Drosophila Orthologs, Expressed Specifically in Courting Zebra Finch Males, are Engaged in Courtship

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Abstract: The comparison of transcriptomes generated from the brains of courting and caring zebra finch males revealed the excessive expression of seven genes in the courting males. Two of the encoded proteins (APPBP2 and CST3) appear to share function with the Amyloid Precursor Protein (APP), and two (ARL2 and GPM6A) are engaged in neurogenesis. The *LOC100228889* gene is expressed in the brain, *DLEC1* is a tumor suppressor gene, and *UBE2O* encodes a ubiquitin-conjugating enzyme. Five out of the seven genes - *ARL2*, *CST3*, *GPM6A*, *APPBP2*, and *UBE2O* - have *Drosophila* orthologs: *Arf84F*, *Cys*, *M6*, *Pat1*, and *CG10254*, respectively. We RNAi-silenced the function of *Arf84F*, *Cys*, *M6*, and *Pat1*- and also that of the *APP* homolog *Dmel\Appl* - in the nervous system of *elav*-Gal4; UAS-RNAi *Drosophila* males revealed that while their courting activities were severely reduced, the locomotor abilities and functions of the sensory system were not affected. It appears that the genes above represent evolutionally conserved gene functions in courtship.

Keywords: Transcriptome, courtship genes, RNAi, APP, zebra finch, *Drosophila*.

INTRODUCTION

Courtship is a highly elaborated, genetically controlled process with a strong genetic basis [1]. Are there courtship-specific genes? If there are, which are those? Are the courtship-specific genes evolutionally conserved? To answer the questions above, we compared brain-derived transcriptomes of courting and non-courting zebra finch (*Taeniopygia guttata*) males that care for their offspring. Zebra finch was chosen as a model species for some reasons: the ease of its handling, its frequent use in neurobiological studies, and its genome has been determined [2].

Although a number of developmental and behavioral features have been analyzed recently using high-throughput methods, the genes controlling courtship and/or caring behavior have not been identified in zebra finches, thus far [3]. Comparative genomic approaches represent successful strategies to explore the genes and gene networks behind an interesting biological phenomenon. For example, several candidate genes behind human osteoporosis were revealed by comparing the transcriptomes of red deer (*Cervus elaphus*) osteoporotic and regenerating rib bone samples [4, 5]. In the present study we followed the experimental strategy above, and made use of the plaque-based competitive hybridization (PBCH) technique which was successfully used to identify e.g. the genes regulating red deer antler development [6]. The PBCH technique is an alternative method to procedures such as applied fragment length comparisons and DNA chip technology to identify differentially expressed genes [5-8]. In the present study we compared transcriptomes by analyzing about 600,000 cDNA clones from both the courting and caring zebra finch male brain libraries using the PBCH method. This technique is especially useful when libraries with a few differences are to be analyzed, just as was presumed between the transcriptomes of the courting and caring zebra finch libraries. Another advantage of PBCH technique is that in addition to providing information about the differentially expressed genes, the identified cDNA clones are accessible for further analyses, and cloning is not necessary (Villányi *et al.*, 2008) [6].

In the present study, the experimental design was the following: lambda phage cDNA libraries were constructed from brain samples of both courting and caring Zebra finch males and plated approximately 600,000 plaques on ten-ten plates from each sample. The twenty plates containing 60,000 plaques each were replica plated onto nitrocellulose membranes. Both cDNA libraries were PCR amplified and labeled at the same time and then hybridized onto the unlabeled libraries to the replica membrane of the other library. (For example, the amplified courting library was hybridized to the replica membrane prepared from the caring library and vice versa.) This way, after washing the filters, only the clones that represent specifically expressed transcripts remained single stranded. These clones were made visible on the filter by hybridizing with the labeled cDNA library identical with the plated library. The positive clones were isolated, converted to plasmids, and sequenced. We identified the clones plate by plate, amplified and added the already identified clones to the unlabeled "saturating" hybridization to avoid the identification of the same clones.

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Seven genes expressed predominantly in brains of the courting zebra finch males compared to the caring control were identified. Five of these genes - *ADP-ribosylation factor-like 2* (*ARL2*), *cystatin C* (*CST3*), *glycoprotein M6A* (*GPM6A*), *amyloid precursor protein binding protein 2* (*APPBP2*) and *ubiquitin conjugating enzyme 2O* (*UBE2O*) - have *Drosophila* orthologs, and most of them have been shown to be engaged in brain functions. Since both *APPBP2* and *CST3* are interacting partners of the amyloid precursor protein (*APP*), we included the *APP* gene in the functional analyses of the *Drosophila* orthologs. The UBE2O gene, a component of the proteasome system, was not included in the present study, since it does not appear to possess brain-specific functions, and silencing its function would lead to pleiotropic effects [9]. We silenced "One by one" the functions of the other five candidate courtship genes in the *Drosophila* brain using the RNAi technique, and report herein that silencing of the five genes above significantly reduces the courtship behavior of *Drosophila* males without reducing their locomotor abilities or sensory functions. A previous genome-wide study on *Drosophila* showed that the expression of 16 genes changed only while a male was courting a female [10]. Interestingly, none of the five genes identified in the present work were found to be differentially expressed in that study, however, silencing their functions clearly reveals their involvement in courtship.

The comparative approach applied in the present study made it possible to identify evolutionally conserved courtship-specific genes that are differentially expressed in zebra finches but not in Drosophila. It appears that the genes studied herein represent conserved genes involved in the control of courtship behavior that became differentially expressed in zebra finches during the course of evolution.

RESULTS AND DISCUSSION

Zebra Finch "Courting Genes"

In order to identify the genes engaged in courtship, we compared the mRNA populations isolated from brain samples of courting zebra finch males with those of zebra finch males that were not courting but caring for their offspring. The caring males appear to be ideal controls for studying the molecular mechanisms of courtship behavior as they never court and, therefore, the genes responsible for courtship behavior are most likely turned off in their brains. A transcriptome comparison of three courting and three caring zebra finch male brain samples - using the PBCH technique and comparing about 1,200,000 clones - identified seven candidate genes with prominent expression in the courting zebra finch male brains (Table **1**).

Functions of two of the seven genes have not been identified before: *DLEC1* is deleted in lung and esophageal cancer; *LOC100228889* encodes a protein with a ribosome binding domain. The five genes with known functions are as follows: *ADP-ribosylation factor-like 2* (*ARL2*), *cystatin C* (*CST3*), *glycoprotein M6A* (*GPM6A*), *amyloid precursor protein binding protein 2* (*APPBP2*), and *ubiquitin-conjugating enzyme E2O* (*UBE2O*) (Table **1**).

In order to confirm the results of the PBCH experiments, we prepared reverse Northern blots with the clones representing the putative courtship genes (Fig. (**1**), Table **1**). The analysis confirmed the overexpression of the seven zebra finch genes in the brains of courting males as compared to the caring ones. The results of the reverse Northern blots are summarized in Table **1**. We cannot exclude a possible effect of inbreeding on differential gene expression. It is possible that the inbred Zebra finch line analyzed in the present study is either expresses different genes or the same in a different way than wild type Zebra finches. Such bias can lead to the identification of false positive courtship candidate genes. To elucidate this possibility we performed functional analysis in Drosophila males. As some of the identified genes were evolutionally conserved, we investigated the role of the orthologous genes in courtship behaviour by silencing them in the brain of Drosophila.

The *Drosophila* Orthologs

The level of similarity between the amino acid sequences of zebra finch and *Drosophila* ARL2 (*Dmel\Arf84F*), GPM6A (*Dmel\M6*), APPBP2 (*Dmel\Pat1*), and APP

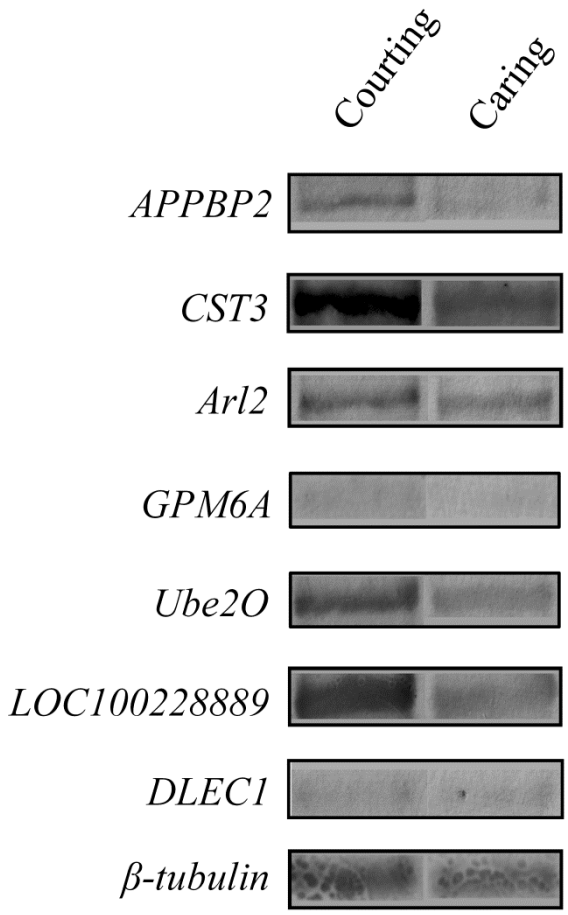

Fig. (1). Reverse Northern hybridization of expression differences of zebra finch genes identified by plaque-based competitive hybridization.

APPBP2 is expressed ≈ 1.8-fold, *CST3* ≈1.7-fold, *ARL2* ≈1.4-fold, *GPM6A* ≈2.3-fold, *UBE2O* ≈2-fold, *LOC100228889* ≈2.3-fold and *DLEC1* ≈1.3-fold in the courting zebra finch male brains compared to caring zebra finch male brains as revealed by the ImageJ software. Values are normalized to *β-tubulin 1* gene expression.

Table 1. Features of the Zebra Finch Genes with Prominent Expression in the Brains of Courting Males as Compared to those of the Caring Males – as Revealed by Reverse Northern Blot

Zebra Finch Gene	Gene Function in Humans	Transcript Levels in Males[1]	
		Courting	Caring
Amyloid precursor protein (*APP*)	APP is an integral membrane protein. It is concentrated in the synapses of neurons indicating its functions in synapse formation and neural plasticity. Its abnormal proteolysis generates β-amyloid peptide, the primary component of the amyloid plaques that appear in the brains of Alzheimer's disease patients. APP is an iron oxidase which promotes the export of iron out of cells [41]. *APPL*, the *Drosophila APP* ortholog, is involved in long-lasting memory. Its formation requires *de novo* protein synthesis and is thought to require synaptic structural plasticity [42].	-	-
Amyloid precursor protein binding protein 2 (*APPBP2*)	It is functionally associated with β-amyloid precursor protein transport and/or processing and is thought to play a role in the pathogenesis of Alzheimer's disease. Its *Drosophila* homolog (*Dmel\Pat1*) interacts with the basolateral sorting signal of APP and is required for the efficient motility of kinesin heavy chain movement along microtubules [43].	33.6±1.9**	15.8±2.2
Cystatin C (*CST3*)	Cystatin C is one of the most important extracellular inhibitors of the cysteine proteinase inhibitor superfamily. Since it binds β-amyloid, reduces its aggregation and deposition, cystatin C seems to play a role in brain disorders, including Alzheimer's disease [44]. Its *Drosophila* homolog, *Dmel\Cys*, has not been analyzed yet.	167.6±9.1**	84.1±4.3
ADP-ribosylation factor-like 2 (*ARL2*)	ARL2 is a member of the small GTP-binding proteins of the RAS superfamily, which was suggested to regulate tubulin polymerization at the centrosomes [28]. *Dmel\Arf84F*, its *Drosophila* homolog, appears to be involved in neurogenesis [45].	38.7±3.9*	21.7±0.6
Glycoprotein M6A (*GPM6A*)	M6a, a membrane glycoprotein, is expressed exclusively by neurons in the central nervous system and is localized in axonal membranes [46]. Targeted depletion of endogenous M6a expression in hippocampal cultures of rat embryos using small inhibitory RNA attenuates neurite outgrowth and impairs synapse formation [27]. The molecular function of its *Drosophila* homolog, *Dmel\M6* is unknown.	17.3±1.6**	6.1±1.0
Ubiquitin-conjugating enzyme E2O (*UBE2O*)	It is a ubiquitin-conjugating enzyme, which catalyzes the covalent attachment of ubiquitin to other proteins. The *Drosophila* homolog (CG10254) has not been studied yet.	57.6±3.9*	24.2±1.2
LOC100228889	Encodes a protein with a ribosome-binding domain. It is expressed in the brain. Its function is not known, and it does not seem to have a *Drosophila* homolog.	104.9±5.4**	39.4±2.8
Deleted in lung and esophageal cancer 1 (*DLEC1*)	*DLEC1* is a functional tumor suppressor [47]. It may act as a tumor suppressor by inhibiting cell proliferation. There are no known *Drosophila* homologs.	22.4±4.7	14.9±1.9
The internal control: β-tubulin, class VI	The major tubulin expressed in platelets.	61.7±5.7	51±4.0

Notes
- *APP* was included due to its interaction with *APPBP2* and *CST3*.
[1]The transcript levels are expressed in arbitrary units as quantified from reverse Northern blots. β-tubulin 1 was used as the internal control.
- * and ** represent significant differences in transcription levels between courting and caring males at P<0.01 and P<0.001, respectively.

(*Dmel\Appl*) is 68%, 50%, 66%, and 53%, respectively. Considering the phylogenic distance between zebra finches and fruit flies these values are quite high. The sequence similarity is not apparent between zebra finch CST3 and the corresponding *Drosophila* protein. Interestingly, the sequence similarity is only 57.5% between *Drosophila melanogaster* and the *Drosophila virilis* CST3 orthologous proteins.

To determine the possible evolutionally conserved roles of the five genes above in courtship, we RNAi-silenced - one by one - the corresponding *Drosophila* genes, and determined the mating success of the males. Using the Gal4/UAS-RNAi system for this analysis [11-13], we silenced the functions of the genes in the nervous system, and analyzed the behavior of the *Drosophila* males. Copulation frequency was determined as described by Basso and Valente (2001) [14]. As Fig. (**2**) and Table **2** show, although RNAi-silencing

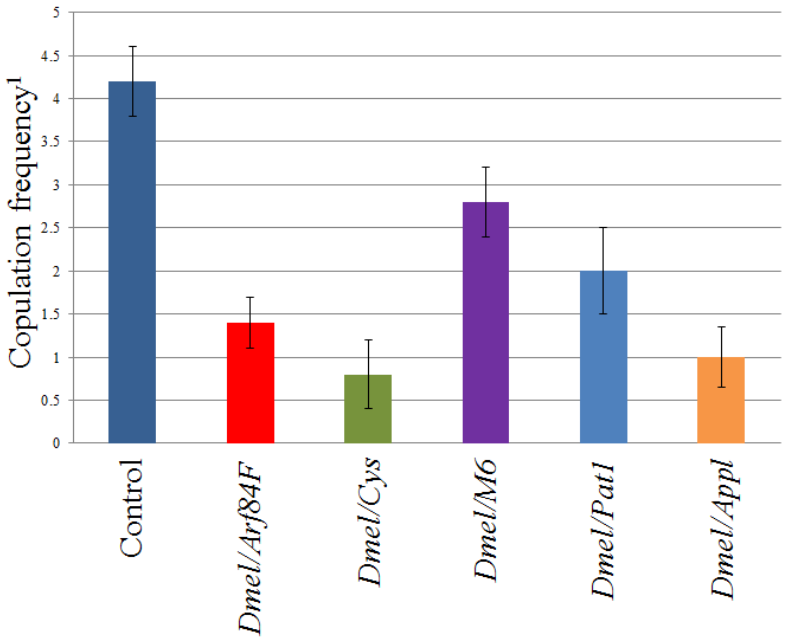

Fig. (2). Copulation frequency of the *elav*-Gal4; UAS-RNAi *Drosophila* males.
[1] Twenty 3-5-day-old "virgin" *elav*-Gal4; UAS-RNAi *Drosophila* males – for each of the five RNAi lines and the silenced genes – were transferred into vials with twenty 2-3-day-old *w* homozygous virgin females, and monitored for 30 min (the control males only carried the *elav*-Gal4 driver.) Five "mating groups" were set up for each line analyzed. The copulating pairs were removed from the vials during the observation period. The mating success of the mating groups of different genotypes was defined as the proportion of copulating males.

Table 2. Copulation Frequency and Geotaxis Activity of the *Elav*-Gal4; UAS-RNAi *Drosophila* Males

Zebra Finch Gene	Silenced *Drosophila* Gene	Copulation Frequency[2]	Geotaxis Activity[3]
-	Control[1]	4.2±0.8	79.4±7.7
ADP-ribosylation factor-like 2 (*ARL2*)	*Dmel\Arf84F* CG7435	1.4±0.6**	79.0±6.5
Cystatin C (*CST3*)	*Dmel\Cys* CG8050	0.8±0.8***	76.0±9.4
Glycoprotein M6A (*GPM6A*)	*Dmel\M6* CG7540	2.8±0.8*	69.9±11.5
Amyloid precursor protein binding protein 2 (*APPBP2*)	*Dmel\Pat1* CG10695	2.0±1.0**	65.9±12.2
Amyloid precursor protein (*APP*)	*Dmel\Appl CG7727*	1.0±0.7***	66.7±8.2

[1] The control males carried the *elav*-Gal4 driver only.
[2] Average ± standard deviation; 5×20 males were analyzed in each experiment.
[3] Average score ± standard deviation.
The differences were significant between the control (elav-Gal4) and *elav*-Gal4; RNAi experimental groups (*P<0.02; **P<0.002; ***P<0.0002). Note that no significant differences were found in the geotaxis experiments.

of the five genes significantly reduced the courtship activities of the *Drosophila* males, it did not abolish their mating ability completely. Moreover, the males in all of the experiments were fertile. The most severe reduction in male fertility emerged upon silencing the *APP* and the *CST3* *Drosophila* orthologous genes.

The defects in courtship could be a secondary consequence of a general lassitude of the central nervous system following gene silencing there. In order to examine this possibility, we conducted a series of geotaxis experiments, and tested the locomotor activities and sensory system functioning of the gene-silenced males using the method of Benzer (1967) [15] following the protocol of Boyles *et al.* (2010) [16]. The results of the experiments summarized in Table **2** and Fig. (**3**) clearly show that the differences in the geotaxis response between the gene-silenced and the control males are not significant, suggesting that the differences in courtship activities are really specific and not related to sluggishness.

APP, *APPBP2*, and *CST3* have previously been connected with courtship behavior in other species. Intracellular *APP* was observed in brain regions involved in the reproductive behavior of the senescent spawning salmon [17]. *APPBP2* was reported to be expressed dominantly in the brains of male *Leporinus macrocephalus* (a gonochoristic fish species) as compared to that of females [18], and *CST3* (*CG8050*) was reported to be downregulated upon mating in *Drosophila* females [19].

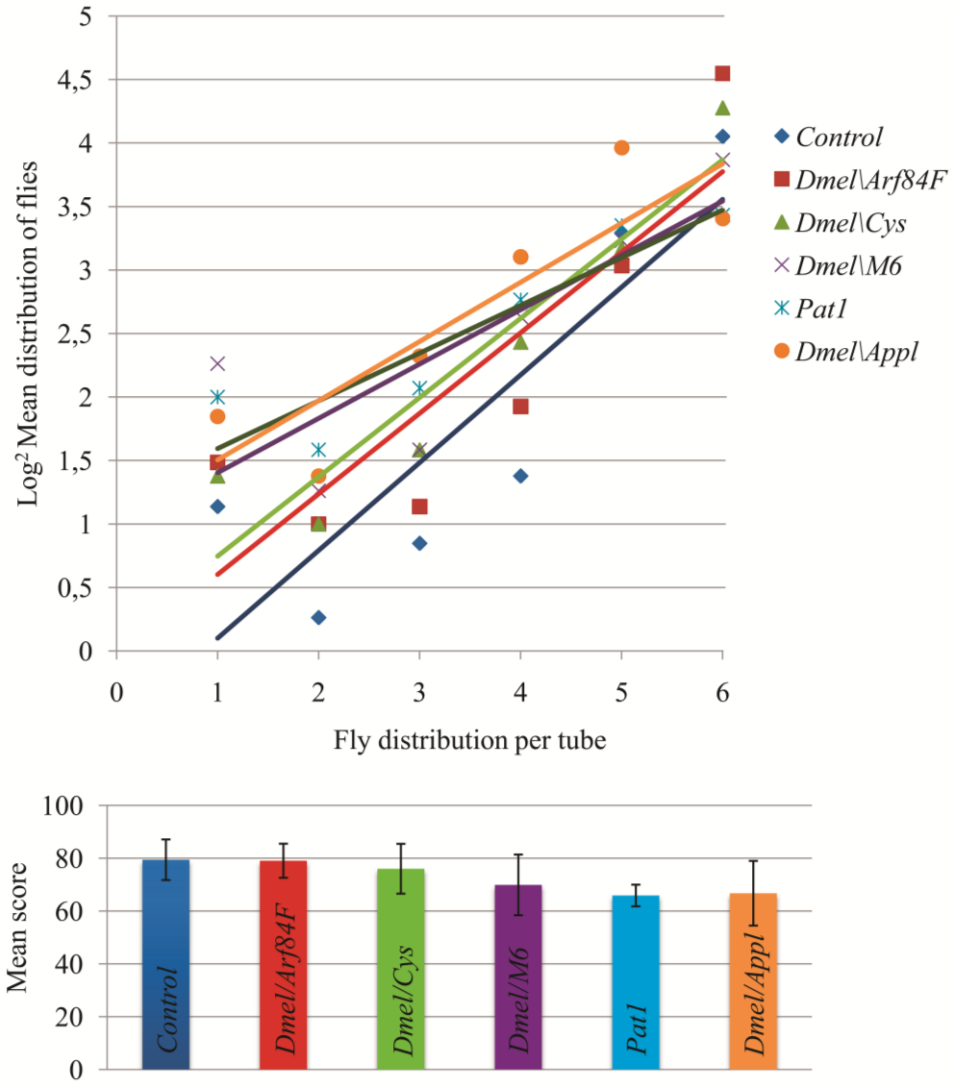

Fig. (3). Results of the geotaxis experiments.
A: Values on the Y axis represent the $\log^2$ mean distribution of approximately 45 flies within each tube after five successive trials. The tube distribution for each group is presented by a best-fit line based on a linear regression. The slopes of both the experimental group (*elav-gal4; UAS-RNAi*) and the control group (*elav-gal4* only) indicate that the majority of the cohorts accumulated in the last few sets of tubes. **B**: The mean score values on the Y axis represent the mean score for each group; no significant difference was found between the experimental groups and the control group (P>0.02).

Products of the *APPBP2* and *CST3* genes were reported to interact with amyloid-β precursor protein (APP). Mutant alleles of the *APP* gene in humans are known to be implicated in Alzheimer's disease [20, 21]. The three genes mentioned above are probably involved in the same pathway, and can also be linked to courtship behavior, as the silencing of any of these genes in *Drosophila* resulted in a decrease in mating frequency, whilst the sensory and motor functions remained intact (Fig. (**3**), Table **2**). The other two evolutionally conserved genes identified as being overexpressed in the brains of courting male Zebra finches were *GPM6A* and *ARL2*. *GPM6A* was suggested to function in modulating synaptic activities in the murine retina and to regulate neurite extension [22]. Two interacting partners of the other identified gene, *ARL2*, are PDE6D and UNC119 which, like ARL2 itself, are connected with phototransduction, visual perception, and related to synaptic transmission [23-25]. Visual cues are important stimuli in birds to trigger courtship. A recent finding provides evidence that the courtship pathway is also primarily vision-dependent in *Drosophila* [26]. The GPM6A protein is a key modulator of neurite outgrowth and filopodium/spine formation [27]. Overexpression of *ARL2* causes defects in the ability to polymerize tubulin [28, 29]. Microtubule polymerization and depolymerization are inevitable events in new synapse formation and, thus, ARL2 may be involved in neural plasticity. Synaptic rearrangements may occur in courting males, and ARL2 and GPM6A probably are key factors in the process.

A previous study on *Drosophila* showed that expression of 16 genes changed while a male was courting a female [10]. Interestingly, none of the genes identified in the present study appeared in that study, although the silencing of their functions clearly revealed their involvement in courtship.

Remarkably, despite the fact that *APP*, *APPBP2*, *ARL2*, and *GPM6A* are highly conserved between species, we did not observe dramatic effects on the viability of the flies following RNAi-induced gene silencing. Loss of *GPM6A* function was connected to claustrophobic phenotypes in mice; however, *GPM6A*-deficient mice develop normally [30]. Similarly, *APP* knockout-mice were also reported to be viable and fertile [31]. Although the loss of *APP* and its homologues, which have alleles connected to Alzheimer`s disease, was studied in several model organisms, no clear picture has yet emerged about the function of APP (see Shariati and Strooper, 2013 for a review) [32]. A recent study revealed that the ancestral *APP* gene sequence, which gave rise to an entire gene family with its numerous interaction partners, evolved during metazoic divergence, which happened much earlier than thought previously [33]. The N-terminal domain of the *APP* gene family member *APL-1* is connected with viability in nematodes [34]. The C-terminus of at least one member of the *APP* family is necessary for viability during early embryogenesis [35-37]. It appears that the members of the *APP* family have different domain-specific functions in different species despite the high conservation of the entire gene. This suggests that in the course of evolution the members of the *APP* family gained novel functions involving specific domains in some of the species. However, the ancestral gene might have had a non-essential function that keeps the gene conserved among the species. We propose that this function is connected to courtship behavior and that the *APP* gene and its interacting partner, *APPBP2*, are kept conserved by sexual selection. This may also be true for *ARL2* and *GPM6A*. This hypothesis may explain how non-essential genes are kept conserved, even if APP has the potential to be a key player in the development of Alzheimer's disease.

MATERIALS AND METHODS

Transcriptome Sources: Brains of Courting and Caring Zebra Finch Males

Six zebra finch males were used in the present study. They originated from an inbred line, and were raised in individual breeding cages for about one year from the 45th day of their life. The six males were three pairs of brothers. Once they were 1 year old, three males (one of the brothers) were each brought together with a female and these three pairs were kept in separate cages to breed. Zebra finch females and males share incubation. The incubating and caring males do not court. On the day when their chicks hatched, the females were removed and after an additional day the caring males were sacrificed: their brains were dissected for mRNA isolation. On the day when the chicks of the caring males hatched, a female partner was provided for each of the three other males who started to court and mate instantaneously. These courting males were analyzed one day after courting, just as their caring brothers.

Isolation of mRNA and Construction of cDNA Libraries

Total mRNA was isolated from the whole brains of three courting and three caring (non-courting) zebra finch males using the FastTrack® MAG Maxi mRNA Isolation Kit (Invitrogen™) according to the manufacturer's instructions. Two cDNA libraries (λ) were prepared using the SMART™ cDNA Library Construction Kit (Clontech, Palo Alto, California). Briefly, 1 μg mRNA samples were reverse transcribed with CDSIII oligo (dT) primer and SMART IV oligo provided with the kit using SuperScript II RNaseH-minus reverse transcriptase (Invitrogen). The reactions were carried out under RNase-free conditions at 42 °C for 60 min. Long distance PCRs were performed in a 50 μl reaction volume using 1 μl from the first strand cDNA reaction, 1 μl dNTP mix (10 mM each), 5 μl 10× BD Advantage2 PCR Buffer, 10 μM primers, and 1 μl 50× Advantage Polymerase Mix. The cycling parameters were 94 °C for 5 min (initial denaturation), 25 cycles at 94 °C for 1 min and 68 °C for 7 min. Then, 3 μg double-stranded cDNA samples were digested with *Sfi*I. Size fractionation was performed by electrophoresis followed by isolation and precipitation; the resulting product was ligated into the *Sfi*I-digested, dephosphorylated λTriplEx2 vector. Plaque libraries were created with the aid of Gigapack® III Plus Packaging Extracts (Stratagene®). For *in vitro* packaging, 1 μl ligation reactions were used and 1-2×10^6 recombinant PFU was further propagated in XL1 Blue strain to obtain amplified libraries with 10^9-10^{10} PFU/ml.

Plaque-based Competitive Hybridization

The experiments were performed according to the method of Villányi *et al.* (2008) [6] with modifications. Further details about the modifications are summarized in Villányi *et al.* (2012) [38].

Briefly, cDNAs in the courting library were labeled with the Roche PCR DIG Labeling Kit, and the signals were developed using the DIG Nucleic Acid Detection Kit (Roche). Nine-centimeter in diameter Petri dishes and compatible Hybond-N+ hybridization membranes were used (Amersham Pharmacia Biotech). A 1 ng cDNA library was used in each case as template for PCR to DIG-label the courting and caring libraries. In addition, the 50 μl reaction mixture contained 0.2 μM insert screening primers provided by Clontech, 1 unit DreamTaq™ polymerase (Fermentas), 0.2 mM dATP, dCTP and dGTP, and 130 μM dTTP. The PCR program was as follows: 95 °C for 3 min followed by 24 cycles of: 95 °C for 40 s, 60 °C for 30 s, 72 °C for 3 min and then 72 °C for 5 min (final extension). A TC-24/H(b) PCR device was used (Bioer Technology, Hangzhou, China). The reaction mixture contained 70 μM DIG-11-dUTP. An appropriate dilution was plated in ten replicates from both libraries at a density of about 60,000 PFU each, and replica membranes were prepared with Hybond-N+ hybridization membranes (Amersham Pharmacia Biotech). Library screening was performed according to the standard protocol [39].

Reverse Northern Hybridization

The mRNA samples isolated from the three courting and the three non-courting brains were reverse transcribed to first strand cDNAs and PCR-labeled through 15 cycles with a PCR DIG Labelling Kit. The other PCR parameters and primers were the same as described in the mRNA isolation and cDNA library construction sections.

Transcripts identified by the PBCH technique were used as probes to verify the expression pattern of the encoding genes. Next, the corresponding clones were PCR amplified. The 50 µl reaction mixture contained 1 ng plasmid DNA as a template for PCR, 0.2 µM insert screening primers provided by Clontech, 1 unit DreamTaq™ polymerase (Fermentas), and 0.2 mM dNTPs. The PCR program was as follows: 95 °C for 3 min followed by 30 cycles of 95 °C for 40 s, 60 °C for 30 s, 72 °C for 3 min, and then 72 °C for 5 min (final extension).

The amplified DNAs were resolved on 1.5% agarose gel and transferred onto Hybond-N+ hybridization membranes (Amersham Pharmacia Biotech). The blots were probed using the previously labeled courting and caring cDNA libraries. Signals developed by the DIG Nucleic Acid Detection Kit (Roche) were quantified using Image-J™ software.

Drosophila Tools Used

The *elav*-Gal4 driver (*P{GAL4-elav.L}3*) ensured the expression of the Gal4-driven genes in the *Drosophila* nervous system [40] and was kindly provided by the Bloomington Stock Center. The *UAS-RNAi* lines for *APP* (42673/GD), *APPBP2* (7066/GD), *CST3* (104909/KK), *ARL2* (44333/GD), and *GPM6A* (101757/KK) were purchased from the Vienna Drosophila Research Center (Stock numbers in the VDRC are listed in brackets.) The *elav*-Gal4; UAS-RNAi males were generated in standard genetic crosses. For an explanation of the genetic symbols see the FlyBase at http://flybase.bio.indiana.edu

Mating Success Analyses

The mating success analyses were carried out as described previously by Basso and Valente (2001) [14]. Briefly, twenty 3-5-day-old "virgin" *elav*-Gal4; UAS-RNAi *Drosophila* males – for each of the five RNAi lines and the silenced genes – were transferred into vials with twenty 2-3-day-old *w* homozygous virgin females, and monitored for 30 min (the control males only carried the *elav*-Gal4 driver.) Five "mating groups" were set up for each line analyzed. The copulating pairs were removed from the vials during the observation period. The mating success of the mating groups of different genotypes was defined as the proportion of copulating males. All tests were performed at 25 ºC.

Geotaxis Response Assay

The geotaxis experiments were performed in an apparatus originally described by Benzer (1967) [15]. In conducting the experiments, we followed the protocol of Boyles *et al.* (2010) [16]. Briefly, the geotaxis countercurrent device consisted of six sets of vertically opposed polypropylene tubes (9×1.5 cm). The opposing tubes were held in a solid frame that allowed the tubes to move in unison relative to each other. Between each tube shift the flies were shaken off to the bottom of the tube. Approximately 45 males were used per round. Ten-second intervals were allowed between each tube shift. The flies were given a score based on how far they had advanced in the tubes by the end of the rounds. The score was calculated as follows: the percentage of flies in the farthest tube, tube 6, was multiplied by 1, those in tube 5 by 0.8, those in tube 4 by 0.6, those in tube 3 by 0.4, those in tube 2 by 0.2 and those in the closest tube 1 by 0. The scores were determined for each round and the mean and the standard error were calculated for five different rounds of testing. All RNAi-expressing groups were compared for significance against the control males, which only carried the *elav*-Gal4 driver. The experiments were performed under standard conditions at 25 °C in the morning.

Ethics Statement

The work carried out in relation to the research presented in this article complied with the current laws of the country where it was performed. The research was approved by the Committee for Experimental Animals of the University of Szeged under license I-74-1/2012 MÁB.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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