak at the fledgling stage, then remain relatively constant off as the bird ages until declining in elderly birds (11). This is different than the mammalian pattern, which has a steep drop off of ApoD production following adolescence followed by a slow exponential increase into senility. This could indicate the possibility that not there may be differences in the regulation of ApoD in the songbird brain, but also that ApoD may play a different role in adapting to neuronal age than in mammals. As was viewed in the previous experiment, adult zebra finch seemed to have little if any decline in mental capacity even with advanced age (11). That data viewed in

context with this data suggests that perhaps ApoD levels are held constant throughout the adult lifespan to prevent the accumulation of oxidative damage in neuronal tissue, whereas in mammals, ApoD expression may serve to rescue neural tissue after oxidative damage begins to arise. Several studies provide evidence to support this idea. The overexpression of GLaZ and human ApoD was have shown to prolong lifespan and increase stress resistance in flies (19,20) and the overexpression of human ApoD in rodents provided increased resistance to neurotoxicity (18). These seem to suggest that a consistent and elevated baseline for ApoD expression across the lifespan may prove beneficial. Alternatively it could suggest that there is an entirely separate process independent of ApoD that may help out birds maintain their high mental capacities thus preventing the need for a change in ApoD levels beyond the initial spike during development.

It was surprising that we found no evidence for sex differences in ApoD expression in any brain region. This was most notable in the telencephalic regions, both rostral and caudal, where song system nuclei show extensive changes between the hatchling and juvenile stages, growth in males and or neural losses in females. Possibly, the extensive changes that occur in both sexes, while they may be opposing, both require modification of neural processes that may involve equivalent ApoD expression. Given that the male brain is likely exposed to more estrogen that the brains of females at these ages, it may be that ApoD in the avian brain is insensitive to gonadal hormone regulation. It may be the our dissections that included tissue beyond the song system nuclei themselves might have diluted results; measures of ApoD smaller discrete dissections may be required to better asses sex differences in expression.

To facilitate the investigation of the role ApoD may have in the mediation of the growth of the song nuclei it may prove useful to instead return to adult birds of another species. While certain song birds such as zebra finch are opportunistic breeders which will breed so long as environmental conditions are met, other species experience are steadfast seasonal breeders (24). In the wild, off season the males song nuclei decay and seemingly dissolve until being redeveloped in the next breeding season (24). Thus by identifying and marking these nuclei during breeding season in a manner which their reduced off season locations can be determined, it may be possible to measure ApoD production during different points in the seasonal regrowth of the song nuclei.

It would also be interesting to look at an avian species that uses movement in a sex specific manner much like the zebra finch use song. One such group of birds is the manakins of the neotropics (16,17). They perform highly coordinated and highly athletic dances around a small prepared area to attract mates. The dances include wing snaps, where the wings are brought together over the back so quickly the bones of the wrist collide and make a loud "snap" sound, as well as a variety of twirls, hops, and dives (16,17). It is possible that due to the males needed a more powerful cerebellum to undergo these movements for their mating rituals, and thus may have a more pronounced sexual difference in ApoD production much like we were expecting to see in the song nuclei. This has a few benefits of looking here rather than in the song nuclei. First the regions are wholly separate rather than embedded in the telencephalon and thus easier to remove from younger birds. Furthermore, this area is present in birds of all ages and sexes regardless of developmental stage, and thus removes the guesswork of being unsure if the pre-song nuclei areas of the brain have been removed. Using either the above mentioned organism,

or other sexually dimorphic birds, may allow for a better investigation of sex-linked ApoD functions in the developing brain.

The results of the hypothalamus are intriguing. Though we cannot directly compare the ApoD production changes with age, there does at least seem to be an overall decrease in expression as the birds age from fledgling to juvenile stages. ApoD expression may have been elevated at the earlier age as the hypothalamus is establishing the neural circuits that are the basis of the hypothalamic-pituitary axes. Identification of a control, housekeeping gene, that remains constant with age would prove useful.

What is now clear is that ApoD is expressed in the developing brains of the two highest orders of vertebrates suggesting an important role in ameliorating ROS production, perhaps a significant evolutionary step in the development of larger and more powerful brains. Thus this may also open a possible point of investigation for developmental disorders that show decreased levels of brain growth in development in humans during early life. It may be worth investigating, if possible, the effects of ApoD knockouts on brain development and seeing what the expression of ApoD is in those with various neural developmental disorders compared to their age matched peers.

Figures
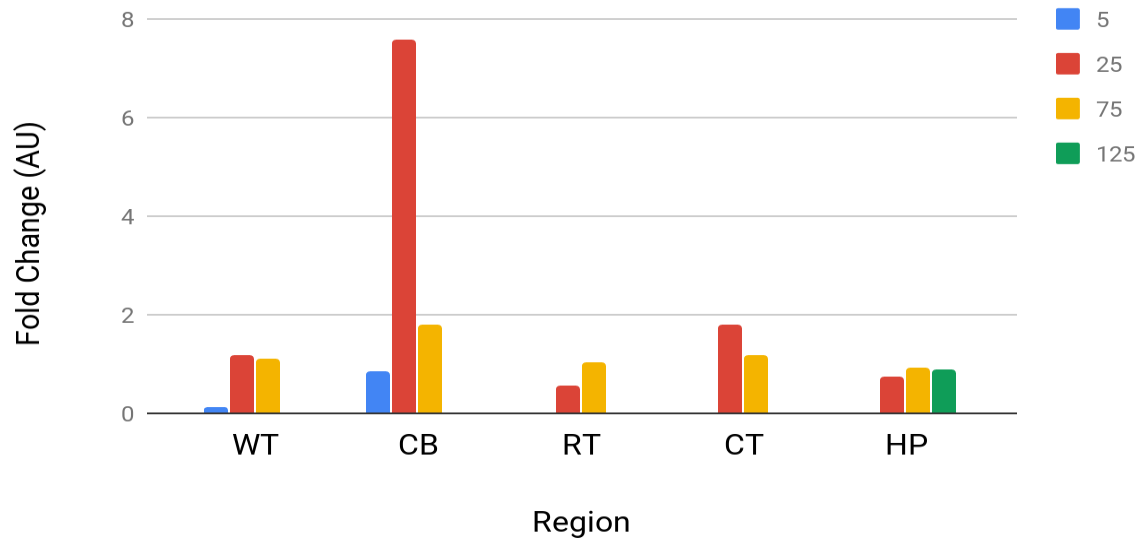
Figure 1

(1) Average Fold Change

Fig. 1. Overall expression data for birds across all regions and ages. Regions from left to right are: whole telencephalon, cerebellum, rostral telencephalon, caudal telencephalon, and hippocampus. Ages are shown in days old.

Figure 2

(2a) Whole Telencephalon ApoD

(2b) Cerebellum ApoD

(2c) Rostral Telencephalon ApoD

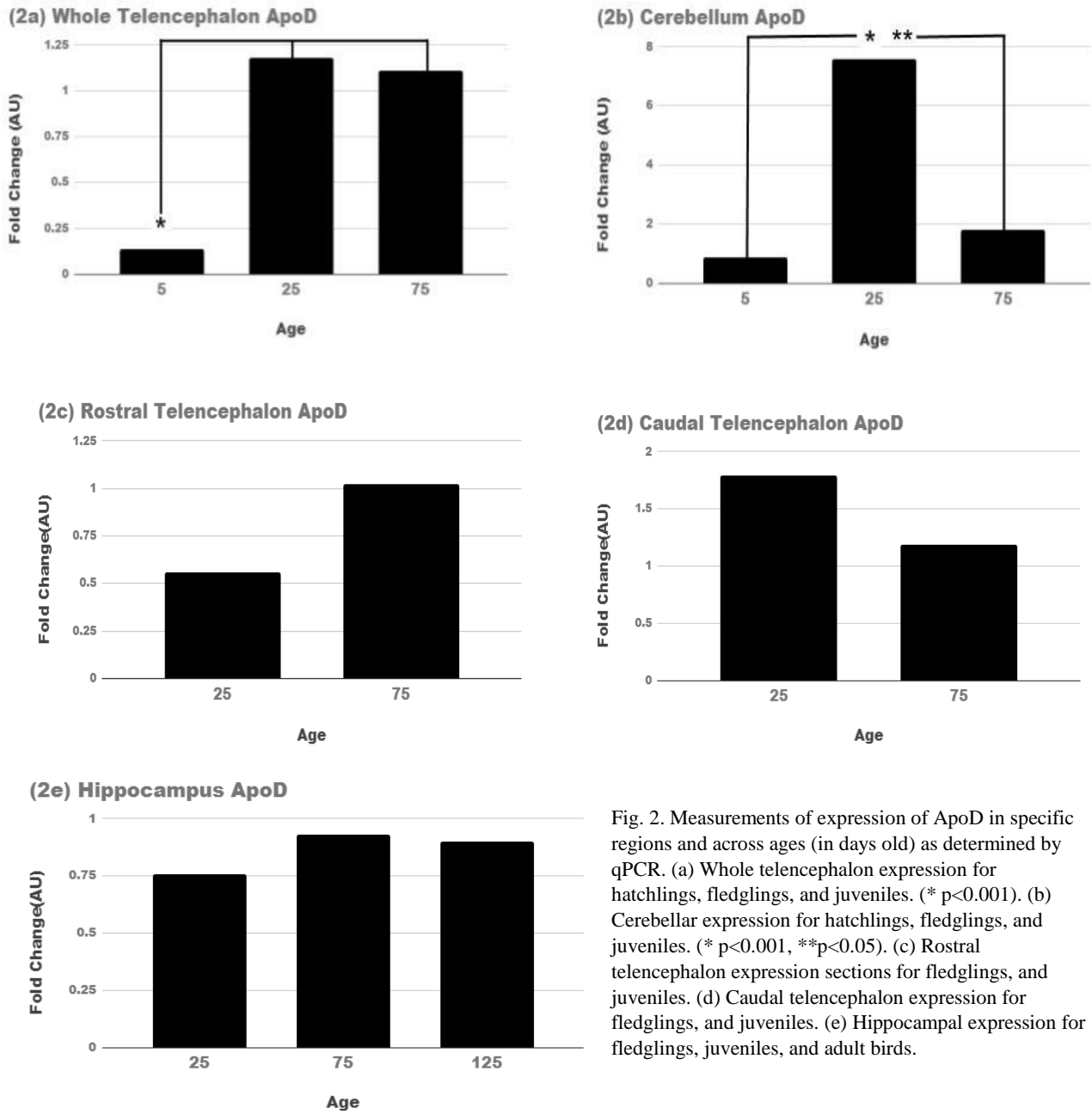(2d) Caudal Telencephalon ApoD

(2e) Hippocampus ApoD

Fig. 2. Measurements of expression of ApoD in specific regions and across ages (in days old) as determined by qPCR. (a) Whole telencephalon expression for hatchlings, fledglings, and juveniles. (* $p<0.001$). (b) Cerebellar expression for hatchlings, fledglings, and juveniles. (* $p<0.001$, **$p<0.05$). (c) Rostral telencephalon expression sections for fledglings, and juveniles. (d) Caudal telencephalon expression for fledglings, and juveniles. (e) Hippocampal expression for fledglings, juveniles, and adult birds.
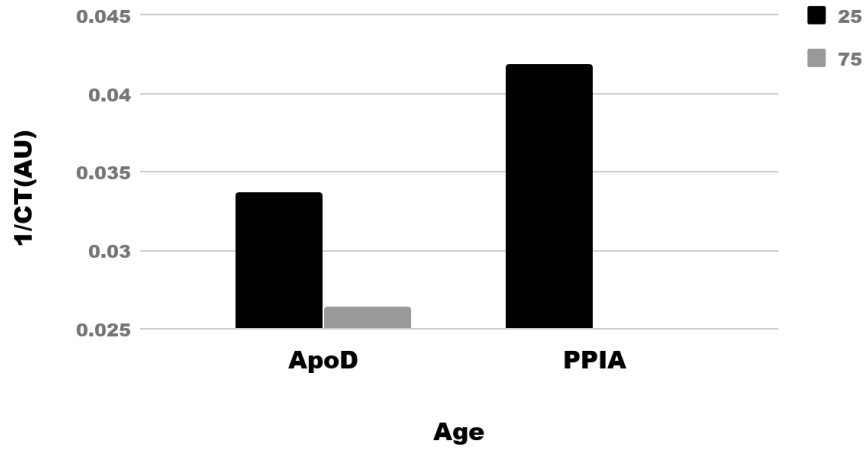
Figure 3

(3) Hypothalamic Apod and PPIA

Fig. 3. Hypothalamic expression of ApoD and PPIA as determined by qPCR. We observed miniscule amounts of ApoD and PPIA for 75 day old showed below threshold expression of PPIA.

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