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EXPRESSION OF AN ARC-IMMUNOREACTIVE PROTEIN IN THE ADULT ZEBRAFISH BRAIN INCREASES IN RESPONSE TO A NOVEL ENVIRONMENT

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ABSTRACT

Zebrafish are a powerful research tool in the field of neuroscience, offering several logistical and physiological advantages over rodents as a research model. However, the molecular dynamics of this model organism, especially with regards to learning and memory, are scarcely known. The current study explored the zebrafish brain for the presence of a protein bearing a similar function to the activity-regulated, cytoskeleton-associated protein (Arc), a critical player in synaptic plasticity. The adult zebrafish brain was found to express a protein with immunoreactivity against the anti-Arc antibody H-300. Immunoreactivity was detected ubiquitously, especially in areas known as adult progenitor cell zones. The protein, termed Arc-immunoreactive protein (AIP), increased in the telencephalon but not the optic tectum 60 min after exposure to a novel environment. Epileptiform brain activity, however, did not induce AIP expression. Evidence presented herein suggests AIP may be the neuropeptide Y receptor rather than a zebrafish homolog of Arc.

Keywords: immediate-early gene, activity-regulated cytoskeleton-associated protein (Arc), *Danio rerio*, adult progenitor cell zones, novel environment, neuropeptide Y receptor

INTRODUCTION

Historically used for studies focusing on developmental biology, *Danio rerio* is now recognized for the plethora of qualities that yield great utility in the neuroscience research arena—their genomes display approximately 70% homology with that of humans (Howe et al. 2013) and gene expression may be modified with consistent success using morpholinos, CRISPR, and other technologies (reviewed in Garcia et al. 2016). Also, they may be readily treated with pharmacology in a noninvasive fashion due to absorption of compounds from surrounding water, and their small size and high fecundity facilitate economical and efficient colony maintenance. Accordingly, zebrafish models have been developed to study several neurodegenerative disorders, such as Parkinson disease, Huntington disease and Alzheimer disease (AD) (reviewed in Xi et al. 2011; Paquet et al. 2010; Santana et al. 2012).

Several research groups have conducted fundamental studies in order to fully utilize the zebrafish model for the study of neurological disease. Anatomically, the small teleost brain shares many features with the mammalian brain, containing a cerebellum, telencephalon, diencephalon, spinal cord, and hypothalamus (Guo 2004; Mueller et al. 2008; Schmidt et al. 2013; Wullimann and Mueller 2004). Within the telencephalon, homologs of the mammalian amgydala (the medial zone of the dorsal telencephalon region, Dm; Lau et al. 2011), striatum (the dorsal nucleus of the ventral telencephalon area, Vd; Lau et al. 2011) and hippocampus (the ventral part of the lateral division of the dorsal telencephalic area, Dlv; Rodríguez-Expósito et al. 2017) have been identified. Conservation of neurotransmitter structures and systems have also been observed, including those for GABA, glutamate, dopamine, serotonin, noradrenaline, histamine, and acetylcholine (Panula et al. 2006; Schmidt et al. 2013). Using pharmacological intervention, Cognato et al. (2012) demonstrated that N-methyl-D-aspartate (NMDA)-sensitive glutamate receptors are required for acquisition and consolidation of spatial memory in a Y-maze, but the contributions of muscarinic acetylcholine receptors are restricted to the acquisition phase. Also, NMDA receptor-dependent long-term potentiation (LTP) (Nam et al. 2004) and muscarinic receptor-mediated long-term depression (LTD) (Park et al. 2008), considered cellular correlates of learning and memory that occur in the rodent hippocampus, occur in the zebrafish telencephalon.

Zebrafish orthologs for rodent proteins that mediate synaptic changes, such as LTP and LTD, have been identified. Known as immediate-early gene products, c-Fos, Egr-1 (early growth response 1), and Arc (activity-regulated cytoskeleton-associated protein), are particularly well characterized in rodent studies. As in rodents, zebrafish neurons rapidly synthesize c-Fos (Chatterjee et al. 2015) and Egr-1 (Boyer et al. 2013) in response to elevated synaptic transmission. For example, elevated brain c-Fos has been observed in zebrafish larvae after pentylenetetrazole-induced seizure (Baraban et al. 2005) and in adults following systemic caffeine administration (Chatterjee et al. 2015). Egr-1, which acts as a transcriptional regulator during LTP (Li et al. 2005), is synthesized in the olfactory bulbs of zebrafish during taste-aversion learning (Boyer et al. 2013).

Neuropeptide Y (NPY) is the most abundant signaling peptide in the vertebrate central nervous system, and its receptor (NPY-R) is likewise widely expressed. NPY-R contributes to a myriad of brain functions—adult neurogenesis, learning and memory (reviewed in Gotzsche and Woldbye 2016), stress resilience (reviewed in Reichmann and Holzer 2016) and neuroprotection against excitotoxicity associated with epilepsy (Silva et al. 2003). With regards to adult neurogenesis, Decressac et al. (2011) showed that exogenous neuropeptide Y promotes hippocampal neurogenesis in the hippocampus of adult mice in vivo, and blocking the NPY-R Y1 isoform via antagonist or genetic knockout prevents NPY-induced cell proliferation. Similar to Arc and other immediate-early genes, expression of the NPY-R Y1 isoform increases in the basolateral amygdala of rats after environmental enrichment (Hendriksen 2012).

In contrast to c-Fos, Egr-1, and NPY-R, an ortholog for the immediate-early gene *Arc* has not been detected in zebrafish. In rodents, Arc is expressed in neocortical and hippocampal neurons, and its activity-dependent expression is absolutely critical for LTP and LTD (Guzowski et al. 1999; Guzowski et al. 2000; Plath et al. 2006). A variety of learning tasks, including taste aversion, novel object recognition, contextual fear conditioning, and the Morris water maze have been shown to elicit or require Arc function (Guzowski et al. 1999; Plath et al. 2006; Dickey et al. 2004; Inoue et al. 2005; Palop et al.

2005). Accordingly, mice lacking the Arc gene perform poorly in several of the aforementioned behavioral tests (Plath et al. 2006). As elevated synaptic transmission directly induces Arc production, its synthesis may be triggered by a variety of stimuli including novel environments (Guzowski et al. 1999; Dickey et al. 2004; Inoue et al. 2005; Palop et al. 2005), pharmacologically-induced seizure, and high-frequency electrical stimulation (Link et al. 1995) *ex vivo*. Given its importance for hippocampal synaptic plasticity and memory, Arc is the subject of intense investigation in relation to Alzheimer disease. Two transgenic mouse models of AD, the APP + PS1 and J20 lines, which overproduce amyloid-β protein, exhibit reduced hippocampal Arc expression and fail to upregulate Arc after exposure to a novel environment (Dickey et al. 2004; Palop et al. 2005). Interestingly, polymorphisms in the Arc gene are associated with reduced risk of AD in humans (Landgren et al. 2012).

The current study was conducted to further characterize the molecular dynamics in the adult zebrafish brain, particularly as they relate to the field of learning and memory. Given the high degree of functional similarity between rodent and zebrafish neurophysiology, we endeavored to explore the zebrafish brain for an Arc-like protein that may be used as a research tool in learning and memory studies. Exploratory experiments revealed the presence of a protein displaying reactivity to the human Arc antibody in all major brain areas of *D. rerio*. Here, we describe the location of the Arc-immunoreactive protein (AIP) and its responsiveness to two forms of stimuli: a novel environment and pharmacologically-induced seizure.

MATERIALS & METHODS

Test Subjects

Adult zebrafish (*Danio rerio)* of a heterogeneous genetic background were purchased from a local Petsmart (Savannah, Georgia) and maintained at 28.0–29.0 °C in a recirculating system (Aquaneering). The recirculating water source consisted of municipal tap water subjected to four-stage filtration (Aqua FX) entailing a 1 μm sediment filter, carbon block filter, 100 GPD reverse osmosis membrane, and an anion exchange deionizing filter. Synthetic sea salts (Instant Ocean) were added to achieve conductivity between 0.6 and 0.8 mS. The pH was adjusted with sodium bicarbonate as needed to maintain a pH ~7.4. Fish were maintained on a light/dark cycle consisting of 14 hr of light/10 hr of darkness. After an acclimation period of no fewer than two weeks after purchase, fish were singly-housed in 1.8 L tanks to facilitate independent treatment and dissection of each fish on specific time intervals. Row positions on the housing rack were rotated daily to control for unseen environmental variables such as light, noise, and vibration. Male and female fish were used. All experimental procedures were approved by the Armstrong State University Institutional Animal Use and Care Committee.

Immunohistochemistry

Fish (n = 3) were anesthetized by hypothermia and decapitated. The entire brain was rapidly removed while submerged in ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in millimoles per liter): 120 NaCl, 3.5 KCl, 2.0 CaCl, 1.3 $MgSO_4$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose. Tissue was fixed by submersion in 4% paraformaldehyde overnight at 4 °C. Whole brains were blocked in 3% agarose and 50 μM horizontal sections were prepared on a vibratome. Floating sections were pretreated with 3% peroxide solution for 3 min, blocked in 2% normal donkey serum for 30 min, followed

by incubation in anti-Arc primary antibody (Santa Cruz, sc-15325) at 1:1000 dilution overnight at 4 °C. Detection occurred by incubation in HRP-conjugated secondary antibody diluted at 1:1000 followed by staining with ImmPACT Nova-RED peroxidase substrate (VECTOR Laboratories).

Sections were mounted on glass slides and imaged using an Olympus BX60 microscope fitted with an Olympus Q Color 5 digital camera connected to a personal computer equipped with Q-Capture Pro 7 (Q-Imaging) software. Brightness and contrast were adjusted digitally for clarity.

Observation Arena

The observation arena consisted of a fully-enclosed cubicle (90 cm wide × 63.5 cm deep × 93 cm high) constructed with ¾-inch plywood. Inside surfaces were painted with satin-white spray paint. Illumination was provided by four 24 inch, 15 W soft-white fluorescent lights (General Electric) mounted vertically in each corner. Wax paper-covered clear acrylic panels were mounted over each light to alleviate glare. The base consisted of a white styrofoam project board covered with a clear acrylic sheet. A vertically-hinged door on the front of the arena was used to insert and remove tanks. For observation, a 2.6 cm hole was cut into the top of the arena and a Logitech HD c525 webcam was mounted and attached by USB cable to a personal computer. Videos were recorded using Logitech Webcam Software.

Handling and Transport

In order to limit the effects of stressors associated with netting, transport, and exposure to a new environment, four consecutive days of handling were performed. The handling procedure was identical for home-tank controls and Y-maze-exposed fish. Each fish was handled separately. The handling protocol consisted of placing the 1.8 L tank in which the experimental subject was housed into an enclosed transport container, transporting the fish to the behavior room (adjacent to the housing room), and placing the home tank in the observation arena with the lid removed for 5 min. After 5 min, fish were immediately transferred via net to a clean 1.8 L tank and returned to the housing facility.

The transport container consisted of a 10 gal rectangular glass tank placed on top of a metal laboratory cart. To limit vibration and shock during transport, a rectangular base constructed from styrofoam project board was mounted inside the tank on top of several layers of styrofoam and soft packaging. Additionally, soft packaging foam resided between the metal cart and the base of the glass tank. To prevent external visual stimuli during the transport procedure, the internal surfaces of the tank were covered with white poster board and a styrofoam lid covered the tank.

Y-Maze (Novel Environment) Apparatus

The Y-maze was constructed using clear acryclic sheets in the University of Alabama at Birmingham machine shop according to the protocol of Cognato et. al (2012) with modifications. The arm dimensions were 25 cm long, 8 cm wide, and 15 cm high. Visual cues consisted of white geometric shapes constructed from vinyl and mounted externally to the walls of the maze arms. Black vinyl panels covered the remaining external surfaces to provide contrast and limit external stimuli. The base of the maze was left uncovered to ensure sufficient contrast between the fish and the base of the arena

such that video monitoring was possible. The maze contained 3.0 L of conditioned housing water warmed to 28 °C.

Y-Maze (Novel Environment) Exposure

Four consecutive days of handling were conducted to alleviate stressor-induced alterations in protein expression prior to Y-maze exposure on the fifth day. Y-maze exposures (n = 6 fish) commenced at 9:00 am and occurred at 30 min intervals. The Y-maze exposure consisted of placing the home tank into the observation arena before transferring a single fish from the home tank into the Y-maze via net, closing the arena door, and allowing the fish to explore the maze for 6 min. Immediately after the 6 min exploration period, the experimental subject was transferred to a clean 1.8 L tank inside the observation arena via net and returned to the housing facility within an enclosed transport container (see *handling and transport* above).

Control (home-tank) fish (n = 6) underwent the same 4 d handling treatment as Y-maze-exposed fish. On day five, home-tank controls remained on the housing system during the periods in which experimental fish were exposed to the Y-maze. In this fashion, tissues from maze-exposed fish were experimentally paired with tissues from home-tank controls, resulting in six pairs of samples.

Between experiments, experimental water was removed by siphon and replaced with 3.5 L of dH2O to rinse the apparatus of secreted factors. The dH2O was then siphoned and replaced with 3.0 L of fresh experimental water.

Pentylenetetrazole (PTZ) Preparation

Aliquots of 10X PTZ stock solution (75 mM) were prepared by dissolving via 60 s vortex 0.311 g of PTZ (Sigma) into 30 mL dH2O. Stock solutions were stored overnight at -20 °C and used the next day. PTZ treatment solutions consisted of 30 mL of 75 mM PTZ diluted into 270 mL of conditioned housing water to reach a final volume of 300 mL and a final concentration of 7.5 mM. Vehicle control solutions consisted of 30 mL of dH2O mixed with 270 mL of conditioned housing water.

PTZ Treatment

Four consecutive days of handling were performed prior to PTZ treatment on the fifth day. Treatments commenced at 9:00 am and occurred at 15 min intervals. Fish were treated for 6 min by immersion into 300 mL of treatment solution (T = 26.5–28.0 °C) contained in a 1.8 L tank identical to that used for housing. After treatment, each fish was moved to a tank containing 300 mL of housing water to rinse the treatment solution from the body surface and then moved to a fresh 1.8 L tank after 30 s. This rinsing procedure prevented PTZ contamination in the recirculating housing system. Each fish was then returned to the housing facility within an enclosed transport container (see *handling and transport* above). PTZ and vehicle treatments occurred in separate tanks in an alternating fashion. Fresh solutions were prepared between experiments, and treatment and rinsing tanks were flushed with tap water rinsed with dH2O.

Dissections and Tissue Collection

At the appropriate time-point following environmental or pharmacological stimulus, each fish was anesthetized in tricaine methanesulfonate (MS 222; 300 μg/mL) for 4 min and then euthanized by decapitation. The head was stabilized in a foam block

contained in a four-well tissue culture plate containing ice-cold ACSF (see *immunohistochemistry* above). The tissue-culture plate was continuously cooled via immersion in ice-cold water during the dissection. The telencephalon and optic tectum were removed and immediately placed into 50 μL or 100 μL, respectively, of homogenization buffer in a 1.5 mL microcentrifuge tube. Homogenization buffer consisted of T-Per Tissue Protein Extraction Reagent (Fisher Scientific) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals). Samples were homogenized using a teflon pestle attached to a Dremel rotary tool for 45 s and immediately moved to a -20 °C freezer for storage.

Immunoblot Analysis

Protein concentration of each sample was measured using a NanoDrop spectrophotometer, and 30 μL samples containing 30 μg of total protein were prepared in SDS sample buffer (Invitrogen) using standard methods. Samples were resolved by SDS-PAGE in 12% polyacrylamide gels and blotted to PVDF membranes by semi-dry transfer. Membranes were blocked in 5% milk/TBST for 1 hr at room temperature prior to application of primary antibody overnight at 4 °C. Chemiluminescent protein detection occurred by application of HRP-conjugated secondary antibody for 1 hr at room temperature followed by treatment with Clarity Western ECL (Bio-Rad) peroxidase substrate. Blot luminescence was digitally imaged using a ChemiDoc MP Imaging System with Image Lab Software Version 5.1 (Bio-Rad). Protein levels were quantified by densitometry using ImageJ software (NCBI). Before detection of loading control proteins, a mild stripping protocol (AbCam) was performed, and the absence of residual antibody was confirmed by digital imaging.

Antibodies for Western Blotting

The primary antibodies were rabbit anti-Arc H-300 (Santa Cruz, sc-15325) diluted in 3% milk/TBST, rabbit anti-tubulin (Tuba1) (GeneTex, GTX124965) diluted at 1:10,000 in 3% milk/TBST, and rabbit anti-beta-actin (Abcam, ab8227) diluted at 1:2000 in 3% milk/TBST. The secondary antibody was HRP-conjugated anti-rabbit (Cell Signaling, 7074S) diluted at 1:1000 in 3% milk/TBST.

Data Analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparison of data from different treatment groups was performed by Student's *t*-test and $P < 0.05$ was considered statistically significant. AIP levels were normalized to loading controls before statistical analysis and graphed with control values set at 1.0.

Bioinformatic Analysis of the Zebrafish NPY-R

A protein-protein alignment search was conducted using the Basic Local Alignment Search Tool [BLAST; National Center for Biotechnology Information (NCBI)] in which the target amino acid sequence of the anti-Arc antibody H-300 was compared to the amino acid sequence of zebrafish NPY-R isoforms.

RESULTS

Exploratory Screen of Zebrafish Proteome and Arc Immunoreactivity

To screen the zebrafish genome for the presence of an Arc-like protein, a protein-protein alignment search was conducted using the Basic Local Alignment Search Tool [BLAST; National Center for Biotechnology Information (NCBI)] in which the amino acid sequence of human Arc (396aa, ACCESSION #: AAFO7185) was compared to the zebrafish proteome published in the NCBI database. A predicted protein, LOC101885994, which displayed 48% amino acid property homology with the query sequence, was returned. In an effort to detect LOC101885994 in the zebrafish brain for further characterization, 50 µm horizontal sections were prepared from adult zebrafish, and immunohistochemical staining using a polyclonal antibody raised against amino acids 1-300 of human Arc (Santa Cruz, H-300; sc-15325) was conducted. This procedure produced widespread immunoreactivity in the zebrafish brain (Figure 1A).

Brain structures that displayed reactivity to the Arc antibody included the brain stem (LX; Figure 1), the internal cellular layer (ICL) of the olfactory bulb (Figure 1B), the dorsal and ventral regions of the telencephalon (Figure 1B), the periventricular granular zone (PGZ; Figure 1D), the optic tectum (TeO; Figure 1D), the hypothalamus and associated mammillary bodies (Figure 1E), the habenula (Ha; Figure 1F), the periventricular pretectal nucleus (PPv; Figure 1G), and the cerebellum (Figure 1H).

A consistent feature regarding the pattern of staining was its localization to periventricular cell populations, which are known to contain neuronal precursor cells in zebrafish. For example, intense somatic staining was evident in cells directly adjacent to the telencephalic ventricles (TelV; Figure 1B), the rhombocephalic ventricles (RV; Figures 1A, 1C), the tectal ventricle (TeV; Figure 1D), and the hypothalamic diencephalic ventricle (DiV; Figure 1E).

Intracellular staining varied from diffuse to punctate depending upon the region. For example, diffuse immunoreactivity was evident in the mammillary bodies (Cm; Figure 1E) and the cerebellum (CCe; Figure 1H); punctate immunoreactivity was commonly evident across the entire rostro-caudal axis, as exemplified in the periventricular pretectal nucleus (PPv; Figure 1).

Using Western blot, we showed that immunoreactivity to zebrafish proteins did not occur in the absence of primary antibody (Additional file 1).

Arc-Immunoreactive Protein Expression after Novel-Environment Exposure

Western blot analysis was performed to investigate if the protein detected by immunohistochemistry in zebrafish using the Arc antibody, which we termed the Arc-immunoreactive protein (AIP), exhibits the same functional properties of rodent Arc. It was predicted that AIP expression would increase in the telencephalon, a structure homologous to the mammalian hippocampus, after exposure to a Y-maze apparatus, which served as a novel environment. Zebrafish taken directly from their home tanks and not exposed to the Y-maze served as nonstimulated controls. In control tissues, it was determined that the molecular weight of AIP is approximately 42 kDa (Figure 2A). After normalization to loading controls, the telencephalon of maze-exposed fish exhibited an increase in AIP expression 70 min after Y-maze exposure relative to home-tank controls (Figures 2B,C; home tank: 1.00 +/- 0.25, n = 6; maze-exposed: 1.70 +/- 0.50, n = 6; P = 0.02, t-test). The increase was consistent, occurring in all six experimental pairs of maze-

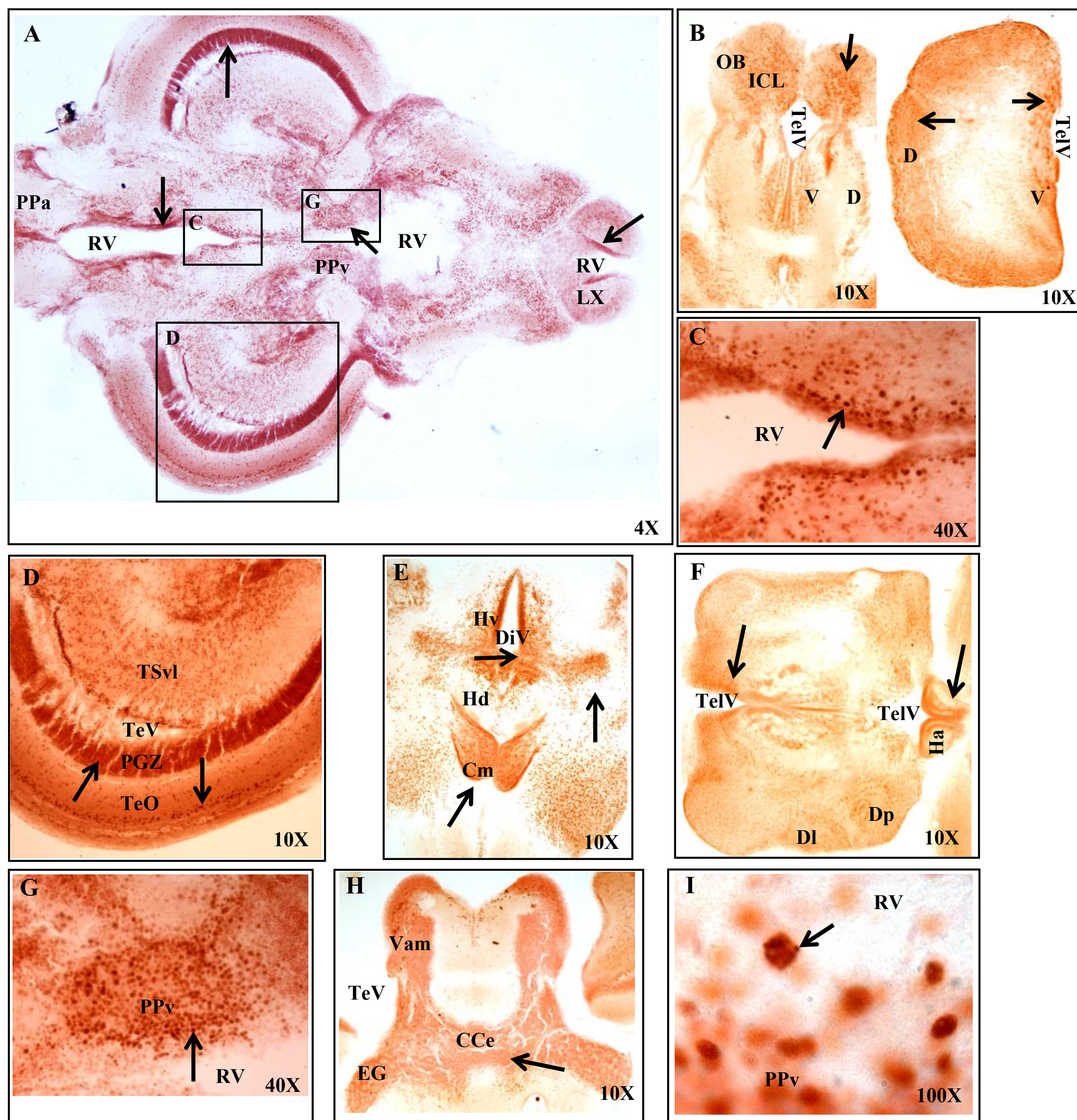

Figure 1. Representative immunohistochemistry images from 50 μM horizontal brain sections showing widespread localization of Arc-immunoreactive protein (AIP) in the zebrafish brain. Arrowheads indicate areas of interest in all panels. ***A.*** Broad distribution of AIP across the rostro-caudal axis, 4X mag. ***B.*** Distribution of AIP in the olfactory bulbs and telencephalon, 10X mag. ***C.*** AIP expressed in the periventricular region of the rhombocephalic ventricle, 40X mag. ***D.*** AIP expressed in the optic tectum and associated structures, 10X mag. ***E.*** AIP distribution in the hypothalamus and mammillary bodies, 10X mag. ***F.*** AIP distribution throughout the telencephalon and habenula, 10X mag. ***G.*** AIP localization in the periventricular pretectal nucleus, 40X mag. ***H.*** AIP distribution in the cerebellum, 10X mag. ***I.*** Punctate intracellular AIP expression, 100X mag. ***Abbreviations:*** **CCe**, corpus cerebelli; **Cm**, corpus mamillare (mammillary bodies); **D**, dorsal telencephalic area; **DiV**, diencephalic ventricle; **Dl**, lateral zone of D; **Dp**, posterior zone of D; **EM**, eminentia granularis; **Ha**, habenula; **Hd**, dorsal zone of the periventricular hypothalamus; **Hv**, ventral zone of the periventricular hypothalamus; **ICL**, internal

cellular layer of the olfactory bulb; **LX**, vagal lobe; **OB**, olfactory bulb; **PGZ**, periventricular granular zone; **PPa,** parvocellular preoptic nucleus, anterior part; **PPv**, periventricular pretectal nucleus; **RV**, rhombocephalic ventricle; **TelV**, telencephalic ventricle; **TeO**, optic tectum; **TeV**, tectal ventricle; **TSvl**, ventrolateral nucleus of torus semicircularis; **V**, ventral telencephalic area; **Vam**, medial division of valvula cerebelli. (Wulliman et al. 1996).

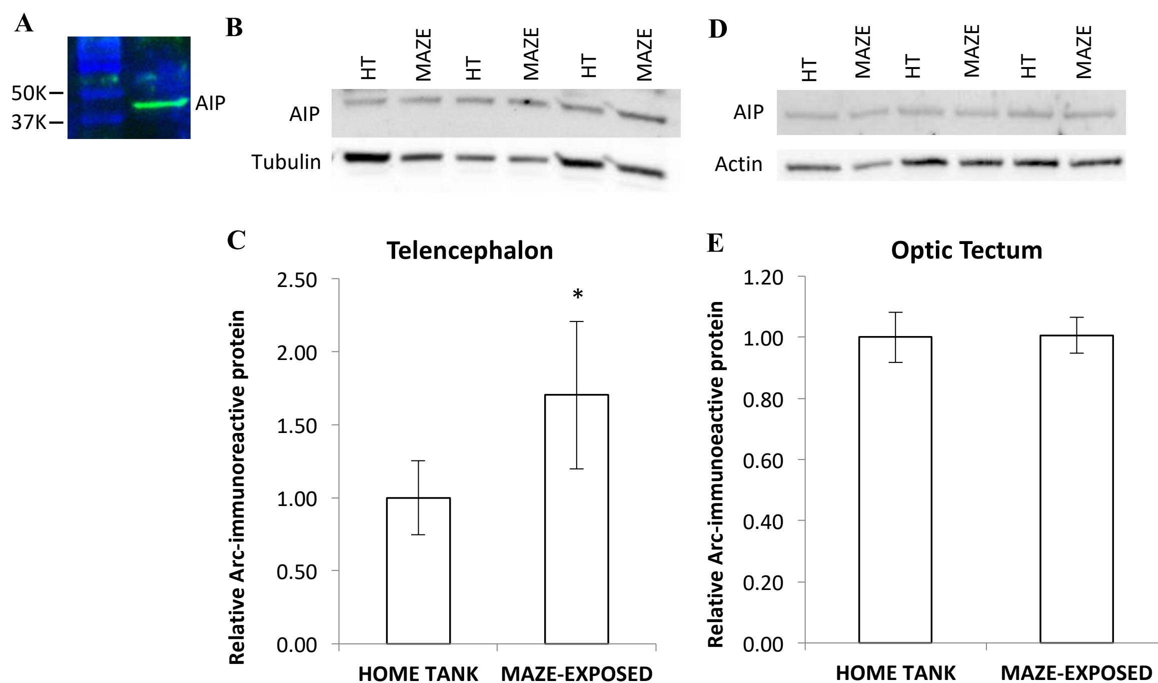

Figure 2. Western blot analysis of Arc-immunoreactive protein (AIP) levels in brain homogenates after novel-environment (Y-maze) exposure. ***A.*** Representative immunoblot image of AIP in the homogenate of telencephalon from a zebrafish taken directly from its home tank and protein standards. ***B.*** Representative immunoblot image of AIP in the homogenate of telencephalon from zebrafish exposed to the Y-maze for 6 min or taken directly from their home tanks. Tubulin was used as a loading control. ***C.*** Densitometric analysis of immunoblots from the telencephalon (normalized by the amount of tubulin) with levels in the home-tank group set as 1.0. Data represent means ± SEM (n = 6 fish per group; $P = 0.02$, * indicates $P < 0.05$). ***D.*** Representative immunoblot image of AIP in the homogenate of optic tectum from zebrafish exposed to the Y-maze for 6 min or taken directly from their home tank. ***E.*** Densitometric analysis of immunoblots from the optic tectum (normalized by the amount of actin) with levels in the home-tank group set as 1.0. Data represent means ± SEM (n = 6 fish per group; $P = 0.46$).

exposed and nonstimulated controls. AIP expression was also measured in the optic tectum, an area of intense immunoreactivity (Figure 1D). In contrast to the telencephalon, AIP expression in the optic tectum (Figures 2D,E) remained unchanged after exposure to the Y-maze (home tank: 1.00 +/- 0.08, n = 6; maze-exposed: 1.01 +/- 0.06; n = 6; $P = 0.46$, *t*-test) compared to the unstimulated control group.

Arc-Immunoreactive Protein Expression after Pentylenetetrazol-Induced Seizure

It was predicted that zebrafish AIP levels would increase after pentylenetetrazol (PTZ)-induced seizure. To test this prediction, the telencephalon was collected from adult zebrafish 2 hr after seizure induced by immersion in PTZ (7.5 mM) for 6 min, a treatment

shown to reliably induce seizure-like activity in adult zebrafish (Mussulini 2013). After PTZ-induced seizure, telencephalic tissue was homogenized and AIP levels were measured relative to vehicle-treated controls. Contrary to our prediction, AIP levels did not increase after seizure (Figure 3; vehicle-treated: 1.00 +/- 0.11, n = 8; PTZ-treated: 0.80 +/- 0.16; n = 6; $P = 0.15$, *t*-test).

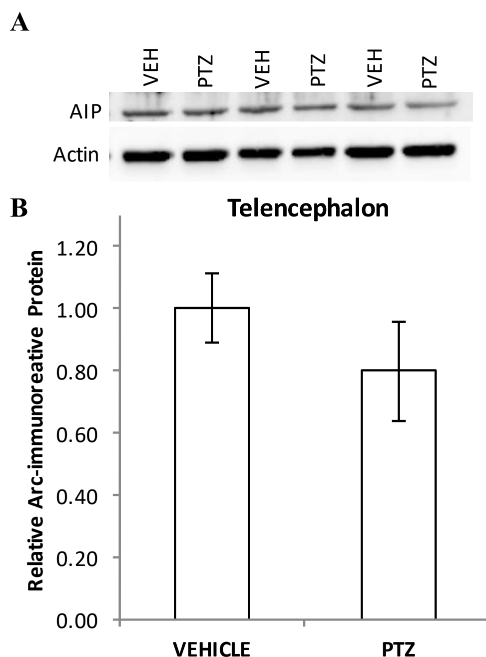

Figure 3. Effect of PTZ exposure on Arc-immunoreactive protein (AIP) in the telencephalon. ***A.*** Representative immunoblot image of AIP in the homogenate of telencephalon from zebrafish exposed to PTZ (7.5 mM) for 6 min or vehicle. Actin was used as a loading control. ***B.*** Densitometric analysis of immunoblots from the telencephalon (normalized by the amount of actin) with levels in the vehicle-treated group set as 1.0. Data represent means ± SEM (n = 6 fish per group; $P = 0.15$).

AIP Sequence Homology

To ascertain the putative identity of AIP, protein-protein BLAST searches were conducted in which the target sequence of the anti-Arc antibody was compared to the zebrafish proteome in the NCBI database. Candidate proteins were then cross-referenced against the properties of AIP revealed in the current study—molecular weight, anatomical localization, and a responsiveness to environmental stimuli. Significant overlap was found between the properties of AIP and the neuropeptide Y receptor (NPY-R). Therefore, the target sequence for the Arc antibody was aligned with the primary structures of several NPY-R isoforms using NCBI BLAST. The results (Table I) indicate homology ranging between 38% and 66% between the Arc antibody and several NPY-R isoforms, including the most abundant isoforms in vertebrate brain—Y1 and Y2.

Table I. Homology between six neuropeptide-Y receptor isoforms in *D. rerio* and the target sequence of the human Arc antibody (H-300) (NCBI BLAST*). Abbreviations: NPY-R, neuropeptide Y receptor.

NPY-R isoform	Amino acid alignment by region (%)	Graphical alignment with target sequence of the human Arc antibody
Y1	48% positives (16/33) 40% positives (6/15) 46% positives (6/13)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240
Y2	52% positives (11/21) 57% positives (8/14)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240
Y4	38% positives (34/88)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240
Y7	66% positives (6/9) 41% positives (6/36) 42% positives (24/56)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240
Y8a	62% positives (10/16) 40% positives (6/15)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240
Y8b	40% positives (6/15)	Color key for alignment scores: <40, 40-50, 50-80, 80-200, >=200; Query 1 60 120 180 240

*Images courtesy of the US National Library of Medicine

DISCUSSION

The current study was conducted to probe the zebrafish brain for an Arc-like protein. To this end, an antibody raised against human Arc was applied to horizontal sections across the entire dorsoventral axis of the adult zebrafish brain, and a putative Arc ortholog (AIP) was evaluated with Western blot. We found that AIP exhibits an Arc-like sensitivity to a novel environment, but a lack of seizure-induced expression was observed. Based upon similarities between AIP and NPY-R in terms of molecular weight, localization to progenitor cell zones, increased expression after novel-environment exposure, and homology to the Arc antibody target sequence, we postulate that AIP may be the neuropeptide Y receptor rather than a zebrafish Arc homolog.

Zebrafish Arc-Immunoreactive Protein Expression Coincides with Progenitor Cell Populations

In our experiments, AIP in zebrafish was ubiquitously expressed in all major divisions of the brain including the olfactory bulbs, telencephalon, optic tectum, cerebellum, medulla, and hypothalamus. Zebrafish display a uniquely-robust volume of adult progenitor cells residing in zones throughout the brain (Grandel et al. 2006; Kizil et al. 2011). Quite notably, AIP displayed robust expression in periventricular zones in all imaged brain regions. Since periventricular localization is a common feature of adult progenitor cells, brain images were further evaluated to determine if AIP expression coincided with progenitor cell zones outside the immediate vicinity of ventricles. Specifically, the optic tectum, the inner cellular layer of olfactory bulbs, the periventricular pretectal nucleus, and the mammillary bodies were examined. Without exception, AIP-positive cells resided within progenitor cell zones. This observation suggests AIP may play a role in adult neurogenesis in the zebrafish brain. However, more in-depth imaging studies utilizing colocalization with progenitor cell-specific markers are necessary to support this assertion.

Comparison of Constitutive AIP Expression in Zebrafish to Arc Expression in Rodents and Frogs

To date, in-depth characterization of adult basal Arc expression in nonzebrafish species has occurred in rodents and frog (*Physalaemus pustulosus*). In rodents, Arc is expressed in calcium/calmodulin-dependent protein kinase II (CamKII)-positive glutamatergic neurons in the hippocampus and neocortex (Vazdarjanova et al. 2006). Ons et al. (2004) detected constitutive Arc expression in a wide range of brain areas, including the cortex, striatum, hippocampus, reticular thalamic nucleus, cerebellar cortex, and brain stem. A similarly widespread distribution of AIP was found in the current study. However, the prevalence of AIP in periventricular areas is not shared by rodent Arc, suggesting a functional difference between the two proteins. Since CamKII expression in adult zebrafish has not been characterized, direct comparison of this characteristic with AIP localization cannot be executed.

In adult frogs, basal Arc expression was found to differ somewhat from that of rodents in that high expression was found in the forebrain, but diminished expression occurred in the midbrain, and none occurred in the hindbrain (Mangiamele et al. 2010). Notably, frog Arc was not expressed in the optic tectum, which contrasts sharply with the intense AIP expression in zebrafish optic tectum. It is therefore likely that AIP and frog Arc, like that of rodents, differ in their functional properties.

Exposure to a Novel Environment Selectively Induces AIP Expression in the Telencephalon

Novel environment-induced expression characterizes the Arc protein, so we evaluated if AIP displays stimulus-induced changes in expression by exposing adult zebrafish to a Y-maze and measuring AIP levels relative to home-tank controls. In an Arc-like fashion, our data indicate AIP increased in the telencephalon 60 min after exposure to a novel environment, but AIP remained unchanged in the optic tectum. Notably, this telencephalon-specific upregulation after an environmental stimulus is consistent with Arc homologs in rodents (Ons et al. 2004), zebra finches (Gilbert and Soderstrom 2013), and frogs (Mangiamele et al. 2010).

These data suggest AIP levels are sensitive to environmental stimuli, but AIP upregulation is region-specific. A closer examination of the subfields within telencephalon may reveal valuable insights into the function of AIP, as homologs of the vertebrate hippocampus, amygdala, and striatum have been identified in that region, and it is possible that environment-induced increases in AIP occurred in a subset of these structures. Alternately, the increase in AIP may have occurred strictly within the periventricular regions of the telencephalon. Follow-up studies may employ an imaging approach to elucidate these differences. Also, a battery of behavioral tests, such as stress-inducing protocols of varying intensities, may induce differential changes in AIP expression, and this may yield further insights into the function of AIP.

Regarding the optic tectum, few studies examining environment-induced gene expression in the central nervous system of teleost species exist that may inform speculation into the selectivity of the increase in AIP. In goldfish, a teleost species closely related to *D. rerio,* Egr1/Zif268 expression increases in the telencephalon after exposure to a spatial-learning paradigm (Rajan et al. 2011), but studies examining the optic tectum in fish have not been published. It is important to consider that AIP expression in the optic tectum is quite ubiquitous across several regions, so an increase in AIP in a subset of cells may have occurred but was undetected due to the inclusion of the entire optic tectum in the Western blot samples. It is also possible that AIP in the optic tectum is unresponsive to the Y-maze stimulus, but alternate environmental stimuli, such as altered light exposure, may induce AIP up-regulation in this brain region.

Increased Synaptic Transmission is Insufficient to Induce AIP Expression in the Telencephalon.

After determining that AIP expression can be induced by exposure to a novel environment, the mechanistic requirements for AIP upregulation were tested. We hypothesized that a true Arc ortholog would display a synaptic-transmission-induced increase in expression. We employed a standard methodology including pharmacologically-induced epileptiform brain activity to test if AIP displays this property. In agreement with Mussulini (2013) all PTZ-treated fish exhibited a robust seizure characterized by erratic burst swimming, loss of body posture, and abnormal whole-body rhythmic muscular contraction. However, it was determined that PTZ-induced seizure does not induce elevated levels of AIP in the telencephalon. These data indicate that elevated synaptic transmission is not the causative factor underlying novel-environment-induced up-regulation of the zebrafish AIP. These results were surprising, because Arc homologs in other animals, such as rodents and *Drosophila* (Mattaliano et al. 2005), share the property of seizure-induced expression. It could be that AIP is a more distantly-related homolog of Arc, and it therefore shares fewer functional properties. In *Drosophila*, for example, Arc expression may be induced by seizure, but Arc does not contribute to synaptic plasticity (Mattaliano et al. 2007). Alternately, AIP is not a zebrafish ortholog of Arc, but instead, it is another protein that displays immuno-reactivity to the Arc antibody.

Similarities Between Arc-Immunoreactive Protein and the Neuropeptide Y Receptor

The properties displayed by AIP in the current study—its ubiquitous expression, especially in proliferative cell zones, its molecular weight of 42 kDa, and its sensitivity to

a novel environmental stimulus, provide valuable criteria for speculating an alternate identity of AIP. According to these criteria, the neuropeptide Y receptor (NPY-R), with a predicted molecular weight identical to that of AIP (NCBI), is a promising candidate.

While the exact localizations of the NPY-R in zebrafish have not been published, NPY-like immunoreactivity is known to be ubiquitous (Matsuda et al. 2012). Importantly, NPY-R is required for adult neurogenesis in rodents (Decressac et al. 2011). Our experiments show AIP intensely localizes to areas associated with adult neurogenesis in zebrafish, namely structures adjacent to ventricles, such as the periventricular pretectal nucleus (Grandel et al. 2006; Kizil et al. 2011). Also, all areas shown to express AIP at high levels in zebrafish—the olfactory bulbs, telencephalon, optic tectum, cerebellum, hypothalamus (including mammillary bodies), and brain stem—have been shown to express the NPY-R in homologous structures in rats (Kopp et al. 2002). Furthermore, NPY-R shares the property of being inducible by exposure to a new environment, as expression of the NPY-R Y1 isoform increased in the basolateral amygdala of rats after environmental enrichment (Hendriksen 2012).

During our experimentation to ascertain the mechanism by which AIP is induced, a trend for a reduction in AIP levels 2 hr after PTZ-induced seizure was detected. Reduced NPY-R levels were observed by Kofler et al. (1997) after kainate-induced seizure in rats. A significant effect on AIP may have been observed in the current study if the seizure-to-dissection interval had been longer, as the study by Kofler (1997) detected a significant effect 6 hr after kainic acid-induced seizures, as opposed to a 2 hr interval in the current study.

Finally, support for the NPY-R as AIP's putative identity hinges upon immunoreactivity between the human Arc antibody and NPY-R. As shown in Table I, several regions of homology are observed between the Arc antibody epitope sequence and several zebrafish isoforms of the NPY-R, including Y1 and Y2, the most abundant isoforms detected in vertebrate brain.

The current study was completed to explore the possibility that an Arc-like protein may be expressed in the zebrafish brain. Using environmental and pharmacological stimuli, a ubiquitously-expressed Arc-immunoreactive protein was shown to be upregulated in the telencephalon after novel-environment exposure, but elevated synaptic transmission alone was insufficient to up-regulate AIP. While these findings refute the presence of an Arc-like protein in zebrafish, they reveal potentially exciting data regarding the molecular dynamics of the zebrafish telencephalon. Namely, AIP shares several properties with the neuropeptide Y receptor, a protein subject to intense investigation in the fields of adult neurogenesis, learning, and memory (reviewed in Gotzsche and Woldbye 2016), stress resilience (reviewed in Reichmann and Holzer 2016) and neuroprotection against excitotoxicity associated with epilepsy (Silva et al. 2003). Follow-up studies to confirm this possibility are severely hampered by the absence of commercially available zebrafish-specific NPY-R antibodies. Alternatively, in situ hybridization or immunoprecipitation and protein sequencing techniques could be used to validate the speculation herein.

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