NeuroToxicology 67 (2018) 46–53

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Orexin receptor expression is increased during mancozeb-induced feeding impairments and neurodegenerative events in a marine fish

Merylin Zizza[a], Mariana Di Lorenzo[b], Vincenza Laforgia[b], Emilia Furia[c], Giovanni Sindona[c], Marcello Canonaco[a], Rosa Maria Facciolo[a,*]

[a] Comparative Neuroanatomy Laboratory, Biology, Ecology and Earth Science Department (DiBEST), University of Calabria, 87036, Arcavacata of Rende (CS), Italy
[b] Department of Biology, Section of Evolutionary and Comparative Biology, University of Naples Federico II, 80134, Naples, Italy
[c] Department of Chemistry and Chemical Technologies (DCTC), University of Calabria, 87036, Arcavacata of Rende (CS), Italy

ARTICLE INFO

Article history:
Received 31 August 2017
Received in revised form 12 April 2018
Accepted 13 April 2018
Available online 16 April 2018

Keywords:
Manganese/zinc-ethylene-bis-dithiocarbamate
Feeding latency
Food intake
Orexin receptor
Diencephalic degeneration

ABSTRACT

Food intake ensures energy resources sufficient for basic metabolism, immune system and reproductive investment. It is already known that food-seeking performances, which are crucially controlled by orexins (ORXs), may be under the influence of environmental factors including pollutants. Among these, mancozeb (mz) is becoming an environmental risk for neurodegenerative diseases. Due to few studies on marine fish exposed to mz, it was our intention to correlate feeding latency, food intake and feeding duration to potential neurodegenerative processes in key diencephalic sites and expression changes of the ORX neuroreceptor (ORXR) in the ornate wrasses (*Thalassoma pavo*). Hence, fish exposed for 4 days (d) to mz 0.2 mg/l (deriving from a 0.07, 0.14, 0.2, 0.3 mg/l screening test) displayed a significant reduction ($p < 0.05$) of food intake compared to controls as early as 1d that became more evident ($p < 0.01$) after 3d. Moreover, significant enhancements of feeding latency were reported after 1d up to 3d ($p < 0.001$) and even feeding duration was enhanced up to 3d ($p < 0.001$), which instead moderately increased after 4d ($p < 0.05$). A reduction (−120%; $p < 0.001$) of mean body weight was also detected at the end of exposure. Likewise, a notable ($p < 0.001$) activation of ORXR protein occurred together with mRNA up-regulations in diencephalic areas such as the diffuse nucleus of the inferior lobe (+48%) that also exhibited evident degenerative neuronal fields. Overall, these results highlight an ORX role as a vital component of the neuroprotective program under environmental conditions that interfere with feeding behaviors.

1. Introduction

Food intake and energy metabolism are essential for the survival of an organism since these processes ensure optimal allocation of energy resources to cover the basic maintenance of metabolism and immune system, somatic growth and reproductive investment (Rønnestad et al., 2017). External factors, such as temperature and photoperiod, stress, predators, and food availability, as well as internal factors, such as genetics, life stage, gut filling, and stored energy tend to play a major role on the success of feeding. In particular, the regulation of food intake is mainly elicited by the hypothalamus, a key diencephalic hub containing neurons that express neuropeptides involved in the regulation of feeding and energy homeostasis via the integration of peripheral signals (Volkoff, 2014). Among orexigenic neuropeptides, great attention has been recently directed to orexins (ORX-A and ORX-B) for their crucial role in appetite stimulation (Facciolo et al., 2011; Gao and Hermes, 2015) and in reward-based feeding behaviors from fish to mammals (Facciolo et al., 2012; Sakurai, 2014). As reported above food-seeking performances may be influenced by a plethora of environmental factors including dangerous pollutants that represent a serious threat for the various behaviors, such as alterations of food detection, feeding frequency and food preferences (Giusi et al., 2010). This is particularly true for fish that are continuously exposed to hazardous toxins, such as agricultural chemicals that may produce consequences on different fish organs (Atamaniuk et al., 2014; Ren et al., 2016) and indirectly on entire fish communities (Giaquinto et al., 2017).

In this regard, studies have shown that carbamates may induce different toxic effects on aquatic vertebrates such as reduction of swimming speed (Shuman-Goodier and Propper, 2016) together with an alteration of some biochemical parameters like enzymes related to neurobehavioral action (cholinesterase), detoxification

* Corresponding author.
E-mail address: rm.facciolo@unical.it (R.M. Facciolo).

https://doi.org/10.1016/j.neuro.2018.04.010
0161-813X/

process (glutathione S-transferase) and antioxidant defense (catalase; Andrade et al., 2016). Among carbamates, mancozeb (manganese (Mn)/zinc (Zn) -ethylene-bis-dithiocarbamate; mz), a fungicide belonging to ethylene-bis-dithiocarbamates (EBCD), is composed of different sub-compounds (Mn, Zn and ethylene thiourea) that together account for multiple toxic mechanisms operating simultaneously during its exposure (Geissen et al., 2010). Indeed, it is known to be a potent endocrine disruptor (Thienpont et al., 2011), as well as an inductor of oxidative damage to lipids/proteins in different organs of fish (Atamaniuk et al., 2014) and a neurotoxicant by inducing both behavioral deficits and neuronal vulnerability (Harrison Brody et al., 2013). Such a pesticide is particularly dangerous for aquatic life since it may reach the sea from the nearby agricultural lands or by means of contaminated rivers. For this reason, mz is recognized as a marine pollutant by the Pesticide Properties Database of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/424.htm). Our recent study highlighted it as an inductor of anxiety-like states and motor disturbances linked to neuronal alterations of the marine fish *Thalassoma pavo* (Zizza et al., 2017a). At date, no toxicological studies have been conducted on any other marine fish contaminated by mz especially regarding neuronal effects associated to vital physiological activities such as feeding.

On this basis, it was our intention to examine effects of a 4 day (d) exposure to mz (0.2 mg/l) on feeding-related performances (i.e. feeding latency, food intake and feeding duration) of the ornate wrasses (*Thalassoma pavo*). This concentration was chosen on the basis of a preliminary screening test of sub-lethal concentrations (0.07, 0.14, 0.2, 0.3 mg/l) handled in our laboratory. These doses are in line with those used in other studies (Jarrard et al., 2004) along with environmentally relevant concentrations detected in waterbodies near agricultural fields (Shenoy et al., 2009). At the same time, potential neurodegenerative processes in key diencephalic sites and expression changes of the ORX neuroreceptor (ORXR) in wrasses were also investigated by using both *in situ* hybridization (*ISH*) and western blotting methods. Results deriving from behavioral and neuromolecular evaluations may provide first evidences about the ability of such a fungicide to influence fish feeding behaviors in relation to neuronal responses of the ORXergic neuroreceptor system, which have been indicated as a key biomarker of stressful conditions (Pavlidis et al., 2015) including neurotoxic pollutants (Zizza et al., 2013, 2014).

2. Materials and methods

2.1. Animal housing

Before starting treatments, only adult female specimens of *Thalassoma pavo* (body weight 7–13 g, body length 8–11 cm) were used since they are smaller than males and so easier to handle. Accordingly, it was necessary to consider fish of about similar body weight and length for toxicological purposes (Zizza et al., 2017a). Fish, obtained from a local commercial supplier (San Lucido, CS, Italy), were acclimated for at least 1 week in 80 l aquaria under 12 h light:12 h dark photoperiod in aerated and filtered seawater. During acclimation fish were fed once a day at 10:00 AM with small pieces of frozen prawns corresponding to 2% of the biomass in the tank (Giusi et al., 2005). Water quality parameters i.e. salinity (35%), density (1.027–1.028 g/cm^3), hardness (100 mg $CaCO_3$/l), dissolved oxygen (8–8.6 mg/l), temperature (20–22 °C) and pH (7.5–8.0) were monitored daily to verify that they remained within the appropriate ranges. Animal maintenance and experimental procedures complied with the law n°116 (27-01-1992) and with European Directive (2010/63/EU) for the correct use of laboratory animals. Efforts were made to minimize animal suffering and reduce number of fish used.

2.2. Mancozeb exposure

The determination of the most effective sub-lethal concentration was based on a screening of different mz (Sigma, Milan-Italy) concentrations (0.07, 0.14, 0.2, 0.3 mg/l) within a 4d period in which food intake was used as a behavioral endpoint. From this screening, fish were exposed for 4d to the nominal sub-lethal concentration of 0.2 mg/l dissolved in seawater (n = 15) and compared to untreated controls (n = 15). Such a concentration also coincided with solubility indications of mz in seawater obtained by an ICP-MS analytical procedure in the same fish species that displayed evident motor alterations (Zizza et al., 2017a) as well as falling within the same range used by others (Jarrard et al., 2004).

A static renewal exposure procedure was chosen in which a daily renewal of the fungicide concentration in seawater was conducted, according to standard procedure guidelines, as previously reported (Zizza et al., 2017a). This type of exposure procedure guarantees a constant pesticide concentration within 24 h since López-Fernández et al. (2017) demonstrated that degradation of mz occurs at a later time, i.e. 39 h, at pH and temperature that are similar to those used in our study. Chemical filters were not used to prevent reduction of mz concentration and aquaria were only equipped with aerator to ensure an optimal oxygen concentration. Water parameters were monitored even during treatment to ensure that they remained within adequate ranges. For this part treated and untreated animals were fed once a day at 10:00 AM, 1 h after water change or mz renewal, at the regular feeding time in which fish were adapted to eat (Volkoff, 2014, 2017).

2.3. Feeding performances

For the behavioral evaluations the following parameters were monitored after 1d–4d of exposure:

- **Food intake:** daily, residual uneaten food was recovered, weighed and compared to the initial quantities supplied to fish in order to evaluate the quantity of food consumed after 2 h upon addition of food as previously reported (Facciolo et al., 2011).
- **Feeding latency:** upon addition of food into the tank, time needed for fish to approach food (Kuz'mina, 2011) was evaluated within a 15 min interval after having assured that all fish reached food sources.
- **Feeding duration:** total time spent executing feeding maneuvers, including both complete and incomplete feeding acts during 1 h observation, commenced upon addition of food into the tank (Abbott and Volkoff, 2011).

In addition, mean body weights of fish per tank were determined at the beginning and at the end of experiments (4d) in both treated and control fish according to the following equation: $\Delta_{body\ weight}$ = final weight-initial weight. The feeding parameters were obtained using a digital camera (SONY, DSC-W310) and videos were analyzed by the behavioral Software Etholog 2.2.5 (Visual basic; Brazil). Values were expressed as mean activity ± standard error of mean (SEM) versus their controls. At the end of the behavioral session and before molecular procedures, fish were checked to assure that only females were used in the study. Morphological observations were necessary since this species is characterized by protogynous hermaphroditism with a first sexual stage that is clearly evident by its distinct sexual dichromatism and, at the occurrence, a sex transition by female-to-male by changing their coloration and size. Even in the absence of macroscopic chromatic changes, an