Brain activation patterns following a cooperation opportunity in a highly social cichlid fish

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ABSTRACT

In highly social species, individuals frequently face opportunities to cooperate. The molecular and neural mechanisms that integrate internal and external information prior to cooperative responses are not well understood. Using expression levels of *egr-1*, a genomic marker of neural activity, we quantified the neural response to an alloparental-care opportunity in a cooperatively breeding fish, a component of cooperative behaviour, across brain regions and time. In this species, alloparental care and submission are considered alternative strategies to appease dominants. We therefore investigated whether brood care and defence as well as submissive displays were associated with *egr-1* expression. Finally, we predicted potential targets of the *egr-1* transcription factor in the cichlid genome. This target prediction suggested that *egr-1* regulates the expression of transcription factors involved in nervous system development, which could be implicated in social memory formation associated with cooperation. *Egr-1* expression levels differed between test and control individuals and across time. Compared to a control, individuals experiencing the cooperation opportunity expressed less *egr-1* in two brain regions, the cerebellum and the telencephalon. This down-regulation was independent of their behavioural reaction, i.e. whether they cooperated or not. However, within the subset of test individuals, *egr-1* expression increased as a function of the amount of submissive behaviours, but not of cooperative behaviours, in the hypothalamus and potentially the telencephalon. These regions host structures that play a role in social decision-making; suggesting that *egr-1* might be a suitable proxy for neural activation due to the social interaction component of the cooperation opportunity, rather than the actual alloparental care component.

1. Introduction

Cooperation is widespread in the animal kingdom, and has evolved several times independently [1]. Research on cooperation mainly centres around questions of its adaptive function [2], whereas the underlying proximate mechanisms of cooperation are hardly understood. Cooperative opportunities, that is, situations requiring a decision whether or not to cooperate, share features of other social interactions, such as the high levels of unpredictability that are inherent to interactions with conspecifics [3]. However, cooperative opportunities also have specific characteristics, for instance a considerable delay and a potentially different currency in pay-off [4]. Thus, more cooperative individuals should possess increased social memory and temporal discounting abilities [5]. While cooperation always occurs in a social context, the particular cooperative behaviours may involve direct social

interactions (e.g. cooperative hunting, [6]) or not (e.g. alloparental care for eggs, [7]). While the telencephalon has been identified as an important hub for modulating social behaviour [8], it is, as of yet, unknown whether these same neural mechanisms underlie decisions to cooperate or not when given the opportunity. First supportive evidence stems from studies reporting neuromolecular correlates of cooperative territory defence in several nuclei of the telencephalon of the cichlid *Astatotilapia burtoni* (Chelsea A [9, 10]). Investigating the molecular and neural responses to cooperative opportunities could substantially contribute to improving our mechanistic understanding of cooperative behaviour [11–14].

One particular kind of cooperative behaviour, helping in the form of alloparental brood care, is shown by subordinates of cooperative breeders (e.g. [15]). The neural response to this particular kind of cooperation opportunity might consist of several components that require

the concerted action of more than one brain region. First, relevant social stimuli, such as features of the social group, dominance relationships or demand of help by dominant individuals, are likely to be perceived and processed in the telencephalon and hypothalamus. Second, the sensory cues obtained from the clutch or dependent young is probably processed in the optic tectum and olfactory bulbs. Third, parental care is regulated by sex-steroid sensitive nuclei in the hypothalamus [16, 17]. Fourth, the sensory input and the spatial motor component of the behaviour is coordinated in the cerebellum [18, 19].

Quantifying the expression level of immediate-early genes (IEGs) in specific brain regions and at a specific time point is often used as a marker of neural activity [20]. One of those IEGs is *early growth response 1* (*egr-1*), which has been established as a pertinent marker of neural activation in fish [21, 22], particularly with regards to cichlids [23, 24]. *Egr-1* codes for a transcription factor that is implicated, among other things, in pathways leading to neural plasticity, long-term potentiation, memory formation and learning [25]. *Egr-1* plays a role in mediating short-term signal transduction cascades [26]. Due to the evolutionary conservation of its sequence and in particular the amino-acid sequence of the DNA-binding domain in vertebrates, *egr-1* is also suitable for measuring neural activation in non-model species, including fishes [27]. Most importantly for this study, *egr-1* has been used as a marker for neural activity induced by social interactions [20]. For instance, e*gr-1* expression is increased in the hypothalamus of individuals that perceive the opportunity to rise in rank (African cichlid *Astatotilapia burtoni*, [28]), and chose a mate (poecilid *Xiphophorus nigrensis*, [21, 29]). *Egr-1* expression is also increased in in the telencephalon and the hypothalamus of individuals that interact aggressively with group members (zebra fish *Danio rerio*, [30]. Since cooperation principally occurs in a social context, we hypothesized that this gene might be a suitable candidate to investigate neural activation induced by a cooperation opportunity. IEG expression has been suggested to peak between 30 and 60 min following either social or pharmacological stimulation [27, 31] and a decrease of their induction is expected only after 120 min [32]. However, since *egr-1* has so far not been used as a marker of responses to cooperative opportunities, characterizing its activity across brain areas would provide novel information on neuronal activity in this particular social context. Furthermore, the time course of neural activation may differ between brain areas, requiring establishing the time structure of *egr-1* gene expression in each area separately. Investigating the spatial and temporal pattern of the *egr-1* response to a cooperation opportunity across regions of the whole brain will thus further our understanding of neural processing patterns in a social context as well as of patterns specific to cooperation.

Egr-1 is widely used as a marker for neural activation in many different species, not only in the social behaviour context, but also in learning and memory as well as cancer and inflammation research. Surprisingly little is known about its targets, and studies often infer its function based on what is known from taxonomically distant species. While the targets of *egr-1* are well defined experimentally in model systems such as humans [33] and mice [34], especially in a biomedical context, a whole-genome catalogue of genes potentially regulated by this transcription factor in most other species is lacking. Most importantly, while the DNA binding domain of the *egr-1* transcription factor is conserved across vertebrates [27], the specific DNA regions where it binds to might differ between taxa [35]. Especially in teleosts those genomic regions involved in the regulation of transcription and development (conserved noncoding elements, CNEs) have substantially diverged from the ancestral form [36]. Thus, identifying potential targets of *egr-1* in a fish species will help define its functional effect in non-mammalian systems.

Here, we aimed to quantify neural activation in a cooperative-breeding context using the immediate early gene *egr-1* as a marker. To this end we used a well-established model system for the study of cooperative behaviour, the highly social cichlid fish *Neolamprologus pulcher* [37, 38]. In this cooperatively breeding species, juveniles and smaller adults perform helping in the form of alloparental brood care or participation in defence and maintenance of the group's territory, which are considered components of cooperative behaviour because helpers delay their own reproduction, thereby incurring fitness costs [37, 38]. With regard to alloparental care, subordinate *N. pulcher* develop distinct behavioural 'helper' types during early life [39, 40]. Some individuals specialize in direct alloparental brood care, such as the tending of eggs and larvae, whereas others contribute less to direct allocare but invest strongly in submissive displays towards dominant group members. Both strategies are thought to serve as appeasement of dominants so that subordinates attract less aggression and remain accepted in the territory [41] and might be mutually exclusive in the sense that subordinates invest either in direct egg care or in submissive displays, but rarely in both. In order to investigate the broad-scale spatial pattern of neural activation during a helping opportunity across the whole brain, our study pursued three aims. First, we aimed to identify which major brain regions (telencephalon, optic tectum, hypothalamus and cerebellum) are involved in the processing of stimuli that lead to the expression of alloparental care ('helping') or submissive behaviours using the quantification of *egr-1* gene expression in individuals after facing a cooperation opportunity compared to controls. We also examined the time structure of the neural activation after the cooperation opportunity by studying gene expression at three different time intervals (30, 45 and 60 min) after the onset of the opportunity. Second, we investigated whether behaviours performed in the cooperative breeding context, particularly brood care, brood defence and submission towards dominants, influenced *egr-1* gene expression levels. Third, we used the human DNA-binding motif for *egr-1* and searched for binding targets for the *egr-1* transcription product in the genome of a closely related species, the Nile tilapia, *Oreochromis niloticus* [42, 43] in order to predict the downstream cellular effects of *egr-1* expression in cichlids.

2. Material and methods

2.1. 1 – Egr-1 expression

2.1.1. Study animals

The dominant breeder pair in *Neolamprologus pulcher* (Poll) [44] groups monopolizes reproduction, and several sexually mature and immature helpers of both sexes delay dispersal and assist the dominants with brood care, territory maintenance and defence [45, 46]. *N. pulcher* are substrate brooders that spawn their clutches in breeding cavities. As a direct form of brood care, small, young helpers clean the dominants' eggs in the breeding cavity by nibbling off bacterial and fungal overgrowth. Furthermore, they perform indirect brood care by defending the clutch against egg predators, for instance the sympatric cichlid *Telmatochromis vittatus* (Boulenger), that is considered an unspecialized egg predator [47, 48]. These two major cooperative behaviours can be elicited experimentally in a controlled laboratory setting.

Our focal fish were offspring of 16 pairs that were lab-bred descendants of wild-caught fish from Kasakalawe Point, Lake Tanganyika, Zambia. Water temperature was kept at 27 $\pm$ 1 °C and the light regime was set to 13:11 L:D to mimic natural conditions. Adult pairs were fed commercial cichlid flake food five days a week and thawed frozen food (*Cyclops* spp., shrimps, *Artemia* spp., mosquito larvae) once a week. When *N. pulcher* larvae reach the free-swimming fry stage, they are independent of direct brood care. This happened 8–10 d after spawning, which was defined as experimental 'day 0'. At day 0, we removed the parents and transferred them to laboratory stock tanks. Focal offspring were kept in groups of full-siblings until behavioural testing, and were fed six times a week with Tetramin® 'Baby' food. On day 85, six individuals of each sibling group were randomly chosen and housed in 20 l tanks equipped with clay flowerpot halves serving as shelters together with a larger, unrelated conspecific for 14 days (mean size difference 7.9 mm $\pm$ 2.7 s.d.). As *N. pulcher* have a linear size hierarchy, the larger fish became immediately dominant over the smaller focal
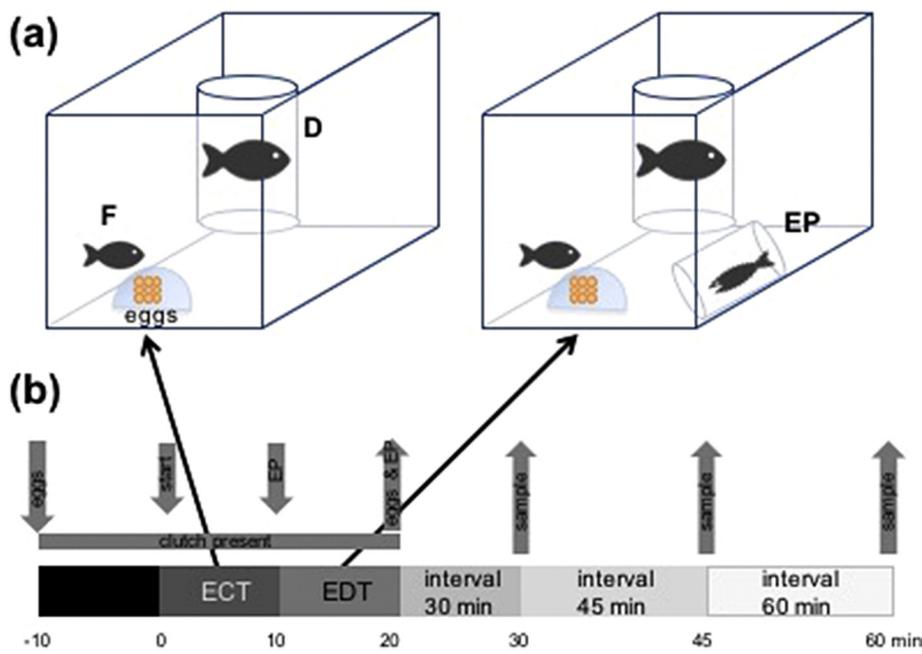
Fig. 1. (a) Sketch of the tank during the behavioural test. Left: the egg cleaning task, F: focal fish, D: dominant territory owner. Right: the egg defence task, EP: egg predator. (b) Sequence of the helping opportunities and subsequent brain sampling. Arrows represent the events and bars the durations of the single units of the trial. The clutch to be cleaned by the test fish was inserted up to ten minutes before the observation began. After 10 min in the egg-cleaning task (ECT), the egg predator individual (EP) was introduced and the observations continued until minute 20 (egg defence task, EDT), when both eggs and egg predator were removed. Test fish for the sampling intervals of 30, 45 and 60 min were left in the tank for another 10, 25 and 40 min, respectively, and then sacrificed.

fish. The presence of a dominant fish during the cooperation opportunity was necessary, because only subordinate fish show direct egg care behaviour, whereas dominant fish cannibalize eggs that are not their own [49] (Fig. 1a). We kept additional breeder pairs, which were unrelated to our focal fish, in 50 l tanks with breeding shelters lined with plastic sheets. Clutches spawned on the sheets could then be easily transferred to the experimental tanks where they were used as stimulus to elicit egg cleaning behaviour (nibbling off bacterial and fungal overgrowth) by the focal fish. *Telmatochromis vittatus* was used as stimulus in the behavioural test to elicit defence behaviour by the focal fish. We presented six different *T. vittatus* individuals during the behavioural tests that were bred from wild-caught parents in the laboratory (mean SL = 45.7 mm ± 3.7 s.d.). In *N. pulcher* sex usually cannot be determined before sexual maturation, which occurs around a standard length of 3.5 cm. Hence, the sex of our test fish, ranging between 1.7 and 2.2 cm length, was unknown. All procedures were conducted under the license 52/12 of the Veterinäramt Bern and adhered to the guidelines of the Association for the Study of Animal Behaviour.

2.1.2. Helping opportunity

The test fish were given a helping opportunity on experimental 'day 100', during which the dominant individual was confined to a transparent plastic tube in the back of the experimental tank, allowing visual contact and social interactions between the two fish (Fig. 1a). We first fixed a portion of a clutch spawned on a plastic sheet by unrelated breeder pairs (20.9 ± 5.3 eggs, mean ± s.d.) to the inside of the upper part of the shelter ('test' condition; Fig. 1a and b). For the 'control' condition, we manipulated the shelter in the same way, but the plastic sheet did not contain a clutch. We started the behavioural recordings either as soon as the test fish remained in the shelter for 5 s continuously (in a pilot experiment this time span had been sufficient to permit the detection of the clutch by the focal fish) or 10 min after the insertion of the clutch, whichever happened first. Using the event-logging software Observer (Noldus Information Technology), we directly recorded all instances of egg cleaning and submissive behaviour towards the dominant in the course of 10 min (egg cleaning task, Fig. 1b). Previous experiments have shown that not all individuals engage in egg-cleaning behaviours when given the opportunity [40], but that non-cleaners tend to show larger amounts of submissive behaviour towards dominant conspecifics [39]. After the first 10 min of a recording, a live egg predator individual in a transparent plastic container was

introduced in the 'test' condition and we recorded all aggressive displays directed towards the plastic box containing the egg predator in the second 10 min of a trial (egg defence task, Fig. 1b). In the 'control' condition we inserted an empty plastic container. No behavioural recordings were done in the control conditions of the first and second 10 min, as the fish could not show egg-cleaning behaviour in this situation. Since we were also interested in the neural response to the opportunity itself, which confronts the fish with the decision whether to help or not, in addition to influences of the performed helping and social behaviours on gene expression, we grouped together individuals that were given the opportunity to cooperate regardless of their actual behavioural performance for the initial analyses. However, for the analyses of the association of gene expression and behaviours we analysed only test individuals, using the respective part of the test (egg cleaning task for cleaning and submissive behaviour, egg defence task for defence behaviours), but pooling over all time intervals.

2.1.3. Brain sampling

For the timeline of the expression of *egr-1*, we sacrificed 29 test and 17 control individuals 30, 45 and 60 min after the start of the test (Table S1). We analysed four different brain regions separately (optic tectum, hypothalamus, cerebellum and telencephalon), similar to previously applied approaches of ([21, 24]. This allowed us to provide a coarse resolution of neural activation over the total brain of helpers while still being able to process a sufficient number of individuals for each time point. We swiftly caught the fish with a hand net to minimize stress and euthanized them in a solution of 1 g of Ethyl-3-amino-benzoate methanesulfonate salt (MS222, tricaine; Sigma Aldrich, Buchs, Switzerland) in 100 ml of water. MS222 is widely used as anesthetic in studies of brain gene expression in poikilotherms, and also when measuring egr-1 expression [24, 30]. Since the exact effects of MS222 on egr-1 expression are unknown, we aimed at reducing its potential impact by minimizing the time interval between respiratory arrest and RNA fixation to approximately 30 s. An experiment inducing *egr-1* expression by injecting kainic acid, a glutamate receptor agonist, has been conducted in a closely related species previously (*Astatotilapia burtoni*, [27, 50]). Another study on *N. pulcher* reported *egr-1* expression differences in the telencephalon and hypothalamus [24]. We therefore assumed that *egr-1* is, in principle, inducible in brain tissue in this species. More specifically, after cutting the fish in half sagittally, we immediately added a drop of RNALater (Sigma Aldrich, Buchs,

Switzerland) to the brain to stabilize mRNAs by deactivating RNases, and transferred it to a small glass Petri dish. Under a stereoscope with 16-fold zoom (Wild M3C, Heerbrugg, Switzerland) we dissected the major brain regions telencephalon, cerebellum, optic tectum, hypothalamus, and hindbrain within 10 min post mortem, according to the brain atlas of a closely related species, *Oreochromis mossambicus* [51]. As we were mainly interested in broad-scale expression patterns across the whole brain and no detailed brain atlas of *N. pulcher* exists to date, we did not attempt to divide the major brain regions further into functional nuclei. We stored the brain parts of each individual separately in 1.4 ml Eppendorf tubes filled with RNALater at +4 °C over night and transferred them to −20 °C on the next day where they were kept until RNA extraction.

2.1.4. RNA extraction and real-time quantitative PCR

We extracted RNA from optic tectum, hypothalamus, cerebellum and telencephalon samples that fulfilled the following two quality control criteria at brain sampling: First, brain extractions and subsequent storage in the RNA-stabilizing solution had to be finalized within 10 min post mortem to prevent mRNA degradation by RNases. Second, the quality of the dissection had to be satisfactory to ensure that regions could be clearly identified. We only used brain samples fulfilling these criteria for the further analyses, resulting in a total of 180 samples for which we extracted RNA (Table S2). We used the miRNeasy Micro Kit (Qiagen, Basel, Switzerland) and performed a DNase treatment (RNase-free DNase Set, Qiagen, Basel, Switzerland) on the columns during the extraction to remove any contaminating genomic DNA. We assessed the quality and concentration of the extracted RNA with NanoDrop (Thermo Fisher Scientific) after each protocol run, and we checked the RNA integrity number (RIN) of 14 randomly selected samples on a BioAnalyzer (Agilent) chip. The median RIN was 8.45 (inter-quartile range of 7.98, 8.9). Eleven RNA extracts had to be excluded from further analysis because of their low RNA concentration and 5 because of poor NanoDrop diagnostics (Table S2). Subsequently, we reverse-transcribed mRNA of 164 samples to cDNA using ultraclean oligo(dT) primers (GoScript Reverse Transcription Kit, Promega, Dübendorf, Switzerland). Samples of the telencephalon as well as the cerebellum were all brought to a concentration of 100 ng/μl, those of the optic tectum and the hypothalamus to a concentration of 250 ng/μl and 144 ng/μl, respectively. We then performed a quantitative real-time PCR of the target gene, *egr-1*, and a reference gene, *18S*, on a 384-well plate qRT-PCR machine (7500 FAST RT-PCR System, Applied Biosystems) using 5× HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis BioDyne, Tartu, Estonia) with 95 °C and 57 °C cycling temperature. We used gene-specific primers for *egr-1 (*also called NGFI-A, zif268, ZENK, TIS8 or Krox-24), the target gene, and *18S,* as reported in [24]. To confirm the specificity of primers, we performed a PCR and gel electrophoresis for both genes, which resulted in a single product each with the expected length (*egr-1:* 115 bp, *18S:* 146 bp). This was supported by an assessment of melt curves performed after the amplification cycles in the quantitative real time PCR analysis. For each of the genes, we determined the amplification efficiency (E) with a dilution curve for 5×10-fold dilutions of pooled samples in duplicates. The efficiency was calculated according to the equation $E = 10^{\frac{-1}{slope}}$ [52]. The slope was determined with a linear model of the relationship between the log cDNA concentration of a sample and its quantification cycle. We measured gene expression in triplicates of the biological sample using the comparative C_T method ($2^{-\Delta\Delta C_T}$, [53, 54]) and we report the expression of *egr-1* relative to *18S*.

2.1.5. Statistical analysis

All analyses were conducted in R version 3.2.0 [55]. We removed one outlier identified by the Grubbs test in the outliers package 0.15 ($G = 6.81$, $U = 0.71$, $p = 2.5 \times 10^{-11}$, [56] while being blind to the experimental condition, brain region and time interval of data points

(Table S2). Prior to analysis, values for gene expression levels were Box-Cox power transformed to meet normality and homoscedasticity requirements. Using the MASS package 7.3–45 [57], we plotted a range of potential power parameters against their log-likelihood and visually identified the maximal value (λ = 0.07). First, we conducted an overall linear mixed-effects model using the lme4 package V 1.1.-12 [58] and the multcomp package 1.4–6 for post-hoc comparisons [59], including the full dataset to analyse the influence of test condition, brain region and time interval on gene expression levels. We accounted for repeated measurements of individuals and full siblings by including individual identity and sibship identity as random effects. The statistical significance of these variance components was assessed with a simulation-based likelihood ratio test in the RLRsim package 3.1–3 [60]. Since neither sibship nor individual identity explained an important amount of the variance, we omitted those random effects from the further analyses. Second, in order to investigate the effect of sampling time and test condition on *egr-1* expression, we conducted linear models in each brain region separately after Box-Cox transformation (cerebellum: λ = 0.1, telencephalon: λ = 0.16, hypothalamus: λ = 0.09, optic tectum: λ = 0.08). Third, to assess whether the amount of *egr-1* expressed was associated with differences in the amount of egg cleaning (first 10 min of trials) or defence behaviours (second 10 min of trials) between individuals, we carried out linear models on the subset of responders, i.e. individuals showing the respective behaviour. In the first 10 min, we additionally investigated the effect of submission on *egr-1* expression in the subset of non-cleaners because it has been suggested that submissive behaviours represent an alternative appeasement strategy in this type [39]. For these analyses, we pooled the data over all time intervals to achieve sufficient power. We present raw *p*-values obtained from likelihood-ratio tests comparing models containing the relevant predictor to models without the predictor of models including interactions, using the Chi-square test for linear mixed-effect models and the F-test for linear models. Initially, all interaction terms were included in the models. In case they were not significant, we removed them in a step-wise fashion (first the three-way, then the two-way interactions) and present likelihood-ratio tests for the lower-order terms of the reduced models. Given the exploratory character of this study we encourage the reader to interpret *p*-values with caution (see [61]) for an extended discussion).

2.2. 2 - Egr-1 target prediction

To search for the potential sequence-specific binding sites that could be targets of *egr-1,* we used the genome of the Nile tilapia *Oreochromis niloticus* (Linnaeus) genome as a reference. While the genomes of some species that are phylogenetically closer to *N. pulcher* have been assembled (see for instance BouillaBase, http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html), only the detailed genomic resources available for the Nile tilapia allow a meaningful search for potential binding sites of transcription factors. More specifically, a list of 13,053 evolutionarily conserved noncoding elements (CNEs) has been published for this species [42, 43]. In general, African cichlid species show high synteny, strong chromosomal conservation and no major genomic rearrangements [42, 43, 62](O'Connor, Marsh-Rollo, Ghio, Balshine, & [11]). Therefore, a high correspondence of *egr-1* targets can be expected, whereas the downstream effects of the genes regulated by *egr-1* are expected to differ, since *O. niloticus* and *N. pulcher* differ in offspring care behaviours. *O. niloticus* is a maternal mouth brooder [63, 64] and hence does not perform the same types of behaviours when caring for eggs. CNEs are expected to be enriched in regulatory elements based on functional assays [65], and hence provide a well-suited starting point for searching DNA-binding motifs of transcription factors. The genomic sequences of these sites were identified with the program samtools (version 1.2; [66]. *Egr-1* binding sites consensus motif was obtained from the Jaspar database (July 2016, [67]), where only data of human and mouse orthologs were available. We

decided to employ the human frequency matrix, based on a chromatin immunoprecipitation with promoter array (ChIP-chip) experiment a in human THP-1 leukemia cell line in the context of monocyte differentiation [33], which is similar to the mouse frequency matrix derived from a bacterial 1-hybrid system. The software FIMO from the MEME suite (version 4.11.2; [68] was used to filter for CNEs with at least one match of the *egr-1* motif. The gene annotation was based on the first version of the genome (oreNil1) on Ensembl (release 84). These co-ordinates had to be converted, by means of a custom Python script, to the newer version (oreNil2; see Supplementary Material for scripts). This conversion was based on the description of the linkage groups present on the ENA website (id: GCA_000188235.2). To restrict our search we focused only on CNEs that were in close proximity to a gene (1 kb), a region that most likely contains active promoters in *O. niloticus* [69]. This approach could potentially exclude a few promoters that are located outside this range or even on another chromosome. A functional enrichment analysis of the GO terms associated with the selected genes was carried out by means of the R package topGO (version 2.26.0, [70]), using Fisher's exact test and 'weight01' algorithm. For a comparison of putative *egr-1* targets between tilapia and humans we conducted a similar search on data for humans obtained from the Broad Institute's Molecular Signatures Database (MSigDB, http://software.broadinstitute.org/gsea/msigdb/cards/EGR1_01, accessed in July 2018). We focused on genes that have, according to Ensembl, a one-to-one orthology relationship between the two species. This reduced the total number of testable genes to 6524 genes and the two target sets to 89 genes for tilapia and 104 for humans.

3. Results

3.1. 1 - Egr-1 expression following a helping opportunity

3.1.1. Gene expression in brain areas across time

The full linear mixed-effect model including data from all brain regions and time points showed that *egr-1* gene expression differed significantly between brain regions (linear mixed-effect model, $\chi^2 = 12$, df = 3, $p = 0.007$, Table 1). *Egr-1* was 2.6 times less expressed in the hypothalamus than in the cerebellum, (Tukey post-hoc pairwise comparison, z = −3.49, $p = 0.003$). Test and control subjects did not differ in *egr-1* expression across brain regions and across time intervals elapsed since the onset of the stimulus presentation (Table 1). All interactions were non-significant, indicating that no strong signal of expression differences between control and test individuals in any brain region at any given point in time. Sibship did not explain any of the variance, and we removed this random effect without further testing. The amount of variance explained by individual identity was 2%, which was not significant (likelihood ratio test, $\chi^2 = 0$, $p = 0.368$). We therefore did not include individual identity in the subsequent models.

Table 1
Model comparisons for brain regions and time intervals using likelihood ratio tests and the Chi-squared statistic. 'Cond' refers to the experimental condition (test or control), 'time' to the time elapsed since start of experiment (30, 45 or 60 min), and 'region' to the brain region (cerebellum, telencephalon, hypothalamus or optic tectum). *P*-values < .05 are highlighted in bold.

Term	χ^2	df	p
Cond × region × time	8.98	6	0.175
Cond × time[a]	0.20	2	0.906
Cond × region[a]	5.60	3	0.133
Time × region[a]	10.51	6	0.105
Cond[b]	1.53	1	0.216
Time[b]	3.18	2	0.204
Region[b]	12.00	3	**0.007**

[a] after removal of non-significant 3-way interaction term.
[b] after removal of non-significant 2-way interaction term.

Table 2
Model comparisons for the time course of *egr-1* expression in the single brain regions (cerebellum, telencephalon, hypothalamus or optic tectum) in test and control individuals using F-tests. 'Cond' refers to the experimental condition (test or control), and 'time' to the time interval after start of experiment (30, 45 or 60 min). P-values < .05 are highlighted in bold.

brain region	term	SS	RSS	df	p
Telencephalon	cond × time	0.10	1.20	2	0.179
	cond[a]	0.11	1.31	1	0.063
	time[a]	0.12	1.33	2	0.134
Hypothalamus	cond × time	0.03	0.38	2	0.183
	cond[a]	0.00	0.38	1	0.671
	time[a]	0.02	0.40	2	0.387
Optic tectum	cond × time	0.01	0.52	2	0.772
	cond[a]	0.00	0.53	1	0.687
	time[a]	0.00	0.53	2	0.971
Cerebellum	cond × time	0.04	0.61	2	0.286
	cond[a]	0.07	0.68	1	**0.037**
	time[a]	0.15	0.76	2	**0.014**

SS: sum of squares, RSS: residual sum of squares, df: degrees of freedom.
[a] computed after removal of non-sigificant 2-way interaction term.

3.1.2. Gene-expression pattern within brain regions

In order to describe more subtle patterns of *egr-1* expression within single brain regions, and since regions differed significantly in gene expression, we analysed the time course and its potential interaction with the test condition separately for each brain area. Test and control individuals did not display differences in the time course; thus all interaction terms were removed from the models for further analyses (Table 2). Irrespective of the time of sampling, test and control individuals differed in their *egr-1* expression in the cerebellum and the telencephalon (linear model; cerebellum: SS = 0.07, RSS = 0.68, df = 1, $p = 0.037$ and telencephalon: SS = 0.11, RSS = 1.31, df = 1, $p = 0.063$), but not in the hypothalamus and the optic tectum. In both cerebellum and telencephalon, control subjects had higher *egr-1* expression than test subjects, but the difference was non-significant for the telencephalon (Table 2, Fig. 2). In the cerebellum, but in none of the other brain regions, we found evidence that *egr-1* expression decreased over the time elapsed since the onset of the helping opportunity (linear model, SS = 0.15, RSS = 0.76, df = 2, $p = 0.014$). However, the lack of a significant interaction with experimental condition indicates that this reduction was similar for individuals confronted with the helping opportunity and controls (Table 2).

3.1.3. Association of gene expression and behaviours

Of the 29 individuals that were given a helping opportunity, 17 individuals (59%) responded with direct brood care in the egg cleaning trial and were considered as 'cleaners' whereas 12 did not clean eggs ('non-cleaners', Table S1). Most individuals (93%) showed defence behaviour in the brood defence trial (Table S1). Neither egg cleaning nor brood defence behaviours showed a significant association with *egr-1* expression in any brain region (Table 3). Only 2 of the 17 'cleaners' but 8 of 12 'non-cleaners' were submissive towards the dominant in the egg-cleaning trial. Hence, the probability of showing submissive behaviour towards the dominant was significantly higher in individuals that did not clean eggs (Pearson's Chi-squared test: $\chi^2 = 9.39$, simulated *p*-value = 0.005). This suggests the existence of mutually exclusive 'cleaner' and 'submission' types. Thus, we explored whether submissive behaviours of non-cleaners were associated with *egr-1* expression. Within the telencephalon and the hypothalamus, the amount of submission of 'non-cleaners' directed towards dominants, pooled over an hour following the stimulus presentation, was significantly positively associated with *egr-1* expression levels (Table 3, Fig. 3).
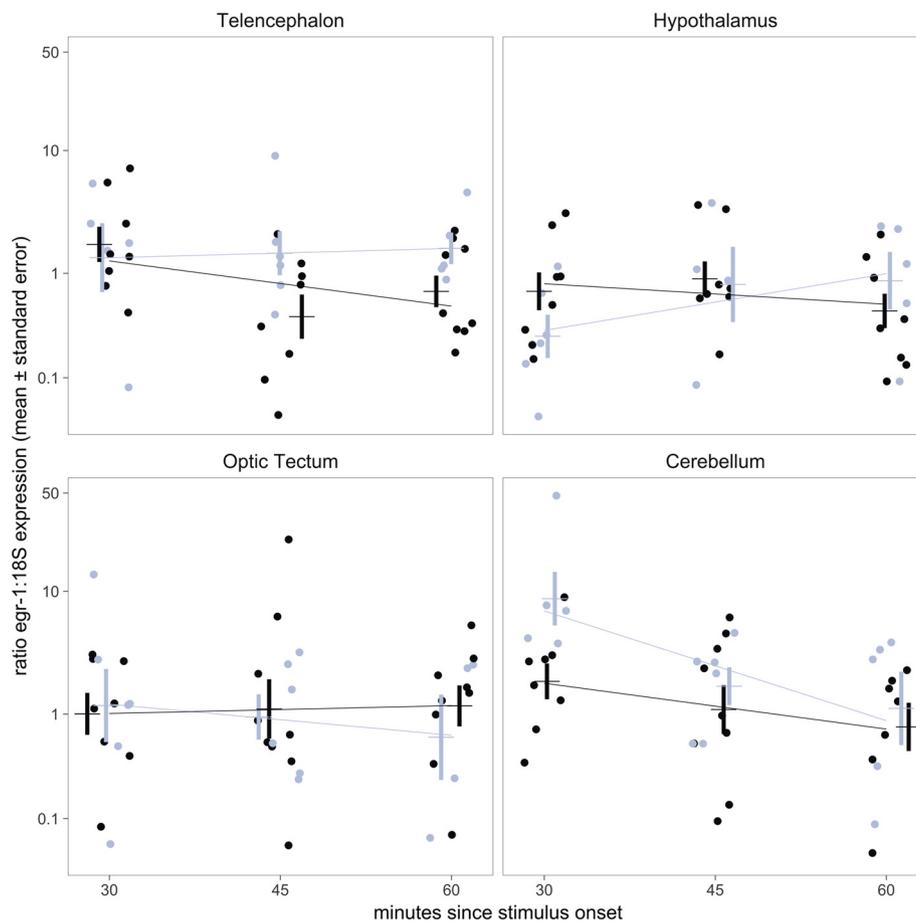
Fig. 2. *Egr-1* expression across brain regions and time for control (grey circles) and test (black circles) subjects. Crosses indicate means; and vertical bars are standard errors of the mean. Linear regression lines for control and test are plotted in the respective grey shades.

Table 3

Association between egg cleaning and submissive display frequencies in the egg-cleaning trial as well as defence behaviour frequencies in the brood-defence trial and *egr-1* expression across brain regions using the F-test. P-values < .05 and 0.05 < *p* < 0.1 are highlighted in bold and italics, respectively. Sample sizes differ between brain regions due to the exclusion of some samples for quality reasons and within brain regions due to the subsetting into non-cleaners, for which we explored the association of submissive behaviours and egr-1 expression.

Brain region	Behaviour	N	F	df	p
Telencephalon	Egg cleaning	14	1.24	1,12	0.288
	Defence	23	0.04	1,21	0.840
	Submission	*11*	*4.57*	*1,9*	*0.057*
Hypothalamus	Egg cleaning	16	0.43	1,14	0.522
	Defence	24	0.75	1,22	0.395
	Submission	9	10.22	1,7	**0.015**
Optic tectum	Egg cleaning	14	0.06	1,12	0.808
	Defence	24	0.01	1,22	0.917
	Submission	12	0.11	1,10	0.745
Cerebellum	Egg cleaning	14	1.06	1,12	0.323
	Defence	22	0.01	1,20	0.910
	Submission	10	0.23	1,8	0.644

SS: sum of squares, RSS: residual sum of squares, df: degrees of freedom.

3.2. 2 – Egr-1 target prediction in cichlids

The search for the DNA binding site of *egr-1* in the 13,053 conserved noncoding elements (CNEs) of the Nile tilapia genome yielded a list of 229 genes that had a motif located within 1 kb of the transcription start site and could thus be considered as potential targets of *egr-1* in cichlid fishes (Table S3). Of these 229 genes, 161 were functionally annotated, and a total of 394 GO terms were associated with them. 27 GO terms were significantly enriched in this list, compared to the whole Nile tilapia genome. 65 of the potential target genes of *egr-1* were linked to these GO terms (Table 4). 26 different genes were implicated in the biological processes 'transcription, DNA templated' (GO:0006351), 'regulation of transcription, DNA templated' (GO:0006355) and 'transcription factor activity, sequence-specific DNA binding' (GO:0003700) (Table 4A) and 17 further genes in the molecular functions 'nucleic acid binding' (GO:0003676), 'DNA binding' (GO:0003677), 'sequence specific DNA binding' (GO:0043565) and 'chromatin binding' (GO:0003682) (Table 4B). These two main functional groups included genes implicated in embryonic development, for instance 6 homeobox genes (*dlx6a, evx1, evx2, irx1a, nkx2.3, nkx3–1*), 5 forkhead box genes (*foxa, foxb2, foxj3, foxp1b, foxp2*), and one paired box gene (*pax10*). Ten GO terms were developmental processes (Table 4C), mainly the 'regulation of signal transduction' (GO:0009966, including 'steroid hormone mediated signalling pathway', GO:0043401, and 'steroid receptor activity, GO:0003707). The 'peripheral (nervous) system development' was also enriched (GO:0007422, GO:0048666, GO:0031290), as well as 'adenohypophysis development' (GO:0021984), 'inner ear development' (GO:0042472, GO:0071599) and 'retina morphogenesis' (GO:0060042). Those terms included a further 9 genes (Table 4C). The remaining GO terms were a combination of metabolic processes and various other biological processes (Table 4D), which included another 12 genes that were not present in the other groups. Several of the aforementioned functions take place in the nucleus, as indicated by an enrichment of the cellular component 'nucleus' (GO:0005634, Table 4E). However, we would like to add a note of caution since these
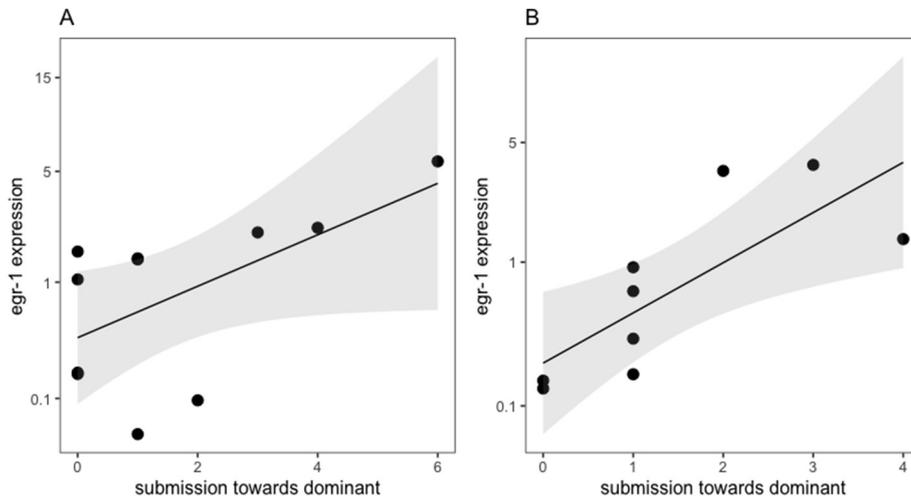
Fig. 3. *Egr-1* expression in relation to submissive behaviour towards the dominant in individuals that did not perform direct brood care in the egg-cleaning trial in the telencephalon (A) and the hypothalamus (B). Linear regression lines with standard errors (shaded areas) are shown.

Gene Ontology terms were established in genetic model species in a biomedical context. Hence, it is unclear how to interpret the biological function of genes in ecological and evolutionary studies.

The comparison of putative *egr-1* target orthologs in tilapia and humans resulted in a shared set of four genes between the two species, *ITGB8*, a fibronectin receptor, *PTCH1* that transduces hedgehog's protein signals, as well as *SP1* and *ZFPM1*, which are other zinc-finger

transcription factors. This overlap is, despite the low number of genes, significantly higher than that of two random sets (hypergeometric test: 2.8-fold enrichment, *p*-value = 0.04). We also determined GO terms that were enriched in human EGR1 targets. Of the 27 functional categories identified in *egr-1* targets in tilapia, five were significantly enriched in humans as well: 'transcription, DNA-templated' and its regulation (GO:0006351, GO:0006355), 'regulation of signal transduction'

Table 4
The 27 significantly enriched GO terms associated with the 161 genes that are potential targets of *egr-1*, ordered by functional category. A: biological processes including transcriptional regulation, B: molecular functions encompassing the binding to nucleic acids, C: developmental processes, D: metabolic and various other biological processes, E: cellular component 'nucleus'. GO terms and gene names in bold indicate the overlapping genes and functions between humans and tilapia in our own analyses. Asterisks (*) denote the exact molecular functions and biological processes that were also reported for human cell lines [33].

	GO term	no genes	p-value	description	genes
A	**GO:0006351**	9	2.36E-02	transcription,_DNA-templated	*esrrga, nr2e1, foxp1b, nr1h5, foxj3, foxb2, foxp2, eomesb, EBF4*
	GO:0006355	25	2.22E-05	regulation_of_transcription,_DNA-templated	*esrrga, nr2e1, foxp1b, irx1a, nr1h5, mta2, foxj3, foxb2, foxp2, eomesb, gata2a, nkx2.3, EBF4, evx1, **sp1**, dlx6a, pax10, foxa, gli1, evx2, nkx3–1, myca, wt1a, cbfa2t2, irf3*
	GO:0003700	16	8.03E-05	transcription_factor_activity,_sequence-specific_DNA_binding	*esrrga, nr2e1, foxp1b, nr1h5, mta2, foxj3, foxb2, foxp2, eomesb, gata2a, **sp1**, foxa, myca, cbfa2t2, irf3, purba*
B	GO:0003676*	20	1.89E-02	nucleic_acid_binding	*foxp1b, **sp1**, gli1, wt1a, sall4, exd3, rev3l, snai1b, MEX3A, esrp2, nars2, cpeb1b, pabpc1a, znf516, znf532, klf15, **zfpm1**, srek1, MEX3C, hivep2a*
	GO:0003677*	21	6.84E-03	DNA_binding	*esrrga, nr2e1, foxp1b, irx1a, nr1h5, mta2, foxj3, foxb2, foxp2, eomesb, nkx2.3, EBF4, evx1, dlx6a, pax10, foxa, evx2, nkx3–1, rev3l, bahcc1, purba*
	GO:0043565	17	3.88E-05	sequence-specific_DNA_binding	*esrrga, nr2e1, foxp1b, irx1a, nr1h5, mta2, foxj3, foxb2, foxp2, gata2a, nkx2.3, evx1, dlx6a, pax10, foxa, evx2, nkx3–1*
	GO:0003682	3	4.80E-03	chromatin_binding	*mta2, sall4, bahcc1*
C	**GO:0009966**	2	1.87E-02	regulation_of_signal_transduction	*nr1h5, si:ch211-26b3.4, cnksr2a*
	GO:0043401	3	3.88E-02	steroid_hormone_mediated_signaling_pathway	*esrrga, nr2e1, nr1h5*
	GO:0003707	4	7.01E-03	steroid_hormone_receptor_activity	*esrrga, nr2e1, nr1h5, paqr7b*
	GO:0007422	2	1.10E-02	peripheral_nervous_system_development	*fgf3, ntf3*
	GO:0048666	2	2.04E-02	neuron_development	*fgf3, lingo1b*
	GO:0031290	2	4.34E-02	retinal_ganglion_cell_axon_guidance	*gli1, alcamb*
	GO:0021984	2	5.23E-03	adenohypophysis_development	*fgf3, gli1*
	GO:0042472	2	6.23E-03	inner_ear_morphogenesis	*irx1a, fgf3*
	GO:0071599	2	7.31E-03	otic_vesicle_development	*esrrga, fgf3*
	GO:0060042	2	2.22E-02	retina_morphogenesis_in_camera-type_eye	*irx1a, dctn1a*
D	GO:0006094	2	2.71E-03	gluconeogenesis	*gpib, pck1*
	GO:0006139*	2	4.10E-02	nucleobase-containing_compound_metabolic_process	*exd3, zgc:110540*
	GO:0008408	2	2.71E-03	3′-5′_exonuclease_activity	*exd3, rev3l*
	GO:0048821	2	1.24E-02	erythrocyte_development	*gata2a, nkx3–1, melk*
	GO:0001525	3	5.00E-02	angiogenesis	*amotl2a, **itgb8**, rspo1*
	GO:0001935	2	5.97E-04	endothelial_cell_proliferation	*amotl2a, **itgb8***
	GO:0008078	2	3.47E-02	mesodermal_cell_migration	*snai1b, has2*
	GO:0009880	2	1.24E-02	embryonic_pattern_specification	*fgf3, amotl2a*
	GO:0035118	3	1.54E-03	embryonic_pectoral_fin_morphogenesis	*irx1a, sall4, hand2*
E	GO:0005634	23	1.83E-02	nucleus	*esrrga, nr2e1, foxp1b, irx1a, mta2, foxj3, foxb2, foxp2, eomesb, gata2a, nkx2.3, EBF4, evx1, **sp1**, dlx6a, pax10, evx2, myca, wt1a, bnc2, kpna1*

(GO:0009966), 'neuron development' (GO:0048666) and 'nucleobase-containing compound metabolic process' (GO:0006139, Table 4). This functional overlap is larger than that of individual genes and it shows that *egr-1* regulates similar pathways, even in very distant species.

4. Discussion

This study aimed to investigate whether neural activation following an alloparental care opportunity could be measured by quantifying the expression of the immediate early gene *egr-1*. We expected to find an increase in *egr-1* expression following the experimentally generated opportunity for alloparental care relative to control individuals. Instead, we found that *egr-1* expression was lower in two brain areas, the telencephalon and the cerebellum. Neither direct brood care nor brood defence correlated with *egr-1* expression. However, in individuals not performing direct brood care, *egr-1* expression in the hypothalamus and potentially in the telencephalon increased with the amount of submissive displays. Finally, we predicted that *egr-1* regulates the expression of other transcription factors, and is likely to be involved in steroid hormone signalling, neuroplasticity and the development of the nervous system.

4.1. Down-regulation of egr-1 expression following helping opportunity

Previous studies in African cichlids and swordtails reported an increase in *egr-1* expression following experimentally generated social opportunities. *Egr-1* expression in the telencephalons of swordtail females were elevated after interacting with preferred males [29], and in the hypothalamus of African cichlids [28]. These findings suggest that the modulation of *egr-1* expression could be a key component of the molecular mechanism that transduces the external stimulus of a social opportunity into an intracellular signal, which eventually results in enhanced neuroplasticity [25], especially in situations that necessitate rapid physiological and behavioural changes like reproduction [71]. In this study, we found lower *egr-1* expression in the cerebellum and telencephalon of fish facing a helping opportunity than in control individuals at all time points surveyed. In other studies reporting down-regulation of *egr-1* and other genes with neural functions following social challenges [24, 72], this was associated with an inhibition of behavioural output [21, 73] and might be a consequence of environmental deprivation or chronic stress [73–75]. A recent study on the neural response to cooperative territory defence in the cichlid fish *A. burtoni* found a negative correlation of the IEG *c-fos* in the Dm-1, which is the putative fish homolog of the mammalian basolateral amygdala [10]. As the amygdala plays an inhibitory role in the behavioural response to threatening situations and dangers, a decreased activation of this brain area might enable the fish to actually perform cooperative behaviours. In general, the exposure to a specific stimulus can have opposing effects on different kinds of IEGs [76], and the direction of gene expression response to a stimulus can vary notably across neighbouring brain regions on a relatively small spatial scale [77–79]. This suggests that social behaviour is modulated by region-specific transcriptional regulatory networks. Furthermore, in the same context the same pathways might be used differently depending on previous experience [24]. Our target prediction showed that *egr-1* potentially regulates the expression of several developmental genes, *lingo1b* [80] [81], *irx1a* [82] and *fgf3* [83], which modulate neurogenesis or could even act as repressors of neurogenesis. Thus, the down-regulation of *egr-1* we observe in individuals that were given the opportunity to co-operate might even result in enhanced neuroplasticity because inhibitors are not expressed [84]. Interestingly, in a similar study using a whole-transcriptome approach we detected a significant up-regulation of *egr-1* in the telencephalon during the helping opportunity compared to a control group, independent of the performance of helping behaviours. However, this increase was only significant in individuals that did not perform cleaning behaviours, and after experiencing the helping

opportunity for the second time [85]. This indicates that, in a helping opportunity as presented in the present study, *egr-1* expression can be, in principle, induced in *N. pulcher*. However, the pattern of egr-1 expression in this context might be less clear-cut than previously assumed.

Our result that the *egr-1* was downregulated in the cerebellum indicates that brain regions that are rarely studied in association with social and cooperative behaviours might also have an important function in these contexts. In line with this finding, a study comparing transcriptomes of threespine sticklebacks (*Gasterosteus aculeatus*) housed either individually or socially for one week revealed a stronger genomic response to this long-term social experience in the cerebellum than in other brain regions [84]. Another study investigating the transcriptional response to a territorial intrusion in threespine sticklebacks reported almost an equal number of differentially expressed genes in the cerebellum and the diencephalon (hypothalamus and preoptic area; [78]). As the cerebellum has a main function in processing sensory input and spatial motor coordination [18, 19], these results indicate that the cerebellum takes an important role in processing of sensory information from social interactions and may also coordinate the social behavioural output. A meta-analysis revealed that the cerebellum plays an important role in human social cognition, probably as a modulator of cognitive processes that updates information and feeds back to the cerebral cortex, thereby increasing the efficiency of other brain structures [86]. In both treatment conditions, *egr-1* expression in the cerebellum decreased significantly over time elapsed since the onset of the trial. This temporal pattern might be caused by a neural activation elicited by some aspect of the experimental manipulation that took place in both conditions, such as the manipulation of shelters or the presentation tube, which may have induced an increased expression during or shortly after the manipulations (i.e. before the first gene expression measurement), followed by a decline of the response. Since IEGs start to be induced rapidly and transiently [87], within minutes of sustained neural activity, the possibility exists that *egr-1* expression might have changed at earlier timepoints than sampled in our study. Moreover, the promotor region of *egr-1* itself contains an *egr-1* binding site, and it can down-regulate its own gene expression [88]. Thus, it is conceivable that *egr-1* levels have decreased already by the time of our first sampling interval at 30 min due to a negative feedback mechanism. However, a recently published timeline of a range of IEGs suggest that *egr-1* expression peaks at around 60 min after sustained depolarization and its expression is expected to be still increased after even 120 min (Fig. 1 in [32].

4.2. Association between egr-1 expression and behaviours

IEG expression is one of the first steps following the reception of an opportunity, but is not necessarily predictive of between-individual differences in behaviour. In some previous studies, an increase in *egr-1* expression was associated with the presence of a preferred mate [21] or dominance behaviour [28]. However, others reported an increase in *egr-1* expression following a social opportunity that was uncorrelated with subsequent nursing behaviour in rats [89], calls uttered in Túngara frogs [90], or agonistic or submissive behaviours in *N. pulcher* [24]. In the present study, differences in the amount of helping behaviours our subjects performed, both during the egg-cleaning task (first 10 min of trials) and in the defence task (second 10 min), were not associated with the level of *egr-1* expression in any of the brain regions. Thus, cooperative behaviours in cichlids might covary with the expression levels of other genes, either downstream of *egr-1* or different IEGs, which should be targeted in future studies either with a candidate gene or a whole-transcriptome approach.

In our study, almost half of the individuals that were given the opportunity to clean eggs did not do so, but instead displayed submissive behaviour towards the dominant individual. Submissive displays from subordinates towards higher-ranking group members take a key role in the sociality of *N. pulcher,* being the main behavioural

mechanism to mediate conflict and stabilize the group hierarchy. The difference in the probability to behave submissively between cleaner and non-cleaner types indicates that the investment in either direct alloparental care or submission might indeed be alternative social strategies, which may both help securing group membership in this cooperatively breeding species (see [39, 41]). Thus, apart from the non-social component of direct alloparental brood care and brood defence, the helping opportunity in our experiment also presented a social opportunity, as test subjects could swim close to the partition confining the dominant individual and direct submissive displays towards it. In the 'non-cleaner' subset, *egr-1* expression increased with the amount of submission displayed in those two brain areas that hold most nodes of the brain network implicated in social decision-making, the telencephalon and the hypothalamus [17]. Taken together, these results suggest that *egr-1* might play a role in the social component of the helping opportunity, but not in helping behaviours directly.

4.3. Egr-1 targets in cichlids

The binding motif of *egr-1* is well defined in humans [33] and mice [34], but not in other taxa, especially not on a whole-genome level. Therefore, the functions of *egr-1* in fish were previously unknown, highlighting the importance of the identification of *egr-1* targets in the particular taxonomic group of interest. Apart from the present study, this list of targets can be of interest for other studies using *egr-1* quantification in cichlids, and can be used as a tool for comparisons with other taxa. Our search for potential targets of *egr-1* in the CNEs of the Nile tilapia, a species closely related to *N. pulcher*, suggested that the most apparent function of *egr-1* is the regulation of the expression of other transcription factors. To investigate the neural substrate specific to the helping opportunity, we focused on biological function categories related to steroid hormone signalling pathways and the development of the central nervous system. The first category comprised three receptors that transduce signals from steroid hormones [91, 92] and an estrogen as well as a progestin receptor, which regulates gonad maturation [93]. We found five potential targets of *egr-1* in cichlids implicated in nervous system development. Two of those genes, GLI family zinc finger 1 transcription factor (*gli1*) and neurotropin-3 (*ntf3*), are clearly transcriptional activators and one gene, leucine rich repeat and lg domain containing 1b (*lingo1b*), apparently suppresses neuroplasticity. The neuroplasticity-activating or inhibiting functions of the remaining two genes, iroquois homeobox 1a (*irx1a*) and fibroblast growth factor 3 (*fgf3*), are not well understood and might be strongly context-dependent [94, 95]. These genes have important functions not only in embryonic neural development, but are also expressed in the adult brain where they regulate synaptic strength and plasticity [96, 97]. These putative *egr-1* targets could be important for an adequate response to the helping opportunity because of the role *egr-1* plays in long-term potentiation and long-term memory formation [98]. Since cooperation might pose special demands on social memory formation [5], these *egr-1*-mediated processes could be candidate mechanisms.

It has to be noted that the target search was performed on the genome of a closely related species with a different form of brood care. However, we did not expect that the immediate targets of *egr-1* will differ importantly between tilapia and *N. pulcher* for the following reasons. Time of divergence between *O. niloticus* and *N. pulcher* has been estimated as approximately 25 MYA (median from 17 studies at http://timetree.org) and a high level of synteny has been demonstrated among cichlid species [42, 43, 62]. Moreover, the coding sequences of isotocin and arginine vasotocin and their receptors, which are candidate hormone pathways for cooperative behaviours, are highly conserved between eight cichlid species, regardless of their social system (cooperative breeders or non-social species). Hence, differences in gene expression rather than sequence differences seem to give rise to different social systems in cichlids [99]. In general, the amino-acid sequence and thus the structure of the *egr-1* protein is highly conserved in

vertebrates [50], but, to our knowledge, no systematic comparisons have investigated the degree of conservation of its targets. We found a very small but significant set of putative cichlid *egr-1* targets that overlaps with those of humans, which were enriched for GO terms related to transcriptional regulation and neural functions, similarly to the Nile tilapia targets. Finding only a small overlap of genes between humans and tilapia is not surprising due to the estimated time since divergence of 432 MYA (median from 25 studies at http://timetree.org) and gene duplication events that have occurred in teleosts. However, given the evolutionary distance between these species, our analysis indicates that the motif search we performed can indeed be a robust method to identify *egr-1* targets in cichlids. However, motif searches have several limitations compared to recent high-throughput techniques like ChIP-seq and the identified targets might thus not represent a complete and exact list of targets. For example, the binding of a transcription factor to its motif can be prevented by the chromatin state of the DNA region where it is located. Our analysis did not take into account this possibility due to the unavailability of such data. *Egr-1* can also serve as a co-factor and can regulate gene expression indirectly by interacting with other transcription factors such as *c-Fos* or *c-Jun* [100–102]. Here, we only considered direct targets of *egr-1* that have a DNA binding motif for it, hence those indirect targets are missing from our search.

Taken together, our results suggest that *egr-1* is likely to regulate the expression of genes implicated in steroid hormone signalling, neuroplasticity and neural development pathways. We found that *egr-1* expression was down-regulated in individuals receiving the helping opportunity as compared to a control group, suggesting that downstream genes with inhibitory effects on neuroplasticity might be less expressed. Finally, similar to previous studies that established a link between *egr-1* expression and the performance of social behaviours, *egr-1* expression was positively associated with the amount of submissive displays towards a dominant in our study. However, gene expression was not influenced by helping behaviours performed. This indicates that, while *egr-1* expression might be a predictor for social interactions shown in a cooperation context, it seems to be a less suitable marker for neural activation in cooperative behaviours not involving social interactions. Hence, future studies should aim to identify additional neurogenetic markers for neural activation during cooperation opportunities.

Author contributions

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2018.07.025.

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