The aversive brain system of teleosts: Implications for neuroscience and biological psychiatry

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Abstract

Defensive behavior is a function of specific survival circuits, the "aversive brain system", that are thought to be conserved across vertebrates, and involve threat detection and the organization of defensive responses to reduce or eliminate threat. In mammals, these circuits involve amygdalar and hypothalamic subnuclei and midbrain circuits. The increased interest in teleost fishes as model organisms in neuroscience created a demand to understand which brain circuits are involved in defensive behavior. Telencephalic and habenular circuits represent a "high road" for threat processing and organization of responses, being important to mounting appropriate coping responses. Specific hypothalamic circuits organize neuroendocrine and neurovegetative outputs, but are the less well-studied in fish. A "low road" is represented by projections to interneurons in the optic tectum which mediate fast escape responses via projections to the central gray and/or the brainstem escape network (not shown). Threatening stimuli (especially visual stimuli) can bypass the "high road" and directly activate this system, initiating escape responses. Increased attention to these circuits in an evolutionary framework is still needed.

Keywords: Fear; Anxiety; Aversive brain system; Comparative neuroanatomy; Teleost fish

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1. Introduction

Defensive behavior is a function of specific survival circuits that are highly conserved across vertebrates (LeDoux, 2012), and involve threat detection, as well as the organization of species-specific defensive responses to reduce or eliminate threat (LeDoux, 2012). In mammals, these circuits involve sensory systems that detect conditioned and unconditioned threatening stimuli, amygdalar circuits that select appropriate behavioral and neuroendocrine responses and promote aversive learning, hypothalamic circuits for both defensive behavior and neuroendocrine responses, and midbrain circuits that control the output (LeDoux, 2012). This survival circuit for defensive behavior links this appraisal phase, in which the global organismic state is determined based on threat detection, to a response phase, in which "the instrumental physiological and behavioural responses are determined and executed" (Andersen et al., 2016, p. 5). Different threat levels restrict attention "in the current global organismic state [to make] the organism focus on a short-term motive to […] survive a threat" (Andersen et al., 2016, p. 6).

In fish, defensive circuits have not been thoroughly described, but there is and increasing awareness that defensive behavior is a fundamental function in these animals (Andersen et al., 2016; Kalueff et al., 2012; Kittilsen, 2013). As a result, fish are increasingly being used in neurobehavioral research (Gerlai, 2014; Hall et al., 2014; Kalueff et al., 2014b; Stewart et al., 2015). A framework for establishing homologies for defensive circuits in fish, therefore, is useful for this enterprise. Classical neuroscientific approaches to describing behavioral circuits involve the demonstration that a given structure is activated (or inhibited) by specific stimuli or behavioral paradigms, as well as the effects of lesioning (or silencing) and stimulating the structure. Even though there are many technical difficulties in performing functional neuroanatomy in the diminutive zebrafish, novel genetic techniques (Friedrich et al., 2010) have allowed important discoveries regarding the limbic system.

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The increase in the use of zebrafish (Kalueff et al., 2014a) and other species (Hall et al., 2014) as organisms in behavioral models for anxiety- and fear-like behavior also prompted the need to describe the neural structures that are involved in these behaviors (Guo et al., 2012). While the unspoken assumption in the field appears to be one of "multiple realizability" (Bickle, 2010) – including the idea that it is the behavioral function that needs to be similar, and not the brain circuits underlying it, in order to use these assays as models in psychopathology (Wright, 2002) –, at least from an evolutionary point of view it is interesting to assess whether the putative conservation of behavioral responses to aversive stimuli is accompanied by conservation of the neural substratum (Striedter et al., 2014). The present article reviews the circuitry that is involved in detecting, identifying, processing, and responding to threat in fish, with a special focus on zebrafish models.

2. Discovering neural circuits for aversive behavior in fish

An important starting point in determining the aversive behavior network in zebrafish is activation studies. Typically, this is done by examining immediate early gene products, such as the *cfos* gene and its protein, c-Fos. A network comprised of the medial nucleus of the dorsal telencephalon (Dm), ventral nucleus of the ventral telencephalon (Vv), supracommissural nucleus of the ventral telencephalon (Vs), and preoptic region (POA) shows upregulated *cfos* expression after exposure to conspecific alarm substance [CAS] (Faustino et al., 2017). The expression of c-Fos during CAS exposure appears to be specific, as no increases were observed in the lateral nucleus of the dorsal telencephalon (Dl), dorsal nucleus of the ventral telencephalon (Vd), habenula, or thalamus (Ruhl et al., 2017). Dm is also activated after exposure to the light/dark test (Lau et al., 2011; von Trotha et al., 2014), an anxiogenic stimulus for zebrafish (Maximino et al., 2010a). Thus, it appears that a network involving Dm, Vv, Vs, and POA is involved in detecting and/or processing aversive stimuli and/or selecting behavioral strategies in zebrafish. A network for the retrieval of fear memo-

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ries include Dm, Dl, and thalamus, as these structures showed c-Fos expression during presentation of a red light that has been previously associated with CAS (Ruhl et al., 2017).

Other aversive stimuli have also been used to investigate threat detection and processing in zebrafish larvae. A looming stimulus, which simulates predatory attack, activates neurons in the optic tectum (Dunn et al., 2016; Temizer et al., 2015). Tyrosine hydroxylase-positive neurons in the caudal hypothalamus (Hc) respond to aversive stimuli, including pH change, high ammonia, and handling stress (Semenova et al., 2014). Using immunohistochemical detection of phosphorylated extracellular signal-regulated kinase (pERK), Randlett et al. (2015) mapped the activity of nuclei in the larval brain in response to different aversive stimuli (exposure to mustard oil, dish taps, heated water, electric shocks). The authors found that while each stimulus promoted pERK activity in specific regions (due to the diversity of sensory channels tapped by each stimulus), all four activated the locus coeruleus, caudal hindbrain, Hc, POA, and subpallium. It is not clear whether the subpallial regions observed in this experiment overlap with those found in CAS-exposed adults (i.e., Vv and Vs); nonetheless, these results suggest a wider network that is specialized in the detection of aversive stimuli which includes pallial and subpallial components of the amygdala homologues, the neuroendocrine hypothalamus, optic tectum, locus coeruleus, and the brainstem escape network.

The focus of this review will now turn to each of these foci. We will quickly review some of the evidence for proposing these areas as full or partial homologues of specific mammalian nuclei which have been implicated in defensive behavior. Importantly, we will review evidence for the participation of these regions in aversive behavior in fish, and delineate, when possible, the circuitry that is involved in these roles.

3. Pallial and subpallial components of the teleostean amygdala

The fact that the teleostean telencephalon is everted precluded, for a long time, the easy establishment of homologies for telencephalic structures (Nieuwenhuys, 2011). Different eversion

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models lead to different proposed final topologies of pallial structures in adult fish (Braford, 2009; Butler, 2000; Mueller et al., 2011; Nieuwenhuys, 2011; Northcutt, 2008; Wullimann and Mueller, 2004; Yamamoto et al., 2007);as a result, different propositions have been made regarding amygdala homologues in zebrafish. Based on topology and topography, cytoarchitectonics, neurochemistry, expression of developmental regulatory genes, and behavioral data, we will follow our previous proposal that the medial nucleus of the dorsal telencephalon (Dm) is homologous to the "limbic associative" amygdaloid system (lateral and basolateral amygdala of mammals), while the subcommissural and postcommissural nuclei of the ventral telencephalon (Vs and Vp, respectively) are homologous to the "autonomic" amygdaloid system (central extended amygdala [CEXA]) (Maximino et al., 2013a).

Lesion studies implicated the Dm in associative learning of aversive memories in goldfish. Ablation of the Dm, but not of the Dl, impair the acquisition and maintenance of two-way (active) avoidance acquisition in this species (Portavella et al., 2004b, 2004a, 2002; Portavella and Vargas, 2005). c-Fos studies with zebrafish have also suggested a role for the Dm in aversive conditioning: the association between a red light and CAS produces conditioned responses to the light that are very similar to the innate repertoire of zebrafish (Hall and Suboski, 1995a, 1995b; Ruhl et al., 2017). Interestingly, during the acquisition phase (when the red light and the CAS are presented together), the number of c-Fos-positive cells is higher in the Dm, but not in the lateral nucleus of the dorsal telencephalon (Dl, the hippocampal homologue; Goodson & Kingsbury, 2013) or in the dorsal nucleus of the ventral telencephalon (Vd, the striatum homologue; Ganz et al., 2011). 24 h later, during the retrieval phase (when only the red light is presented), c-Fos-positive cells are increased in the Dm, Dl, and thalamus (Ruhl et al., 2017). Thus, it appears that the Dm is involved in both the acquisition and the maintenance of aversive memories in cyprinids.

The majority of neurons in pallial regions are glutamatergic (von Trotha et al., 2014), with some GABAergic interneurons. Lal et al. (2018) produced *Gal4FF* zebrafish lineages driving the

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expression of the botulinum toxin B light chain (BoTxBLC) gene that showed deficits in active avoidance conditioning. Among these lineages, two showed expression limited to the Dm, and in both the trapped gene was *emx3*. In addition to showing deficits in conditioned aversive behavior, these animals also showed changes in their response to CAS, with decreased freezing but increased erratic swimming. Only 16% of the cells in the Dm express the Gal4FF construct, and the majority are neurons; 94% of these neurons are glutamatergic. These neurons project heavily to the hypothalamus, including anterior tuberal nucleus (ATN), lateral hypothalamic nucleus (LH), and dorsal zone of the periventricular hypothalamus, as well as to telencephalic regions (entopeduncular nucleus, preoptic area, Vd, and Vs), suggesting neural networks involved in both conditioned and unconditioned aversive behavior (Lal et al., 2018).

The role of the Dm in unconditioned behavior has also been assessed. In zebrafish, exposure to the light/dark test increases c-Fos expression in the Dm (Lau et al., 2011; von Trotha et al., 2014). Moreover, CAS exposure also increases *cfos* in the Dm of zebrafish (Faustino et al., 2017), suggesting that ethologically-relevant aversive stimuli recruit these cells even when conditioning is not involved. As already discussed, expression of BoTxBLC in a subset of Dm neurons in zebrafish decreases freezing responses and increases erratic swimming after CAS exposure (Lal et al., 2018). A participation in restraint stress-induced behavioral responses has also been suggested. In Nile tilapia (*Oreochromis niloticus*), restraint stress increases 5-HT metabolism in the Dm (Silva et al., 2014). Likewise, acute stress (lowering water levels) increases *htr1aa* and *htr1ab* expression in the Dm of Rainbow trout (Vindas et al., 2017); interestingly, 5-HIAA levels were increased in the Dm only in those animals which were classified as “proactive” in relation to a behavioral response to hypoxia (Vindas et al., 2017), suggesting that 5-HT acts in this nucleus to mount active responses to remove stressors and restore homeostasis. Consistent with the hypothesis of a role of the Dm in active responses to aversive stimuli, inhibition of the Dm in *Leporinus macrocephalus* by injecting midazolam abolished stress-induced analgesia (Wolkers et al., 2015).

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If there is considerable evidence for the homology between Dm and the limbic associative amygdala of mammals, this is not the case for the homology between Vs/Vp and the autonomic amygdala; in fact, it is more probable that these regions are only *partially* homologous to the CEXA (Goodson and Kingsbury, 2013; Maximino et al., 2013a). In mammals, the CEXA has been proposed to mediate the selection of vegetative and behavioral responses to threatening stimuli (Gozzi et al., 2010; LeDoux and Pine, 2016), as well as conditioned fear (Ciocchi et al., 2010; Haubensak et al., 2010; LeDoux, 1998). The circuit acts by tonically inhibiting downstream responses mediated by the periaqueductal gray area (PAG) and hypothalamus (freezing, flight), or by the basal forebrain (risk assessment); inhibition of these circuits (by, e.g., signals from the "limbic associative" amygdala) release behavior in one of these two streams, producing appropriate active or passive responses to the threatening stimulus (Maximino, 2012). In zebrafish, these neurons are also GABAergic (Mueller and Guo, 2009), suggesting a similar circuit.

Almost no behavioral evidence exists for the role of Vs/Vp in defensive behavior. One of the possible reasons is that the Vs and Vp are continuous with the Vd in a rostrocaudal axis, leading authors to report effects (or lack thereof) of interventions in wrongly identified structures. One important exception is the observation that CAS increases *cfos* expression in the Vs (Faustino et al., 2017). Interestingly, as reported above, this work also found increased *cfos* in the Dm, Vv, and POA. Importantly, control animals showed functional connectivity between these regions, while exposure to CAS decreased these correlations; in fact, animals exposed to CAS showed only co-activation of Dm-Vs and Vv-Vs (Faustino et al., 2017). The precise mechanism for this decreased coherence is yet to be described; however, considering the equivalent mammalian circuit, it is possible that activation of the Dm activates inhibitory neurons from the Vs, which could be responsible for decreased connectivity with Vv and POA, while at the same time disinhibiting downstream (hypothalamic and mesencephalic) mechanisms for response emission (Figure 1).

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In summary, the Dm appears to be important for both conditioned and unconditioned aversive responses, as well as to mounting appropriate (active vs. passive) responses. The Vs also appears to participate in processing unconditioned threat, but its role in response selection and learning is still unknown.

3. Does the habenula participate in defense?

The habenulae are paired structures located in the roof of the rostral diencephalon of fishes, divided classically into a dorsal, asymmetric portion (dHb) and a ventral, symmetric portion (vHb). While subdivisions from the vHb have not yet been identified, the dHb can be further subvidided into medial and lateral subnuclei (Aizawa et al., 2011; Okamoto et al., 2011, 2008). The vHb receives (putatively GABAergic) projections from the ventral portion of the entopeduncular nucleus (Okamoto et al., 2011), which by its turn receive excitatory projections from the Dm (Lal et al., 2018).

The difference in size between left and right habenulae is due mainly to the enlargement of the lateral subnucleus (dHbL) in the left habenula in relation to the right, which shows an enlarged medial subnucleus (dHbM) (Amo et al., 2010; Okamoto et al., 2011). This difference correlates with parapineal asymmetry (Gamse et al., 2003) and is concordant with the lateralization of viscera (Barth et al., 2005; Domenichini et al., 2011) and subsets of behavioral functions (Barth et al., 2005; Dadda et al., 2010; Facchin et al., 2009). In the left dHb, high levels of the potassium channel tetramerization domain-containing protein 12.1 (*kctd12.1/leftover/lov*) are expressed, while low levels are observed in the right dHb. Conversely, two other members of the KTCD family, *kctd12.2* (*right on/ron*) and *kctd8* (*dexter/dex*), are expressed exclusively in the right dHb (Beretta et al., 2012; Gamse et al., 2005; Y. Kuan et al., 2007; Roussigné et al., 2011). These genes have been used as markers for the neuroanatomical divisions of the dHb.

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While the dHb is asymmetric, the ventral habenula (vHb) is symmetric (Amo et al., 2010; Okamoto et al., 2011). The vHb is characterized by the expression of *diamine oxidase* (*dao*), *lov* and *protocadherin 10a* (*pcdh10a*) mRNA, in a complementary fashion with the expression of *POU domain, class 4, transcription factor 1* (*brn3a*) which is expressed exclusively in the medial subnucleus (Amo et al., 2010). Again, these genes have been used as markers for the neuroanatomical subdivision of the vHb.

The neuroanatomical asymmetry of the dHb has produced an interesting literature on its behavioral correlates, especially in zebrafish. The neuroanatomical asymmetry correlates with behavioral asymmetries in different assays. Animals with left parapineal position (L-PPO) tend to use their right eye when viewing a mirror and swim preferentially in a clockwise direction, while animals with right parapineal (R-PPO) do not show this preference; conversely, R-PPO zebrafish use the right eye to inspect a live predator, while L-PPO animals show no preference (Taylor et al., 2011). Importantly, while these animals show preference for eye use for a stimulus which elicits aggression (mirror image) or defensive behavior (predator), no eye preference whatsoever is observed towards “neutral” stimuli (Y.-S. Kuan et al., 2007) In addition to the lateralization of behavior, some important differences are observed in adult behavior in other domains. In the first minutes of an open tank test, R-PPO fish show less thigmotaxis than L-PPO fish; likewise, R-PPO fish spend more time near a predator towards the end of the task (Aizawa et al., 2011; Beretta et al., 2012; Concha et al., 2009; Okamoto et al., 2011). In the *frequent-situs-inversus* lineage, larvae with a left-lateralized habenula (LH) begin to view their mirror image with the right eye, but then change to the left eye by the end of a five-minute period; in larvae with a right-lateralized habenula (RH), this pattern is reversed (Dadda et al., 2010; Domenichini et al., 2011). When adults are confronted with a two-choice bead test, LH *fsi* fish bite the right target, while RHT *fsi* fish bite the left target. In an emergence test, when LH *fsi* fish are confronted with a black stripe they show progressively increasing latencies to change compartment; while this effect is observed in RH *fsi* fish, it is much

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smaller (Barth et al., 2005). These results seem to suggest that a right-lateralized habenula decreases responsiveness to novel and threatening stimuli.

Contrary to this hypothesis, however, zebrafish expressing the tetanus toxin light chain in the dHbL [Tg(*narp:GAL4*^{VP16}; UAS:TeTxLC) lineage] show more bottom-dwelling in a novel tank, as well as increased freezing responses to an alarm substance combined with the presentation of a moving shadow above the tank (Mathuru and Jesuthasan, 2013); nonetheless, *c-fos* or *egr1* expression is not significantly changed in any Hb nuclei after exposure to an alarm substance in zebrafish (DeCarvalho et al., 2013). On the other hand, in larvae, expression of botullin toxin light chain [Tg(*gng8: Gal4*; UAS:BoTxBLC-GFP)] in the dHbL (but not in the dHbR) impairs light preference (Zhang et al., 2017), consistent with decreased anxiety (Steenbergen et al., 2011). Adult animals with silenced dHbL show increased dark preference (Zhang et al., 2017), consistent with *increased* anxiety (Maximino et al., 2010a). Moreover, exposure to the light/dark assay, a model of anxiety-like behavior, increased *cfos* mRNA expression in the dHb of zebrafish, but only if they are handled before the experiment (Lau et al., 2011). These results suggest that the zebrafish dHbL is an important center in defensive behavior, being necessary to mount an adaptive response to innate aversive stimuli; in adults, dHbL appears to inhibit anxiety- and fear-like behavior, while in larvae it appears to increase it.

Other important pharmacogenetic work attempted to establish the role of the habenula in stimulus appraisal and behavioral control in zebrafish. In the first work, Agetsuma and colleagues (2010) expressed the tetanus toxin light chain [Tg(*narp:GAL4*^{VP16}*; UAS:TeTxLC*)] or a nitroreductase-mCherry fusion protein in dHbL neurons, and were thus able to block synaptic transmission in that area. When they reached maturity, animals were trained in a cued fear conditioning task; while controls showed increased flight behaviors to the cue after conditioning, dHbL-silenced fish instead showed persistent freezing to the cue. In another experiment, Lee et al. (2010) expressed the photo-sensitizer KillerRed in a ventral telencephalon-habenula projection, and

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photobleaching on the left habenula led to deficits in the acquisition of two-way avoidance and hyperarousal after light onset before conditioning. They also expressed TeTxLC in the dHbL, obtaining the same result regarding avoidance conditioning deficit.

Interestingly, the dHbL has also been implicated in social conflict resolution. Chou et al. (2016) showed that, after the establishment of social dominance, loser (socially submissive) fish show intense activity in the ventral IPN and median raphe and a very reduced responsiveness of the dorsal IPN after acute electrical stimulation of the Hb. These results suggested a participation of different dHB-IPN pathways in the behavioral plasticity that is associated with the losing experience. Indeed, zebrafish expressing the tetanus neurotoxin in the dHBM [Tg*(gpr151:GAL4VP16; brn3a-hsp70:GFP-Cre; UAS:loxP-DsRed-loxP-GFP-TeNT)*] show a consistent trend to win fights, while dHBL-silenced [Tg*(narp:GALVP16; UAS:TeNT)*] fish showed a consistent trend to lose fights (Chou et al., 2016). The authors proposed that the reduction in the dHBL-iIPN-GC pathway could switch behavior from offensive behavior to defensive behavior (i.e., from attacking to fleeing), resulting in losing the fight. Conversely, reduction in the dHBM-vIPN-MRN pathway results in winning the fight by disinhibiting the MRN, which would tend to increase resilience to aversive stimuli.

The role of the vHb has been less extensively studied. An interesting exception is the work of Amo et al. (2014), which demonstrated two types of responses in vHb neurons after fear conditioning. One type responds phasically to an unconditioned stimulus (US) before conditioning, a response which is gradually substituted by sustained firing for all duration of the presentation of the conditioned stimulus (CS). A second type responds phasically to the US and gradually substitute this response to phasic firing to the CS. Amo et al. (2014) suggested that these responses code for aversive expectation value and prediction error, respectively. Multi-unit activity of the vHb increases in the early stages of active avoidance conditioning and later returns to normal, suggesting that the vHb encodes the negative reward expectation value in active avoidance learning (Amo et

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al., 2014). Expression of TeTxLC in the vHb abolishes active avoidance learning without alterations in basal anxiety-like behavior or fear conditioning, strengthening the hypothesis of a specific role in active avoidance conditioning; finally, pairing the optogenetic activation of the vHb with a specific tank compartment elicits avoidance of that compartment only when the stimulation is tonic, but not when it is phasic. Overall, these results suggest that tonic responses in the zebrafish vHb represent an aversive expectation value, participating in a larger vEP-vHb-MRN circuit (Amo et al., 2014).

Another important evidence for the role of the vHb comes from work with kisspeptin, a system of peptides associated with reproductive behavior. In zebrafish, the neuropeptide Kiss1 is expressed only in the vHb, while Kiss2 is expressed in the hypothalamus (Servili et al., 2011). These Kiss1-positive co-express glutamate, and project to glutamatergic interneurons in the IPN and MRN (Nathan et al., 2015a). Interestingly, kissr1 receptors are not found in the raphe, but can be found in the vHb and the IPN (Ogawa et al., 2012). Intracranial administration of Kiss1 increases c-Fos expression in the vHb and MRN, and increases the expression of serotonergic system-related genes (*pet1* and *slc6a4a*) (Ogawa et al., 2014, 2012). Importantly, Kiss1 blocks responses to CAS (Ogawa et al., 2014), an effect which is mediated by 5-HT_{1A} and 5-HT_2 receptors (Nathan et al., 2015b). These results underline a mechanism by which the vHb-MRN circuit works: aversive stimuli Kiss1/glutamatergic neurons in the vHb, which project to excitatory interneurons in the IPN and raphe. This feed-forward mechanism induces the activation of the raphe, encoding expectations of dangerous outcomes. These expectations can be compared to real outcomes by the activation of 5-HT_{1A} and 5-HT_2 receptors, which have been shown to be important in controlling aversive behavior in zebrafish (Maximino et al., 2014, 2013b; Nowicki et al., 2014).

4. Hypothalamic circuits for defense

The hypothalamus of teleosts fish presents pair of ventrolaterally extending hypothalamic lobes. These lobes ranges from moderately elevated lobes up to hemisphere-like corpora reaching

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almost the size of the optic tectum (Senn, 1981). The use of diverse model systems to study hypothalamus development has provided evidence that the molecular pathways regulating hypothalamic induction and patterning are generally conserved from fish to mammals. In addition, the basic hypothalamic cell types and the codes of gene expression that specify them are also highly homologous throughout vertebrate species (Xie and Dorsky, 2017). The fish hypothalamus contains equivalents to most if not all of the mammalian hypothalamic cell types. The hypothalamic neurons are all located in stereotypical clusters within the ventral diencephalon hypothalamic and neuronal populations that control the pituitary in fish have been conclusively shown to be functionally analogous to their mammalian counterparts (Machluf et al., 2011).

In teleost fish, partial homologies for hypothalamic nuclei that are relevant for defensive behavior have been proposed: the preoptic area (POA), for example, is composed of cells of different sizes that form clusters considered homologous to mammalian hypothalamic nuclei (Goodson and Kingsbury, 2013). Groups of magnocellular and gigantocellular cells are considered homologous to the supraoptic nucleus, while the cluster of parvocellular cells is supposed to be homologous to the paraventricular nucleus (Moore and Lowry, 1998). Between the POA and ventral hypothalamic region there is a transition zone called ventral tuberal region (VTN) that is thought as homologous to the mammalian AH, and in the ventrocaudal part of the hypothalamus is located the anterior tuberal nucleus (ATN), a putative homolog of the mammalian VMH (Forlano et al., 2005; Forlano and Bass, 2011; Goodson, 2005; O'Connell and Hofmann, 2011). The AH is a source of vasopressin in the mammalian brain, and activation of these vasopressinergic neurons increases aggressive behavior in rodents (Gobrogge et al., 2007).

Hodology also supports the homology of these regions. Both the ATN Several hypothalamic regions of teleosts, including ATN and VTN, are connected to the Vs (Folgueira et al., 2004), which has been proposed as homologous to the MeA (Biechl et al., 2017) or to the extended CeA (Maximino et al., 2013a) – regions which, as discussed above, participate in the processing of

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aversive stimuli and defensive responses of both mammals and teleosts. Thus, it is possible that these connections of hypothalamic regions to Vs are related to regulation of defensive behavior in fish.

Unfortunately, no functional studies have been made assessing the role of he ATN and VTN in behavioral responses. In teleosts, vasotocin (AVT, the homologue to vasopressin) is also expressed in the VTN (Rodriguez-Santiago et al., 2017), and manipulating AVT levels decreases antipredator behavior in zebrafish (Braida et al., 2012). However, AVT is also expressed in other brain regions, including the POA and pallial and subpallial amygdala (Rodriguez-Santiago et al., 2017), precluding any speculation as to the role of the VTN. The ATN of teleosts also expresses sex steroid hormone receptors, as is the case of the VTN (O'Connell and Hofmann, 2012). Stimulation of the ATN elicits reproduction-related vocalizations in male midshipman fish *Porichthys notatus* (Goodson and Bass, 2000), suggesting a role in reproductive behavior; however, a role in defensive behavior has not yet been determined.

Indirect evidence for a participation of the hypothalamus in defensive behavior is stronger in relation to neuroendocrine endpoints, especially cortisol responses, given that these responses are under descending hypothalamic control. Aversive stimuli which have been shown to elicit cortisol responses in zebrafish include acute chasing stress (de Abreu et al., 2016; Idalencio et al., 2017; Tran et al., 2014), acute restraint stress (Abreu et al., 2017a; Ghisleni et al., 2012), unpredictable chronic stress (Piato et al., 2011), exposure to the novel tank or the light/dark test (Kysil et al., 2017), and subordinate-dominant interactions (Pavlidis et al., 2011), . CAS also elicits increases in cortisol responses in Nile tilapia (Silva et al., 2015) and zebrafish (Abreu et al., 2017a; Schirmer et al., 2013); interestingly, cortisol responses are also observed after visual contact with a predator in zebrafish (Barcellos et al., 2010, 2007), and *D. rerio* also appears to be able to communicate predation risk to conspecifics, since cortisol responses are observed after seeing a shoalmate displaying antipredator behavior (Oliveira et al., 2017, 2013). However, presently little is known

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about either a causal relationship between cortisol and behavioral responses to aversive stimuli in fish, or about which hypothalamic regions are involved in these responses.

5. Tectal circuits for detection of visual threatening stimuli

The most prominent structures within the fish tectum are the optic tectum (TO) and torus semicircularis (TS); they are homologous to the mammalian superior and inferior colliculi, respectively (Nieuwenhuys et al., 1998). Zebrafish present at least six easily identifiable tectal layers (from superficial to deeper: marginal [MS], optic [SO], superficial fibrous and gray [SFGS], central gray [SGC], central white [SAC], and periventricular strata [SPV]). It has been observed that, in all vertebrates, the upper layers of the tectum are retinorecipient, while the deeper layers house the projection neurons (Butler and Hodos, 2005). In rodents, information from the upper visual field is represented in the medial optic tectum, while information from the lower visual field is represented in the lateral portion; likewise, stimulation of the lateral portion leads to approach-like and appetitive movements, while stimulation of the medial portion leads to defensive-like behavior (Brandão et al., 2003, 1999). In rodents, these medial regions receive exclusive projections from multimodal and association sensory cortices, visual thalami, hypothalamic nuclei associated with defensive behavior, and a few pretectal nuclei (Comoli et al., 2012). In goldfish, the medial tectal zone seems to be related to orienting responses, the anteromedial zone to goal-directed saccades, the extreme anteromedial zone to eye convergence, and the posterior zone to escape responses (Herrero et al., 1998; Salas et al., 1997).

Many different interneurons have been described in the OT (Figure 2A). Superficial interneurons (SINs) in the superficial layers (SO and SFGS) are GABAergic (Scott and Baier, 2009). About 75% of the cells from the periventricular stratum (SPV) are GABAergic, while 10% are glutamatergic (Nevin et al., 2010; Robles et al., 2011). Glutamatergic cells from the tectum are of the bistratified periventricular (bsPVIN) interneuron type, with somata which locate in the deeper and

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intermediate regions of the SPV, a single apical process that spans the SGC and SFGS, and glutamatergic axons which terminate in the layer between both of these strata (Nevin et al., 2010; Robles et al., 2011). Another group of cells, the non-stratified periventricular (nsPVIN) type, is a small population of GABAergic interneurons located deep in the SPV, arbor in the deeper regions of the SGC and SFGS; they lack the stratification and laminar specificity of bsPVIN cells, with their axons terminating mostly in the SGC and in between this stratum and the overlying SFGS and the underlying SAC (Nevin et al., 2010; Robles et al., 2011; Scott and Baier, 2009). Finally, periventricular projection neurons (PVPNs) are GABAergic cells with a dendritic arbor spanning the regions between SFGS and SGC, the SGC itself, and the region between SGC and SAC, and an axon that forms a sparse arbor of collaterals in the vicinity of the lateral longitudinal fascicle and the hindbrain escape network (Nevin et al., 2010; Robles et al., 2011; Scott and Baier, 2009). Some periventricular projection cells course medially and terminate in the superior raphe (Nevin et al., 2010), but it is not known whether these cells are GABAergic or not; however, lipophilic dye injection into the tectal neuropil marks a much larger population of axons than the GABAergic neurons, suggesting that this is a small and very specialized projection (Nevin et al., 2010; Scott and Baier, 2009).

bsPVINs, nsPVINs and PVPNs have been thought of as representing different parts of a circuit for detecting visual threats and selecting appropriate responses (Fig. 2B). The distribution of the neuropil, as well as the glutamatergic nature of the bsPVIN neuron type suggest that these cells perform superficial to deep information transfer (Nevin et al., 2010; Robles et al., 2011). In contrast, the inhibitory nsPVIN is a GABAergic interneuron, producing feed-forward inhibition to filter the visual information transmitted onto projection neurons (Robles et al., 2011). Moreover, the location of their dendrites primarily in the SGC layer, which receives non-visual afferents from the telencephalon and thalamus, suggest that they might integrate inputs from visual and non-visual areas (Nevin et al., 2010). Since projection cells from the tectum have dendrites in the deeper and intermediate layers of neuropil (but not the superficial layers), this suggests that they are not directly

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retinorecipient (Butler and Hodos, 2005), and therefore must receive information from bsPVIN and nsPVIN cells.

In zebrafish, the role of these circuits in escape responses has been described in a series of elegant experiments in larvae. Zebrafish larvae respond to a moving dot stimulus in a size-dependent way: if the stimulus is relatively small (e.g. potential prey), the animal approaches it, while larger stimuli (e.g., potential predators) are avoided (Barker and Baier, 2015). Silencing tectal neurons completely abolishes both approach and avoidance responses, irrespective of stimulus size. When SINs are ablated, however, large object avoidance is impaired (Barker and Baier, 2015). Interestingly, ablating tectal cells marked in the *Gal4mpn354* line – most of which are glutamatergic nsPVINs – shift behavior from approach to avoidance, with ablated larvae avoiding small stimuli; optogenetic stimulation of these neurons produce the opposite effect (Barker and Baier, 2015). Noteworthy, larvae acutely treated with fluoxetine, therefore increasing serotonergic tone, decrease the probability of avoiding small and medium stimuli, but do not affect responses to larger stimuli (Filosa et al., 2016), suggesting that this neurotransmitter does not participate in the control of escape responses by the OT, but instead participates in shifting from fleeing to foraging.

Using a different stimulus – a “looming” dot that increases in size, simulating a predator strike – it was demonstrated that zebrafish larvae attempt to escape this stimulus (Dunn et al., 2016; Temizer et al., 2015), and that this stimulus activates three specific targets of retinal ganglion cells: arborization fields AF6 and AF8, and the OT (Dunn et al., 2016; Temizer et al., 2015). In the OT, responses were observed in the SFGS and SGS (Temizer et al., 2015); a specific response is observed in SINs (Dunn et al., 2016). It appears that these SINs modulate the inputs from retinal ganglion cells to periventicular projection neurons, “fine-tuning” the motor command produced by these later cells to critical angle (Dunn et al., 2016). The higher density of looming-selective responses in the OT in relation to extra-tectal retinorecipient areas suggest that the OT is involved in processing looming stimuli, while AF6 and AF8 process other visual cues such as whole-field motion and lu-

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minance changes. Indeed, ablation of retinotectal projections – leaving intact projections to other AFs – impaired the ability of larvae to escape the looming stimulus (Temizer et al., 2015). Ablation of the brainstem escape network – one important motor output from OT – resulted in a specific bend deficit in response to looming stimuli, suggesting a participation of these neurons in the escape response elicited by looming stimuli (Dunn et al., 2016).

6. The brainstem escape network

The concept of a "brainstem escape network" was introduced to describe gigantocellular neurons in the brainstem of fish (the Mauthner [M-]cells, its two segmental homologs MiD2cm and MiD3cm, and other identified neurons in the reticulospinal segments adjacent to the Mauthner cells) that activate fast-start responses that are used by fish to escape predatory attacks (Eaton et al., 2001). These reticulospinal system receives massive primary acoustic input as well as sensory inputs from the optic tectum, and synapses on motoneurons that innervate trunk muscle on the contralateral side (Kinkhabwala et al., 2010). The activation of M-cells produces a robust turn of about 45º, leading to the initiation of a very fast response called C-start (Eaton et al., 1977) that is fine-tuned to the angle of stimulation; the participation of the other components of the brainstem escape network code other kinematic features that result in propelling the fish away from the stimulus (Eaton et al., 2001). These responses are very fast; in zebrafish, the latency for a C-start after acoustic stimulation was recorded as about 5 ms (Eaton et al., 1977), and the latency for looming visual stimuli varied from 10-20 ms (Temizer et al., 2015) to a few hundreds of milliseconds (Dunn et al., 2016).

The brainstem escape network receives inputs from many different regions of the teleostean brain. As described, escape responses to visual stimuli are mediated by the optic tectum (Dunn et al., 2016; Temizer et al., 2015), which projects to this system. While telencephalic projections have not yet been described, telencephalic ablation decreases startle probability in goldfish (Collins and

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Waldeck, 2006), suggesting a facilitatory role; whether this is due to ablation of pallial or subpallial amygdalar components is unkown.

Monoaminergic inputs are also important in the modulation of C-starts. In zebrafish larvae, tyrosine hydroxylase and 5-HT immunoreactivity was observed closely apposed to the ventral dendrites of the M-cell, MiD2cm, and MiD3cm, and tyrosine hydroxylase immunoreactivty was observed near the lateral dendrite (McLean and Fetcho, 2004). 5-HT increases inhibitory currents produced by activation of presynaptic pathways, while DA increases the amplitudes of electrical and glutamatergic components of auditorily evoked responses (Korn and Faber, 2005).

While this apparently simple reflex has been considered to be a “fixed action pattern”, with little modulation by upstream structures and therefore little relevance for fear- and anxiety-like states, there is interesting evidence for considerable plasticity of this system (Medan and Preuss, 2014). Larval zebrafish present prepulse inhibition (PPI), in which the probability of a C-start is reduced when it is preceded by a non-startling sound (Burgess and Granato, 2007). This prepulse sound attenuates the synaptic response of M-cells to a subsequent auditory stimulus (Curtin et al., 2013; Medan and Preuss, 2014, 2011). In zebrafish, the PPI is disrupted by apomorphine, a dopaminergic agonist (Burgess and Granato, 2007); in goldfish, apomorphine blocks the prepulse sound-evoked reduction in M-cell membrane resistance (Medan and Preuss, 2011; Neumeister et al., 2008).

5-HT has been implicated in social modulation of startle responses in the African cichlid *Astalotilapia burtoni* (Whitaker et al., 2011). In this species, dominant males show increased startle probability and lower escape thresholds when compared to subordinate males (Neumeister et al., 2010), perhaps as a compensation of the increased conspicuity caused by brighter body coloration and higher activity (Medan and Preuss, 2014; Neumeister et al., 2010). The behavioral increases in startle are accompanied by increased excitability of M-cells to auditory stimuli, as well as a reduction on the inhibitory drive (Neumeister et al., 2010). The 5-HT_2 receptor antagonist ketanserin de-

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creases feedback inhibition in subordinate, but not dominant, African cichlids (Whitaker et al., 2011). These represent presynaptic mechanisms, since only 5-HT_{5A} and 5-HT_6 receptors are expressed in Mauthner cells (Whitaker et al., 2011).

7. Conclusions

Different regions from the rostrocaudal axis appear to be involved in detecting, identifying, processing, and responding to aversive stimuli in fish. In general, threats are detected and processed at the level of the Dm, which may also be responsible for response selection at certain situations. This region is homologous to the mammalian frontotemporal amygalar cluster (Maximino et al., 2013a). The "classical model" of the role of the amygdala in fear involves aversive learning in the frontotemporal amygdala, while the behavioral output would be mediated by the autonomic/limbic amygdala (Vargas et al., 2012). Recent evidence, however, suggests that the frontotemporal amygdala is involved in encoding emotional events (including aversive stimuli and contexts) with reference to particular sensory features, while the autonomic amygdala encodes the motivational or affective significance (Balleine and Killcross, 2006). This is consistent with our hypothesis that the Dm also is responsible for mounting appropriate coping (active vs. passive) responses.

A parallel circuit for negative incentive and coping has been described in the habenula as well. The vHb appears to represent aversive expectation values and modulate aversive behavior via the median raphe nucleus (Amo et al., 2014). Similarly, a dHBM-vIPN-MRN appears to be involved in resilience to aversive stimuli and/or active coping, while the dHBL-iIPN-GC appears to be involved in selecting appropriate responses (Okamoto et al., 2011). So far, it is not known if this circuit is parallel to the (more classical) amygdalar/Dm one, or whether there are neuroanatomical connections between Hb nuclei and Dm and/or Vs, but an indirect projection to the vHb via the ventral entopeduncular nucleus has been described (Lal et al., 2018). Whether this is a disinhibitory cir-

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cuit, as appears to be the case with the Dm-Vs outputs, is unclear. It is possible that both circuits run in parallel and compete (or collaborate) to decision-making in the GC.

In addition to this "slow" pathways (the "high road"), a "quick-and-dirty" pathway for visual aversive stimuli is organized in the optic tectum (the "low road")(Carr, 2015). This "low road" is sensitive to stimulus size and critical angle, and switches from approach (small stimuli probably mean "food") to avoidance (large stimuli arriving at a specific angle probably mean "predator strike"). This circuit projects to motor regions of the medulla and spinal cord, as well as to the GC, initiating and/or modulating escape responses that are highly stereotypical. It is expected that visually threatening stimuli activate this circuit and bypass the Hb-IPN/raphe-GC or the Dm-Vs-GC circuits.

The participation of the habenular pathways in neurovegetative responses is also less clear, but a amygdalar-hypothalamic circuit has been suggested on the basis of c-Fos and p-ERK activity (Faustino et al., 2017; Randlett et al., 2015). Aversive stimuli, including CAS and physical stressors, induce cortisol (Abreu et al., 2017b; Schirmer et al., 2013) and norepinephrine and epinephrine release (Maximino et al., 2014). This is consistent with a classical model of amygdalar control of fear in rodents, in which CEXA-hypothalamic projections regulate the neurovegetative responses to threats (Misslin, 2003). Based on coherence analysis of c-Fos expression to CAS (Faustino et al., 2017), it is suggested that the Dm activates inhibitory Vs neurons, disinhibiting downstream hypothalamic mechanisms.

What is the "switch" that regulates active vs. passive coping? What conditions favor switching from the "high" to the "low" road? While the neural bases of these changes are presently unknown, environmental characteristics which lead to decision-making have been described thoroughly. For example, threat probability and distance vary in a continuum that restricts attention (Andersen et al., 2016), and therefore whether fast or slower responses are needed (Brown et al., 1999; Fanselow and Lester, 1988; Kavaliers and Choleris, 2001; Laundré et al., 2010; McNaughton

and Corr, 2004; Perusini and Fanselow, 2015). Thus, decision-making is biased towards escape (flight) or fight responses when the threat is proximal, while avoidance and freezing are elicited when threat is distal (Fanselow and Lester, 1988; McNaughton and Corr, 2004; Perusini and Fanselow, 2015). Similarly, the decision to freeze or flee is dependent on environmental affordances, such as the availability of escape routes (Blanchard and Blanchard, 1988). It has been suggested that habenular circuits act as comparators for these stimuli, biasing the organism towards careful approach or escape (Okamoto et al., 2011).

Much work is still needed to identify the regions that are responsible for aversive behavior in fish. The present review suggests some of the regions involved and presents a roadmap and framework for future research. The evolutionary relevance is clear, but it is also important to consider the implications of these findings for work in the field of behavioral models and experimental psychopathology: establishing homologies between regions involved in similar behavior strengthens the hypotheses that these behaviors are indeed conserved, an assumption of most models that is rarely tested (Maximino et al., 2010b) and of which depends the construct validity of these models. This implication opens up novel avenues for future research which ought to be prolific.

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Figure 1 – Circuits for threat detection, identification, and processing and response selection in the teleost brain. The dorsomedial telencephalon (Dm) is homologous to the frontotemporal amygdala system, and is the entry for the "high road" that terminates in the mesencephalic central gray (GC) and in the hypothalamic circuits for neurovegetative responses (POA, Hc). An indirect projection is also depicted for the ventral habenula (Hb); this projection involves a glutamatergic projection from the Dm to the entopeduncular nucleus, and a GABAergic projection from there to the ventral habenula. This can represent part of a habenular circuit in the "high road" which projects indirectly to the raphe (MRN) serotonergic neurons via glutamatergic interneurons in the interpeduncular nucleus and raphe (not shown). The "low road" is represented by projections to interneurons in the optic tectum (OT) which mediate fast escape responses via projections to the GC and/or the brainstem escape network (not shown). Threatening stimuli (especially visual stimuli) can bypass the "high road" and directly activate this system, initiating "quick-and-dirty" escape responses.

Abbreviations: Dm: medial nucleus of the dorsal telencephalon; Vs: supracommissural nucleus of the ventral telencephalon; Vv: ventral nucleus of the ventral telencephalon; Vl: lateral nucleus of the ventral telencephalon; Hb: habenula; POA: preoptic area; Hc: caudal hypothalamus; OT: optic tectum; GC: central gray; MRN: median raphe nucleus.

Full black arrows: direct projections; dashed black arrows: indirect projections. Arrows terminating in dimension lines (┤) represent inhibitory projections. Blue arrows represent outputs, while red arrows represent inputs.

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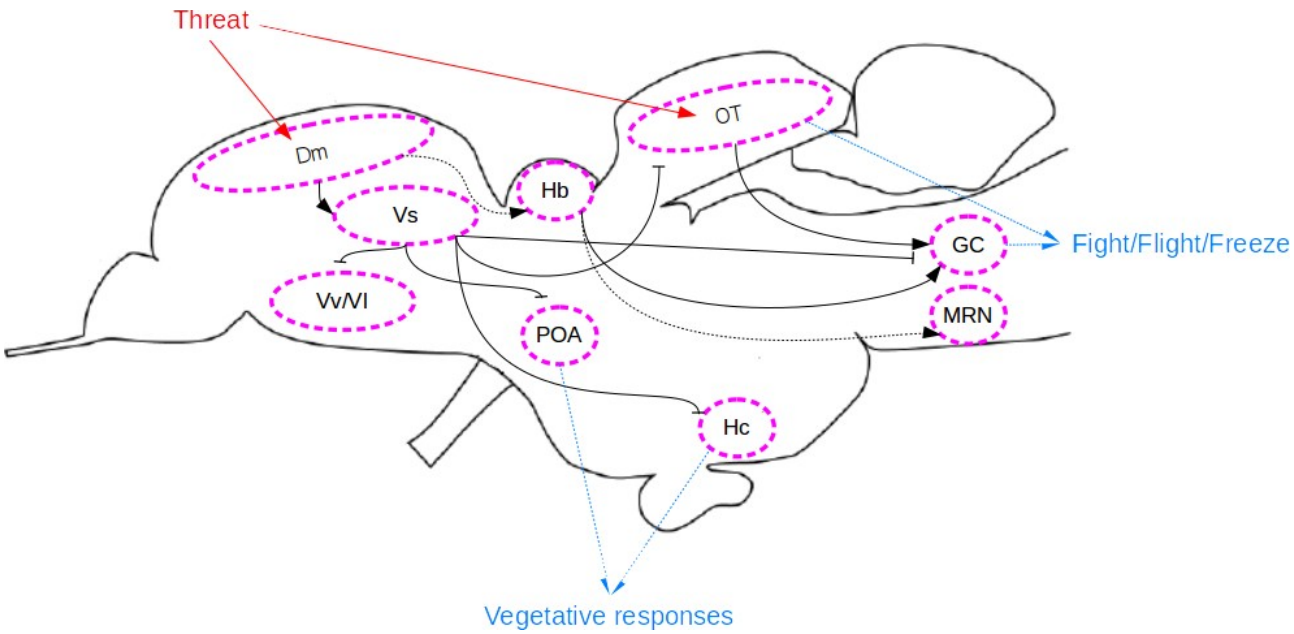

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Figure 2 – (A) Depiction of the tectal interneurons that participate in the escape circuit. The upper layers of the tectum (stratum opticum and stratum fibrosum et griseum superficiale) are retinorecipient, and also receive catecholaminergic (CAs) projections. Interneurons in the deeper layers (stratum griseum centrale, stratum album centrale, stratum periventriculare) participate in decision-making, and receive serotonergic (5-HT) and catecholaminergic projections. Identified interneuron types include the superficial inhibitory interneuron (SIN), as well as a variety of periventricular interneurons (non-stratified [nsPVIN), bistratified [bsPVIN], and mono-stratified [msPVIN] interneurons). Morphologically distinct periventricular projection neurons (PVPNs) have also been identified. (B) Stimulus properties, including size, are coded by retinal ganglion cells (RGCs) bias the circuit towards approach (smaller size) or avoidance (large size). (B) These stimuli activate inhibitory SINs and excitatory bsPVINs. SINs also fine-tune responses of bsPVINs, and disinhibit PVPNs by inhibiting nsPVINs. These projection neurons, in its turn, modulate the activity of the reticular formation, raphe, and medulla.

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