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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

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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, *egr-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 ± 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 ± 2.7 s.d.). As *N. pulcher* have a linear size hierarchy, the larger fish became immediately dominant over the smaller focal