Egr1 Is Necessary for Forebrain Dopaminergic Signaling during Social Behavior

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Abstract

Finding the link between behaviors and their regulatory molecular pathways is a major obstacle in treating neuropsychiatric disorders. The immediate early gene (IEG) EGR1 is implicated in the etiology of neuropsychiatric disorders, and is linked to gene pathways associated with social behavior. Despite extensive knowledge of EGR1 gene regulation at the molecular level, it remains unclear how EGR1 deficits might affect the social component of these disorders. Here, we examined the social behavior of zebrafish with a mutation in the homologous gene egr1. Mutant fish exhibited reduced social approach and orienting, whereas other sensorimotor behaviors were unaffected. On a molecular level, expression of the dopaminergic biosynthetic enzyme, tyrosine hydroxylase (TH), was reduced particularly in dopaminergic neurons of the preoptic area (POA) synaptically connected to neurons already identified within a social circuit. Our study provides evidence of a role for egr1 in establishing social behavior, and adds to our understanding of the underlying circuitry.

Significance Statement

Egr1 is an immediate early gene (IEG) linked to neuropsychiatric disorders, particularly those with social components. However, the role it plays in control of social behavior remains elusive. We found that zebrafish with a mutation in the egr1 gene present deficits in social approach and orienting behavior. Furthermore, egr1 mutants have reduced tyrosine hydroxylase (TH) expression, particularly in dopaminergic neurons of the preoptic area (POA) synaptically connected to neurons already identified within a social circuit. Our study provides evidence of a role for egr1 in establishing social behavior, and adds to our understanding of the underlying circuitry.
tyrosine hydroxylase (TH), was strongly decreased in TH-positive neurons of the anterior parvocellular preoptic nucleus. These neurons are connected with basal forebrain (BF) neurons associated with social behavior. Chemogenetic ablation of around 30% of TH-positive neurons in this preoptic region reduced social attraction to a similar extent as the egr1 mutation. These results demonstrate the requirement of Egr1 and dopamine signaling during social interactions, and identify novel circuitry underlying this behavior.

Key words: dopamine; preoptic area; social behavior; zebrafish

Introduction

Intraspecies interaction, or social behavior, is a widespread phenomenon among animals. It is considered to be beneficial for reasons of predator evasion, reproduction and cooperative foraging (Fernald, 2012). Brain regions, neuronal circuits, as far as they have been characterized, and even genes regulating social behavior are conserved and follow similar basic principles across vertebrates. Therefore, understanding the neuronal circuits and gene networks that underlie social behavior in animal models may provide information pertinent to disorders that compromise human social behavior.

One of the most studied genes with dysregulated expression in disorders with a social component is the immediate early gene (IEG) EGR1. In fact, this gene has been evaluated as a biomarker for certain neuropsychiatric disorders (Cattane et al., 2015; Czéh et al., 2016). EGR1 has been linked to stress-related mood disorders, schizophrenia, and major depressive disorder (Covington et al., 2010; Kimoto et al., 2014). In addition, gene expression studies and gene network modeling have identified EGR1 as part of a synaptic gene node disrupted in brains of individuals with autism spectrum disorder (ASD; Liu et al., 2016). EGR1 can perform this nodal function as it is a critical component, similar to other activity-induced transcription factors such as FOS. They regulate downstream late-response genes involved in neuronal physiology by dynamically activating a molecular response cascade consisting of a wide range of genes (Duclot and Kabbaj, 2017).

Studies in mouse models also point to a role for Egr1 in social behaviors. Social defeat stress, a common depression model, reduces Egr1 expression in the prefrontal cortex (Covington et al., 2010), confirming Egr1 as a biological marker for stress-related depressive disorder. Furthermore, antisense-mediated knock-down of Egr1 reduces male rat social interaction (Stack et al., 2010), identifying a direct contribution of Egr1 to social behavior. Conversely, viral-mediated Egr1 overexpression prevents castration-induced deficits in male rat social interactions (Dossat et al., 2017). Similarly, the optogenetic stimulation of cells in the prefrontal cortex increases Egr1 expression and induces anti-depressant effects (Covington et al., 2010).

In zebrafish, social deprivation results in down-regulation of four genes, including egr1 (Anneser et al., 2020). In addition, in cichlid fish, egr1 is specifically induced in the preoptic area (POA) as a response to recognition of social opportunity and ascension to dominance (Burmeister et al., 2005). Thus, studies in both the human patient population and vertebrate models suggest a role for Egr1 in the organization and functioning of brain circuits that support social behavior.

The Egr1 gene encodes a transcription factor with upregulatory and downregulatory effects on a multitude of target genes. Around 9000 genes, 54% of genes annotated in the Encyclopedia of DNA Elements (ENCODE) project, contain at least one Egr1 binding motif in their promoter region (Duclot and Kabbaj, 2017). Despite this widespread potential to bind to promoters, Egr1 shows a bias toward genes involved in regulating synaptic function and plasticity (Duclot and Kabbaj, 2017). For example, Egr1 is recruited to the PSD-95 promoter in response to NMDA receptor activation (Qin et al., 2015). Also, the presynaptic plasticity gene Synapsin1 has long been known to be regulated by Egr1 (Thiel et al., 1994). In addition to regulating these synaptic proteins, Egr1 binds to the gene encoding tyrosine hydroxylase (TH), a biosynthetic enzyme for catecholaminergic neurotransmitters (NTs), often involved in response to emotional stress (Papanikolaou and Sabban, 2000; Stefano et al., 2006). Further, the expression of th and egr1 are tightly correlated in response to sensory deprivation in zebrafish (Kress and Wullimann, 2012), suggesting that Egr1 modulates expression of TH-synthesized NTs in an activity-dependent manner. Nevertheless, it remains unclear whether Egr1 regulates gene expression in specific neural circuits that contribute to social behavior.

Development of robust social behavior was recently described in the zebrafish (Dreosti et al., 2015; Larsch and Baier, 2018; Stednitz and Washbourne, 2020) and a region of the basal forebrain (BF) necessary for this behavior was identified (Stednitz et al., 2018). Here, we examine...
whether egr1 plays a role in regulating social behavior in this system using established assays, including a high-throughput virtual biological motion assay (Larsch and Baier, 2018). We find that loss of egr1 function compromises zebrafish social behavior, but not other behaviors. BF th expression is also reduced in egr1 mutants, with a ~30% reduction in TH-positive anterior parvocellular preoptic nucleus (PPa) neurons. Chemogenetic ablation of about one third of the identified PPa neurons resulted in social behavior deficits, similar to the reduction observed in egr1 mutants. Our results suggest that egr1 is required for expression of th in a BF population of dopaminergic neurons necessary for normal social interactions, thereby expanding our understanding of the precise circuitry for social behavior.

Materials and Methods

Zebrafish husbandry

All zebrafish embryos, larvae, and adults were raised and maintained at 28.5°C according to standard protocols with a 14/10 h light/dark cycle (Westerfield, 2007). Lines used were AB/Tübingen, egr1sa64, Et y321 [Et (rex2-scp1:gal4ffly321), Et y405 [Et(scp1:gal4ffly405], and c264Tg(14xuas:nfsB-mCherry). The egr1sa64 line, which was generated by ENU mutagenesis during the Sanger Zebrafish Mutation Project (ZMP), was outcrossed to AB/Tübingen for 7 generations before the analysis described. Genotyping was performed for the egr1sa64 allele using a Competitive Allele-Specific PCR SNP genotyping system (KASP, LGC Biosearch Technologies) according to manufacturer’s protocols after behavioral assays and before processing for Western blotting and immunolabeling. Animals with unknown genotypes were excluded from experiments and analysis.

Behavioral assays

Split dyad analysis

Socially-motivated place preference and visually-evoked orienting behavior of 14 d postfertilization (dpf) zebrafish were measured using a split dyad assay (Stednitz and Washbourne, 2020). Roughly size-matched larvae from an incross of egr1sa64 heterozygotes (hets) were placed in paired, isolated tanks (50 mm in length × 20 mm in width × 20 mm in depth) separated by an opaque divider and allowed to habituate for 5 min, then the divider was removed and the animals allowed to interact for an additional 5 min. Both the presocial and social stimulus periods were recorded to determine the baseline exploratory and locomotor behavior. Recordings were obtained from below at 10 fps using a Mightex SME-B050-U camera and illuminated by an overhead white LED panel (Environmental Lights). For numbers of animals and their genotypes see Table 1.

Biological motion social assays

To explore social engagement, we tested social approach using a virtual social assay that is based on detection of biological motion (Larsch and Baier, 2018). The stimuli, which are dots projected below dishes of solitary animals, are followed when they move in bouts with kinetics similar to age matched animals. These assays were performed as described by Larsch and Baier (Larsch and Baier, 2018). Briefly, larvae from an incross of egr1sa64 hets were placed individually in watch glasses filled with facility water. The watch glasses were located in a shallow aquarium on translucent sheets, with a cold mirror and IR LEDs below. A projector, located to the side, projected images onto the translucent sheets by way of the cold mirror. A digital camera with an IR filter was located above the watch glasses. Projector and camera were controlled by bespoke software in Bonsai. Dots of varying sizes were projected intermittently, moving in a synthetic knot shape for a 5-min period. Fish were maintained for 2–3 h in the watch glasses. At each frame, animal and stimulus parameters were streamed to a text file for offline analysis. Fish were tested in facility water at room temperature (25°C). For numbers of animals and their genotypes see Table 1. The numbers for the thigmotaxis and speed analyses are the same as for virtual social at 14 dpf.

Chemogenetic ablation experiments

Fourteen days postfertilization larvae from a cross between [c264Tg(14xuas:nfsB-mCherry)] and [Et(scp1:gal4ffly405)] fish were incubated in the dark for two nights in static facility water with 2.5 μm nifurpirinol (Furanol, JBL), a nitroaromatic antibiotic that is converted into a toxic metabolite by the enzyme nitroreductase. Water flow was restored and larvae were fed during the day. As a control, sibling larvae only expressing GFP, and not the nitroreductase transgene, under the influence of the Et y405 enhancer trap, were analyzed in parallel. Water flow was restored for a minimum of 1 h before biological motion social assays. A total of 60 animals were tested from two experiments with 28 animals expressing GFP only and 32 animals expressing both GFP and nitroreductase.

Predator avoidance tests

Predator avoidance was assessed by measuring visually induced escape responses on presentation of looming stimuli as described previously (Fernandes et al., 2021). Briefly, expanding black dots were projected from below to free-swimming animals using custom scripts in Bonsai to control OpenGL drawing routines. Dots were positioned 10 mm left or right with respect to the center of mass and orientation of each animal at the onset of dot expansion. Dots expanded for 500 ms (15 frames) with a linear increase in diameter to their final size ranging from 0 to 12 mm. Looming stimuli of different sizes were presented left or right of the animals once per minute in random order. A moving grating was presented for 20 s

| Test                        | Age (dpf) | Total | +/- | +/-- | –/-- |
|-----------------------------|-----------|-------|-----|------|------|
| Split dyad                  | 14        | 56    | 9   | 36   | 11   |
| Virtual social              | 14        | 75    | 27  | 36   | 12   |
| Virtual social (pooled)     | 14/16     | 201   | 172 | 29   |
| Predator avoidance          | 14        | 126   | 28  | 77   | 21   |
Table 2: Antibody resources for Western blotting

| Target    | Manufacturer         | Catalog #, clone | Dilution | Species | Immunogen                                                                 | RRID     |
|-----------|----------------------|------------------|----------|---------|---------------------------------------------------------------------------|----------|
| Syn1/2    | Synaptic Systems     | 106002           | 1:1000   | Rb      | Rat peptide, aa 2-28                                                     | AB_2619773|
| TH        | Millipore Sigma      | AB152            | 1:5000   | Rb      | Denatured purified protein from rat                                       | AB_390204|
| β-Actin   | Santa Cruz           | Sc-47778         | 1:1000   | Ms      | Purified chicken protein                                                  | AB_2714189|
| pan MAGUK | NeuroMab             | K28/86           | 1:1000   | Ms      | Human PSD-95 fusion protein (77–289)                                     | AB_2877192|

Table 3: Antibody resources for immunolabeling

| Target    | Manufacturer         | Catalog #, clone | Dilution | Species | Immunogen                                                                 | RRID     |
|-----------|----------------------|------------------|----------|---------|---------------------------------------------------------------------------|----------|
| Syn1/2    | Synaptic Systems     | 106002           | 1:250    | Rb      | Rat peptide, aa 2-28                                                     | AB_2619773|
| Gephyrin  | Abcam                | Ab32206          | 1:1000   | Rb      | Ms Peptide, aa 700-C-term                                                | AB_2112628|
| TH        | Millipore Sigma      | AB152            | 1:500    | Rb      | Denatured purified protein from rat                                       | AB_390204|
| GFP       | Invitrogen           | 3E6              | 1:500    | Ms      | Recombinant GFP                                                           | AB_2313858|

Rb, rabbit; Ms, mouse.

ending 10 s before the presentation of the next loom stimulus to drive larvae toward the center of a dish. At each frame, animal and stimulus parameters were streamed to a text file for offline analysis. Fish were tested at 14 dpf in facility water at room temperature (25°C) for a total of 1 h. For numbers of animals and their genotypes see Table 1.

In situ hybridization (ISH)

RNA ISH was conducted on brain cryostat sections (16 μm) according to standard protocols (Westerfield, 2007) and using the manufacturer recommended protocols for RNAscope (ACD).

Western blotting

Heads were removed by cutting across the trunk at the level of the heart of anesthetized 15 dpf larvae. Heads were homogenized in RIPA buffer, with the protease inhibitors TLCK, PMSF, and pepstatin. Homogenates were incubated under constant motion at 4°C for 1 h before centrifuging at 12,300 g for 20 min at 4°C. The supernatant was removed and the pellet was resuspended in Laemmli sample buffer with 5% β-mercaptoethanol. Samples were heated at 95°C for 5 min, electrophoresed and blotted to nitrocellulose membranes under standard conditions. Antibodies used are listed in Table 2. Secondary antibodies made in donkey and conjugated to IR dyes 680RD and 800CW (LiCor) were revealed with a LiCor Odyssey.

Immunolabeling

Immunolabeling of synaptic markers was performed on brain cryostat sections (16 μm) according to standard protocols (Westerfield, 2007). Antibodies used were to Synapsin 1/2 and Gephyrin (Table 3). Immunolabeling of TH and GFP (Table 3) were performed on paraformaldehyde fixed, dissected heads with jaws and dura removed. Permeabilization, blocking and antibody incubations were performed as described previously (Goode et al., 2020). Primary antibodies were revealed using the following secondary antibodies at a concentration of 1:750: Alexa Fluor goat anti-mouse and rabbit IgG1, IgG2a, and (H+L), ThermoFisher Scientific, coupled to 488, 546, or 633. Images were taken on a Leica SP8 confocal microscope with LasX software with 10×, 20×, or 63× oil immersion objectives.

Quantification and statistics

Size comparisons

Animals were considered “stunted” when they were shorter than a threshold set at 1.5× the SD below average wild-type (wt) size, measured from tip of nose to the end of the trunk (excluding the tail fin). We measured and genotyped 30–32 randomly selected individuals from 8 clutches for the analysis in Figure 1.

Split dyad analysis

The percentage of time in motion was calculated as the number of frames in which the animal moved at least one-third of their total body length per frame. Larvae that spent <10% of the experiment in motion were excluded from subsequent analyses. Larvae were genotyped following the experiment as previously described. In separate analyses, data for wt and het animals was pooled and the genotype of the respective stimulus fish was used as the classifier (Fig. 2E,F). Social interaction is parameterized as the average relative distance from the divider (relative place preference) and the percentage of time spent at 45–90° (% time orienting) using previously described software written in Python (Stednitz et al., 2018). Place preference was calculated as the average relative proximity to the divider across all frames. Behavior was compared statistically using a mixed-model repeated measures ANOVA (mmANOVA) in Python, using genotype as the between-subjects factor and time (presocial and during social stimulus) for each metric as the within-subjects factor.

Biological motion social assay and predator avoidance test analysis

Background subtracted, inverted and thresholded avi files were analyzed using scripts in Jupyter as described.
previously (Larsch and Baier, 2018). The social index (SI) quantifies attraction of the animal toward the biological motion dot stimulus. SI relates the observed animal-dot distance relative to a predicted distance that would result purely from chance encounters with the stimulus. SI was quantified as previously described (Larsch and Baier, 2018) using python scripts: the "real" observed animal-dot distance was calculated for each animal as an average in 5-min chunks (IADr). Next, we shifted the animal trajectories in time relative to the stimulus trajectory by 10 different offsets, 60 s and re-calculated a "shifted" animal-dot distance (IADs) for each time shift. Because of the time shift, IADs reflects the dot-animals distance expected by chance encounters. Mean IADs (mIADs) for all time shifts was used to compute SI as (mIADs-IADr)/mIADs. Significance was determined using JMP software. A Tukey–Kramer HSD for pairwise comparisons of least square means was applied to the three genotypes with a \( p \) threshold of 0.05. Data from within the biological motion social assay was also analyzed for motion and thigmotaxis, as described previously (Larsch and Baier, 2018), and significance was determined using HSD.

**Western blotting**

Images of Western blottings were analyzed using LiCor Image Studio software. We only used intensity data for bands that were within the linear range, i.e., not saturated. Total intensity of bands was divided by the total intensity of in-lane \( \beta \)-actin bands. Resulting intensity ratios were then normalized to the mean of wt larvae. A minimum of two technical replicates were analyzed for four biological replicates. Pools of homogenized heads were of a size 8 clutches, \( n = 248 \) animals.

![Figure 1](image). *egr1* is expressed in the forebrain. **A**, ISH reveals *egr1* expression (blue) in the basal forebrain (BF), optic tectum (OT), hypothalamus (Hyp), and epithelial cells of the pharyngeal pouch (PP) at 6 dpf. *myogenin d* was used as a control probe (red). Anterior to the left. Scale bar: 100 \( \mu \)m. **B**, Magnification of the BF (white box in A) showing expression surrounding the anterior commissure (AC). **C**, Exon organization of the *egr1* mRNA, location of the zinc finger domains (zf), and location of the sa64 mutation (red bar). **D**, *egr1* sa64 mutants (–/–) develop a normal body plan with functional swim bladder, but with size deficits (bottom). **E**, Close to 80% of mutant larvae are stunted (dark gray). **F**, More than 50% of stunted larvae are also wt (+/+, white) and het animals (+/–, light gray). \( N = 8 \) clutches, \( n = 248 \) animals.
minimum of ten heads of each genotype. Significance was determined using JMP software. HSD for pairwise comparisons of least square means was applied to the three genotypes with a $p$ threshold of 0.05.

**Synaptic puncta**

Cropped sections of 63/C2 images were analyzed in ImageJ using the PunctaAnalyzer plug-in for a region of 77 $\mu$m². A minimum of eight images for each genotype was quantified. HSD was applied to the analysis of puncta numbers with a $p$ threshold of 0.05.

**Results**

**Egr1 influences overall growth but not development**

To identify a role for the egr1 gene in social behavior, we first examined the expression pattern of egr1 in developing zebrafish larvae by RNAscope ISH at 6 dpf. This developmental period, during which synaptic circuitry remains dynamic (Goode et al., 2020), occurs one week before the appearance of robust social behavior (Dreosti et al., 2015; Larsch and Baier, 2018; Stednitz and Washbourne, 2020).

Egr1, in addition to being expressed in the pharyngeal pouch (PP; Fig. 1A), is predominantly expressed in a few clusters in the forebrain and midbrain. Given previous evidence for telencephalic contribution to social behavior (Stednitz et al., 2018), we noticed the prominent egr1 expression in the telencephalon, including the BF, in cells adjoining the anterior commissure (AC; Fig. 1B). We conclude that egr1 is expressed in a region that contributes to social behavior at a time when neuronal circuits are being established.

We obtained a mutant from the Sanger ZMP with a point mutation in the egr1 gene (sa64). The C > A mutation generates a premature termination codon in exon 2, likely resulting in a truncated 137-aa protein. As this mutation is located upstream of the functional zinc finger DNA binding motifs (Fig. 1C), we predict that it results in loss of protein function. To eliminate possible additional background mutations, we used egr1sa64 hets that had been outcrossed to wt zebrafish for seven generations.

Larvae derived from an incross of egr1sa64 hets develop normally (Fig. 1D) and homozygous mutants were present at an approximate Mendelian ratio at 15 dpf (27.7 ± 13.6% SD; $N = 4$ clutches, $n = 201$ animals), a developmental time at which social behavior is already robust (Stednitz and Washbourne, 2020). However, we noticed a large proportion of larvae that were much smaller ($\mu$m minus 1.5/SD) than others, which we will call “stunted.” Mutants were significantly smaller than wt and hets on average ($p = 0.002, p = 0.006$), although stunted individuals were found across all genotypes and reached other developmental milestones with their siblings (Fig. 1D, bottom). For example, stunted larvae inflated their swim bladders by the same day (5 dpf) as all other animals, including homozygous egr1sa64 mutants. Although the majority of homozygous egr1 mutants were stunted (78 ± 12.9% SE across 8 clutches; Fig. 1E), almost half of...
the stunted larvae occurred in het and wt larvae (Fig. 1F). This phenotype is similar to the stunted growth reported in the mouse Egr1 gene knock-out mutant (Topilko et al., 1998). Analysis of egr1 using morpholine-modified oligonucleotides (MO) in zebrafish also resulted in reduced growth in morphants (Zhang et al., 2013). Therefore, despite the incomplete penetrance in our system, we conclude that egr1 contributes to size of the animal. Because of the relatively high occurrence of the stunted phenotype in hets, we conclude that this phenotype may be because of a gene interaction with an, as yet, undetermined other gene. We also conclude that egr1sa64 is likely to be a null allele, although we cannot exclude the possibility that it is a hypomorph. Importantly, we considered size as a factor for all additional experiments.

**Egr1 is necessary for social behavior**

To examine social behavior in egr1sa64 mutants of regular-sized and stunted animals, we used a split dyad assay with size-matched animals (Fig. 2A; Stednitz et al., 2018). Social behavior becomes robust at 14 dpf and is visually driven, with animals engaging in stereotypical orienting and approach behavior when they can see an animal of similar size (Dreosti et al., 2015; Stednitz and Washbourne, 2020). egr1 mutants spent less time orienting at angles to the divider between 45° and 90° ($\rho = 0.003$; Fig. 2B), a strong indicator of social engagement (Stednitz et al., 2018), and less time close to the divider (Fig. 2C). Mutants were not less active during the assay period ($\rho = 0.131$; Fig. 2D). We conclude that Egr1 is involved in modulating social behavior.

Consistent with our previous findings on active reciprocity during social interactions in adult and larval zebrafish, wt and heterozygous fish paired with mutants also exhibited reduced social orienting ($\rho = 0.003$; Fig. 2E) and place preference ($\rho < 0.001$; Fig. 2F). Using multiple linear regression, we found that both the genotype of the target fish and the stimulus fish significantly influences orienting behavior ($\rho = 0.021$ and $\rho = 0.040$, respectively). Therefore, the deficit in social behavior of egr1 mutants is detected by wt and heterozygous siblings. However, we also conclude that this social assay provides a variable social stimulus for test larvae that might lead to spurious results, especially with the added size effect.

To explore social engagement without stimulus variability, we tested whether a social deficit could also be detected using a virtual social assay that is based on detection of biological motion (Larsch and Baier, 2018). This assay reduces variability, as the stimulus is identical for each larva. Dots projected below dishes of solitary animals are followed preferentially when the dots move in approximate body size at 4.0 mm for 14 dpf larvae (Fig. 3A). Using multiple linear regression, we found that both the genotype of the target fish and the stimulus fish significantly influence orienting ($\rho = 0.37$, $R^2 = 0.14$, $\rho = 0.0011$; Fig. 3G). This effect was consistent across individual genotypes and at all ages examined. We further explored the influence of size by comparing the SI of animals in groups of similar size. Consistent with previous observations (Larsch and Baier, 2018), larger wt and heterozygote animals have a 67% higher SI than wt stunted animals ($\rho = 0.186$, HSD, $n = 9$ stunted animals; Fig. 3H, +). However, mutants exhibited reduced social behavior both for regular-sized and stunted individuals ($\rho = 0.0001$, HSD; Fig. 3H, −/−). We conclude that genotype, rather than size, is the primary factor influencing social behavior deficits in egr1 mutants.

**Egr1 is necessary for TH2 expression**

The Egr1 transcription factor regulates expression of proteins that play a role in synaptic transmission and NT synthesis (Duclot and Kabbaj, 2017). We used Western blotting of protein extracts from 15 dpf larval heads to explore whether Egr1 promotes social behavior by influencing abundance of such proteins in the brain. Levels of MAGUK proteins (panMAGUK), such as PSD-95, SAP102 and SAP97, and Synapsin proteins 1 and 2 (Synapsin 1/2) were not significantly influenced by egr1 loss of function ($\rho > 0.8$, HSD; Fig. 4A–C). In contrast, an antibody that recognizes two forms of TH, which based on predicted molecular weights likely correspond to TH1 and TH2, revealed that levels of TH2 but not TH1 are compromised by mutation in egr1 (TH1: $\rho = 0.9$, TH2: $\rho = 0.0485$, HSD; Fig. 4A, A–D, E).

Given the bias of Egr1 toward regulating synaptic genes (Duclot and Kabbaj, 2017), we assessed whether Egr1 influences forebrain synapse abundance. We quantified synapses by immunolabeling sections of 15 dpf larval brains with antibodies to Synapsin 1/2 and Gephyrin in egr1sa64 mutants (Fig. 4F). Synapsins are enriched at over 95% of vertebrate synapses (Micheva et al., 2010), where as Gephyrin is a specific marker of inhibitory synapses (Fritschy et al., 2008). Within the BF, a region with high expression of egr1 (Fig. 1A, B) and a region implicated in controlling social behavior (Stednitz et al., 2018), we focused on the neuropil of the AC. Quantification of the number of puncta, an approximation of synapses, in the medial and lateral AC neuropil showed no difference in March/April 2022, 9(2) ENEURO.0035-22.2022 eNeuro.org
total (Synapsin 1/2, \( p = 0.71, \text{HSD} \)) or inhibitory (Gephyrin, \( p = 0.99, \text{HSD} \)) puncta density in \( egr1 \) mutants compared with wt (Fig. 4G). The lack of effect on synapse number is consistent with the Western blotting results above, which demonstrated that levels of the widespread synapse markers Synapsin 1/2 and PSD-95 are unchanged in \( egr1 \) mutants. However, our results do not preclude the possibility that specific synapse populations might be regulated by Egr1. We conclude that Egr1 regulates the abundance of TH2 protein, but does not regulate presynaptic or postsynaptic protein abundance of Synapsin 1/2 or MAGUK proteins, nor general synapse density.
A synaptic connection between TH-positive neurons and cholinergic BF neurons (cBFNs) defined by the enhancer trap line \( \text{Et}^{y321} \) \([\text{Et}^{\text{trex2-scp1:gal4ff}}]^{y321} \) was recently identified (Goode et al., 2020). Chemogenetic ablation of cBFNs disrupts social attraction and orienting behavior (Stednitz et al., 2018). Images from the most basal region of the forebrain (Fig. 5E), including the anterior part of the presumptive PPa, reveal that the TH-positive neurons project to the AC neuropil (Fig. 5A–A'), which is also innervated by cBFN arbors (Goode et al., 2020). TH-positive neurons in the PPa are highly likely to be dopaminergic (Yamamoto et al., 2011), so we will refer to these cells as dPPaNs. Cells in this region have been documented to express both \( \text{th1} \) and \( \text{th2} \) genes in larvae and adults (Semenova et al., 2014).

We explored whether TH expression in dPPaNs requires Egr1 (Fig. 5B–E). The number of TH-positive dPPaNs was significantly reduced in homozygous \( \text{egr1}^{sa64} \) mutants relative to wt siblings at 14 dpf \( (p = 0.049, \text{HSD}; \text{Fig. 5F}) \). The ~28% reduction of TH-positive dPPaNs in mutants was evident as early as 7 dpf (Fig. 5G), a time at which stunted individuals were not detected. Interestingly, we found an intermediate, but not significant, phenotype in TH cell number in heterozygous \( \text{egr1}^{sa64} \) mutants compared with wt at both time points \( (p = 0.362, p = 0.099, \text{HSD}) \), analogous to results from the Western blot experiments (Fig. 4E). This may indicate a haplo-insufficiency at the molecular level, although this is not detected at the behavioral level. Together, our results suggest that the discrepancy in TH expressing cells is established early in development, consistent with the expression of \( \text{egr1} \) in the BF at this developmental stage (Fig. 1B). Given the expression of both \( \text{th1} \) and \( \text{th2} \) in dPPaNs (Semenova et al., 2014), we conclude that our result is because of a decrease in the actual number of dPPaNs, and not because of a detection limit as a consequence of a \( \text{th2} \) expression decrease. Our experiments suggest that

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**Figure 4.** Egr1 is necessary for TH2 expression. A, Western blotting of larval head homogenates at 14 dpf reveals a reduction in expression of TH2 in \( \text{egr1} \) mutants. A', Two color labeling of a Western blot to highlight in-lane normalization to \( \beta \)-actin and technical replicates. B–E, Western blot intensities in A, A' were first normalized to in-lane \( \beta \)-actin, and then to wt average intensity. \( N = 4 \) experiments, \( n = 2 \) technical replicates, \( p < 0.05 \), HSD. F, Immunolabeling of synapses with antibodies to Synapsin 1 and 2 (Syn1/2, magenta) and Gephyrin (Geph, green) in the anterior commissure (AC), lateral view. Insets are magnifications of the cyan box. Scale bar: 5 μm. G, Quantification of synaptic puncta revealed no changes in synapse density in the medial and lateral AC. \( n \geq 5 \) brains per genotype. wt (+/+, white), het (+/−, light gray), mut (−/−, dark gray). Error bars are SEM.
catecholaminergic, presumably dopaminergic, signaling in this part of the larval brain is compromised by loss of Egr1, although it is possible that feedback mechanisms might compensate for the NT deficit.

**BF TH neurons are necessary for social behavior**

To gain genetic access to the TH-positive neuron population and test a possible role for these neurons in social behavior, we examined driver lines that express in the BF. The Tg(galanin:GFP) line expresses in neurons in the PPa, and the neuropeptide galanin has been implicated in various mammalian social behaviors (Wu et al., 2014). Labeling of galanin:GFP transgenic animals with TH antibody revealed that galanin-expressing and TH-positive neurons are distinct populations (Fig. 6A). The enhancer trap line Et y405[Etscp1:gal4ff)y405], in which the construct integrated in the robo2 gene, also drives expression in the PPa. In contrast to the galanin driver, 30% of neurons labeled by the Et y405 driver overlapped with TH-positive dPPaNs (29.2 ± 5.1%, N = 3 brains; Fig. 6B).

We hypothesized that chemogenetic ablation of neurons marked by Et y405 would reduce dPPaNs similar to egr1 loss of function, allowing us to test whether a reduction of dPPaNs might be the cause of reduced social behavior in egr1 mutants. We treated larvae generated by a cross between the Et y405 driver and a nitroreductase expression line with the antibiotic nifurpirinol. The enzymatic activity of nitroreductase on nifurpirinol generates a toxic by-product that kills cells in a cell-autonomous fashion. Indeed, treatment of animals with nifurpirinol resulted in a 31% reduction in dPPaNs when the nitroreductase transgene was expressed (GFP control: 82 neurons ± 2.5, nitroreductase: 56.2 ± 5, n = 11 brains, p < 0.005, t test). Biological motion-based social behavior was significantly reduced in larvae with ablated dPPaNs compared with control animals at dot sizes 2.0, 4.0, 8.0, and 16.0.
Chemogenetic ablation of random neuronal populations in larvae does not always cause a deficit in social behavior (Stednitz et al., 2018), suggesting that the social behavior deficit is specific to the ablated population and not because of general neurologic trauma. We cannot exclude that ablation of neurons in other regions of the brain within the \( \text{Et}^{y405} \) line might contribute to the social deficit, as the \( \text{Et}^{y405} \) enhancer trap drives sparse expression throughout the brain at 16 dpf (Fig. 7). However, our results are consistent with the conclusion that dPPaNs are necessary for robust social behavior.

### Discussion

In this study, we examined the contribution of \( egr1 \), a gene implicated in the etiology of neuropsychiatric disorders, to the establishment of social behavior in zebrafish. Egr1 regulates social behavior as measured by two different assays: a split dyad assay (Fig. 2) and a biological motion-based assay (Fig. 3). Investigation of downstream proteins that might be affected by a loss of this transcription factor revealed a decrease in TH2 protein, but no differences in other known targets of Egr1 (Fig. 4). We examined a population of TH-positive neurons in the PPa that are in synaptic contact with identified “social” neurons of the BF and discovered a reduction in the number of the TH-positive neurons in \( egr1 \) mutants (Fig. 5). Chemogenetic ablation of a similar number of TH-positive neurons in the PPa resulted in a comparable reduction in social behavior (Fig. 6).

Our findings using \( egr1^{sa64} \) suggest two important conclusions: (1) Egr1 is implicated in regulating social behavior, and (2) dPPaNs represent a novel population of neurons implicated in driving social behavior. We discuss these findings further below.

First, the behavioral deficit in \( egr1 \) mutants appears to be relatively specific to social behavior, as it does not affect vision or general motility. This specificity may lie in the \( egr1 \) expression pattern. From the first day of development, \( egr1 \) is specifically expressed in the ventral telencephalon and diencephalon (Close et al., 2002). This expression is puzzling for an IEG, as it appears long before one would predict high levels of neuronal activity. In fact, activity in the BF social circuit presumably starts during the second week of development, as this is when social behavior becomes apparent (Stednitz and Washbourne, 2020). Interestingly, Egr1 has been described to act as a developmental transcription factor in some tissues, for example during development of skeletal muscle (Zhang et al., 2018). Therefore, it is possible that a primary role of Egr1 in the BF is activity-independent regulation of TH expression in dPPaNs. In the...
absence of egr1 expression, dPPaNs presumably synthesize and release less dopamine, reducing critical input to cBFNs and a putative social network in the BF, resulting in aberrant social behavior. Our finding is reminiscent of the loss of another IEG, FosB, in mammals. Most behaviors are largely the same in FosB knock-out mice, suggesting no ongoing deficit in neuronal function across most of the brain. However, nurturing of neonates is almost absent in knock-out dams, which was explained by deficient signaling in the POA (Brown et al., 1996). This may suggest an intriguing role for IEGs in establishing circuitry and/or cell type during development, and in the cases of Egr1 and FosB, particularly within social brain networks.

Indeed, the effect we describe in zebrafish induced by the egr1 mutation is consistent with the identification of EGR1 as a susceptibility gene for neuropsychiatric disorders including schizophrenia and depression (Covington et al., 2010; Kimoto et al., 2014; Cattane et al., 2015). Our results also bolster the idea that EGR1 is a hub for other genes involved in synaptic transmission that are affected in ASD (Liu et al., 2016). Although EGR1 levels are altered in various disorders including schizophrenia (Yamada et al., 2007), it is important to note that mutations in the human population have not yet been identified which directly link EGR1 and these disorders. It is possible that in humans EGR1 mutations are severe or lethal, and that only other mutations or environmental influences that modulate EGR1 activity or expression levels result in neuropsychiatric symptoms. This postulated exclusive mode of EGR1 modulation via other effectors may be unique to humans, as the gene has been implicated in a human-only network of genes aberrant in ASD cases (Liu et al., 2016).

Second, our study of the molecular mechanisms underlying the social phenotype of egr1 mutants implicates a population of neurons, dPPaNs, and their connections in driving social behavior. The PPa region lies within the POA, an integral part of the conserved vertebrate social behavior network (O’Connell and Hofmann, 2012). Consistent with this categorization, the PPa was recently implicated in zebrafish social behavior. Developmental oxytocin-cell ablation, which reduces social behavior, eliminates activation of the PPa by social stimuli (Nunes et al., 2021). Our results now define a precise population of cells within the PPa that

**Figure 7.** Whole-brain expression of Et^y405 at 16 dpf. Maximum Z projection of Et^y405 (y405) brain expressing GFP, with the region of interest from Figure 6B marked with a cyan box. Anterior to the top; * nonspecific labeling of meninges. Tel, telencephalon; PPa, anterior parvocellular POA; OT, optic tectum; Th, thalamus. Scale bar: 200 μm.
play a role in controlling social approach and orienting. Our previous finding that TH-positive terminals synapse on cBFN(arbors suggests that the dPPaNs identified here are presynaptic to cBFNs (Goode et al., 2020). It is yet more intriguing to consider that these terminals increase their Synaptotagmin 2a protein levels when social behavior becomes robust at 14 dpf (Goode et al., 2020), implicating a developmental gain of function in the dPPaNs as the trigger that "turns on" social behavior. This insight now ties two concrete neuronal populations together as a first step in elucidating a social circuit at molecular and cellular resolution.

Finally, based on anatomic marker studies of the developing zebrafish brain, the TH-positive PPaNs are most probably dopaminergic (Yamamoto et al., 2011; Gunaydin and Deisseroth, 2014). The putative dopaminergic identity of dPPaNs raises interesting possibilities, as the dopamine system is affected in neuropsychiatric disorders with social components, such as ASD (Hettinger et al., 2008). Dopamine is generally considered to be part of a reward system; therefore, dPPaNs could be part of a reward circuit for social motivation (Gunaydin and Deisseroth, 2014). Alternatively, dPPaNs could also modulate social cognition (Gunaydin and Deisseroth, 2014). Future studies to discriminate between these two options will be critical for understanding the circuits and mechanisms underlying social approach and orienting behavior. Although further transcriptomic characterization will help to define the molecular identity of dPPaNs, our findings already inform potential pharmacological intervention strategies for the social component of neuropsychiatric disorders.

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