

Late onset of NMDA receptor-mediated ventilatory control during early development in zebrafish (*Danio rerio*)

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Abstract

Increased ventilation frequency (f_V) in response to hypoxia in adult fish depends on ionotropic *N*-methyl-D-aspartate (NMDA) receptors. Nonetheless, the ontogeny of central control mechanisms mediating hypoxic ventilatory chemoreflexes in lower vertebrates has not been studied. Therefore, the aim of this study was to determine when the hypoxic ventilatory response during zebrafish (*Danio rerio*) development is mediated via NMDA receptors, by performing physiological experiments and western blot analysis of NMDA receptor subunits. Zebrafish larvae at stages 4–16 days post-fertilisation (dpf) were exposed to an hypoxic pulse in control groups and in groups treated with MK801 (NMDA receptor antagonist). The hypoxic increase in f_V was present at all larval stages, and it matured during development. The reflex became MK801 sensitive at 8 dpf, but did not completely rely on a glutamatergic transmission until 13 dpf. This, together with changing subunit composition during the different stages (increasing amounts of NMDAR1 subunits and appearance of NMDAR2A subunits in adults), suggests that the amount of functional NMDA receptors needed to achieve a fully developed reflex is not attained until later stages. Furthermore, our results suggest that other non-NMDA receptor mechanisms are responsible for the hypoxia-induced increase in f_V during the earlier developmental stages.

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1. Introduction

An increase in ventilation frequency (f_V) and amplitude are general ventilatory responses during periods of hypoxic challenge in adult vertebrates and it has been shown that these responses depend on central ionotropic glutamate receptors (Mizusawa et al., 1994; Ohtake et al., 1998; Sundin et al., 2003; Turesson and Sundin, 2003). In the shorthorn sculpin (*Myoxocephalus scorpius*) the f_V increase is abolished after *N*-methyl-D-aspartate (NMDA) receptor blockade (Turesson and Sundin, 2003), suggesting that glutamatergic transmission of ventilatory reflexes has been conserved throughout vertebrate evolution.

In fish, the major portion of the sensory receptors responsible for triggering oxygen chemoreflexes is situated in the gills

(Sundin and Nilsson, 2002), where they monitor the oxygen levels in the respired water and/or the blood (Milsom and Brill, 1986; Burleson and Milsom, 1993). The gill however, is not the primary site for respiratory gas exchange in early larval stages. Under normoxic conditions the zebrafish (*Danio rerio*) for instance, obtains a sufficient oxygen supply during the first 14 days post-fertilisation (dpf) simply by oxygen diffusion through the skin (Rombough, 2002). However, since the larvae both have a high overall metabolic demand (Rombough, 1988) and develop in aquatic environments with fluctuating oxygen availability, they may still require additional gas exchange via gill ventilation to maintain homeostasis, as shown in newly hatched bullfrog larvae (Burggren and Doyle, 1986). Hence, it may be necessary for fish to develop ventilatory responses during early larval stages. Due to the inherent difficulties in studying fetuses in utero, there is a lack of in vivo studies on the early development of the formation of functional native NMDA receptor control of ventilatory reflexes in mammals. Therefore, earlier studies have mainly used in vitro techniques. In one study Watanabe et al. (1992) located the presence of mRNA

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coding for NMDA receptor subunits in embryonic mouse brainstems.

The NMDA receptor is assembled from two classes of NMDA receptor subunits, the NMDAR1 and the NMDAR2 subunits, of which the latter are of four types; A, B, C and D (Nakanishi, 1992). Using cloning techniques, somewhat functional NMDA receptors can be expressed when NMDAR1 subunits form homomeric receptors on their own, but the addition of NMDAR2 subunits markedly potentiate the responsiveness of the receptor (for a review see Ozawa et al., 1998). Thus, the heterogenic configuration of the NMDA receptor is important for the formation of functional native NMDA receptors.

Zebrafish have a long history as a model organism in developmental biology, and the major scientific focus has until now been on the establishment of a functional cardiovascular system. The ventilatory system on the other hand, has received relatively little attention. However, with the inherent experimental limitations of assessing the central control mechanisms of ventilation in embryonic mammals in utero, zebrafish may be a powerful tool to reveal the development of ventilatory control mechanisms. The present study was performed to analyse the development of central ventilatory control systems in zebrafish by studying whole animal responses and the presence of NMDA receptor subunits during different larval stages. More specifically, the aim was to study the ventilatory response to hypoxia during development and the onset of a central glutamatergic control of this oxygen chemoreflex via NMDA receptor neurotransmission.

2. Materials and methods

2.1. Animals

Zebrafish larvae (*D. rerio*, Hamilton, 1822) obtained from the stationary breeding colony at the Department of Zoology, Innsbruck University, were used in the experiments (ethical permit no. GZ 66.008/4-BrGT/2004). The parent wild-type animals were raised in aquariums (56 l) containing temperate (28 °C) normoxic tap water. To stimulate spawning, a small open breeding box containing gravel material and artificial plants was placed in the aquarium the day before eggs were required. After fertilised eggs had been collected the next day from the breeding box, they were transferred to small glass beakers (100 ml) positioned afloat in a temperate (28 °C) water bath to assure constant temperature in the beakers. The water in the beakers was exchanged on a regular basis to reduce fungal growth and to remove excess food. Daily micro powder feeding (Cyclops-eeze, Argent Laboratories, supplied by Dohse Aquaristik KG, Grafschaft-Gelsdorf, Germany) commenced around the time of swimbladder filling, which occurred approximately at 5 days post-fertilisation (dpf).

2.2. Western blot protocol

To study the establishment of functional NMDA receptors during development, western blotting was employed. Two

NMDA receptor subunits; NMDAR1 and NMDAR2A, were chosen for evaluation as both are important for the formation of functional NMDA receptors (Nakanishi, 1992). Zebrafish larvae (40 larvae from each stage) and two adult zebrafish were sacrificed and the heads were removed, rinsed in PBS and weighed. As a positive control a rat brain was used, and the same procedure for harvesting protein samples as described for the zebrafish heads was applied. Lysis buffer was added (400 µL/0.1 g tissue) and the tissues were homogenized. The homogenates were centrifuged at 14 000 g for 15 min (at +4 °C) and the supernatants were stored in the freezer (at –40 °C). A BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) was used to determine the protein content in the supernatant of each stage. Thereafter, equal amounts of protein from each stage (20 µg) were loaded onto the gels after being mixed 1:1 with sample buffer (containing e.g. SDS and β-mercaptoethanol) and heated to 95 °C for 5 min. The mixtures were stored at –20 °C when not used instantly. After loading the samples onto the gels (7.5% Tris–HCL, 15 wells, 15 µL, positioned in a Mini-Protean 3 Electrophoresis Module, BioRad, Sundbyberg, Sweden) the electrophoresis ran for 45 min at 200 V. Upon completion, the gels were placed in transfer buffer to remove salts and detergents. Separated proteins were then transferred onto a nitrocellulose (NC) membrane (Trans-Blot® Transfer Medium, BioRad, Sundbyberg, Sweden) using a Mini Trans-blot unit (BioRad). The current was set to 250 mA and the blotting process continued for 60 min. Subsequently the NC membranes were washed in milli-Q water 3 times for 5 min each, and thereafter placed in blocking solution (5% milk in Tween in TBS, slowly shaken) for 60 min. After rinsing in 0.1% Tween in PBS in the same manner as in milli-Q water, the membranes were incubated with mouse anti-NMDAR1 monoclonal antibody (1:1000, Chemicon International) and rabbit anti-NMDAR2A affinity purified polyclonal antibody (1:100, Chemicon), respectively, overnight at +5 °C. As an assurance of the NMDAR1 antibody-specificity a BLAST (Basic Local Alignment Search Tool) search of the protein sequence in rat against that in zebrafish showed 98% similarity. In addition, according to Chemicon International the antibody against NMDAR2A is specific for both rat and fish. After a second rinse in 0.1% Tween in PBS the membranes were then incubated with goat-anti-mouse IgG (H+L)-HRP Conjugated antibody (1:3000) and goat-anti-rabbit IgG (H+L)-HRP Conjugated antibody (1:3000), respectively (BioRad). The membranes were developed onto Hyperfilm™ (Amersham Biosciences, Buckinghamshire, UK) using the ECL™ Western Blotting Analysis System (Amersham) and standard photo development procedures. To estimate the amount of the two NMDA receptor subunits from the different densities of the HRP marked protein band, the Labview based program Densitometry (M. Noll-Hussong, Ludwig-Maximilians University, Germany, and M. Axelsson, Göteborg University, Sweden) was used.

2.3. Animal preparation and experimental protocol

The experimental protocol was designed to study larvae at stages 4 to 16 dpf. Before the experiments began the zebrafish

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