Please cite this article in press as: Cocco A et al. Characterization of the γ-aminobutyric acid signaling system in the zebrafish (*Danio rerio* Hamilton) central nervous system by reverse transcription-quantitative polymerase chain reaction. Neuroscience (2016), http://dx.doi.org/10.1016/j.neuroscience.2016.07.018

Neuroscience xxx (2016) xxx–xxx

CHARACTERIZATION OF THE γ-AMINOBUTYRIC ACID SIGNALING SYSTEM IN THE ZEBRAFISH (*DANIO RERIO* HAMILTON) CENTRAL NERVOUS SYSTEM BY REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION

ARIANNA COCCO, A. M. CAROLINA RÖNNBERG, ZHE JIN, GONÇALO IGREJA ANDRÉ,[1] LAURA E. VOSSEN AMOL K. BHANDAGE, PER-OVE THÖRNQVIST, BRYNDIS BIRNIR AND SVANTE WINBERG *

Department of Neuroscience, Biomedical Centre, Uppsala University, P.O. Box 593, 751 24 Uppsala, Sweden

Abstract—In the vertebrate brain, inhibition is largely mediated by γ-aminobutyric acid (GABA). This neurotransmitter comprises a signaling machinery of $GABA_A$, $GABA_B$ receptors, transporters, glutamate decarboxylases (*gads*) and 4-aminobutyrate aminotransferase (*abat*), and associated proteins. Chloride is intimately related to $GABA_A$ receptor conductance, GABA uptake, and GADs activity. The response of target neurons to GABA stimuli is shaped by chloride-cation co-transporters (CCCs), which strictly control Cl^- gradient across plasma membranes. This research profiled the expression of forty genes involved in GABA signaling in the zebrafish (*Danio rerio*) brain, grouped brain regions and retinas. Primer pairs were developed for reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA levels of the zebrafish GABA system share similarities with that of mammals, and confirm previous studies in non-mammalian species. Proposed $GABA_A$ receptors are $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\delta$, $\alpha_{2b}\beta_3\gamma_2$, $\alpha_{2b}\beta_3\delta$, $\alpha_4\beta_2\gamma_2$, $\alpha_4\beta_2\delta$, $\alpha_{6b}\beta_2\gamma\gamma_2$ and $\alpha_{6b}\beta_2\delta$. Regional brain differences were documented. Retinal hetero- or homomeric ρ-composed $GABA_A$ receptors could exist, accompanying $\alpha_1\beta_y\gamma_1$, $\alpha_1\beta_y\delta$, $\alpha_{6a}\beta_y\gamma_2$, $\alpha_{6a}\beta_y\delta$. Expression patterns of α_{6a} and α_{6b} were opposite, with the former being more abundant in retinas, the latter in brains. Given the stoichiometry $\alpha_{6w}\beta_y\gamma_z$, α_{6a}- or α_{6b}-containing receptors likely have different regulatory mechanisms. Different gene isoforms could originate after the rounds of genome duplication during teleost evolution. This research depicts that one isoform is generally more abundantly expressed than the other. Such observations also apply to $GABA_B$ receptors, GABA transporters, GABA-related enzymes, CCCs and $GABA_A$ receptor-associated proteins, whose presence further strengthens the proof of a GABA system in zebrafish. **© 2016 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).**

Key words: GABA, comparative neuroscience, teleost, zebrafish, neurotransmitter systems, receptors.

INTRODUCTION

The amino acid γ-aminobutyric acid (GABA) is a widely distributed neurotransmitter in the vertebrates' central nervous system (Roberts and Kuriyama, 1968; Farrant and Nusser, 2005). Its main precursor, L-glutamic acid, undergoes decarboxylation at the α-carbon site, a reaction catalyzed, in mammals, by glutamate decarboxylase (GAD) 67 or 65 (Roberts and Kuriyama, 1968; Kaufman et al., 1991). The subcellular localization of those two isoforms is different, with GAD67 found to be almost ubiquitous in GABA-producing neurons and GAD65 specifically located at axon terminals, where it associates with mitochondria and synaptic vesicles (Kaufman et al., 1991; Buddhala et al., 2009). Both GADs function as holoenzymes with pyridoxal phosphate as cofactor, a condition also represented in the GABA-degrading GABA-α-ketoglutarate transaminase (GABA-T, also referred to as 4-aminobutyrate aminotransferase, abat; Roberts and Kuriyama, 1968).

GABAergic neurons and glutamate decarboxylases have also been identified in zebrafish (*Danio rerio* Hamilton) central nervous system (Kim et al., 2004; Delgado and Schmachtenberg, 2008). This fish species has three isoforms of GAD of which two, gad1a and gad1b, resemble the mammalian GAD67 and the third, gad2, is homologous to GAD65. Those enzymes have been localized in the adult fish cerebellum (gad2; Delgado and Schmachtenberg, 2008) and in the forebrain during embryonic development (gad1b; MacDonald et al., 2013).

*Corresponding author.
E-mail addresses: arianna.cocco@neuro.uu.se (A. Cocco), carrr0-@hotmail.com (A. M. Carolina Rönnberg), zhe.jin@neuro.uu.se (Z. Jin), goncalo.igrejaandre@research.uwa.edu.au (G. I. André), laura.vossen@neuro.uu.se (L. E. Vossen), amol.bhandage@neuro.uu.se (A. K. Bhandage), per-ove.thornqvist@neuro.uu.se (P.-O. Thörnqvist), bryndis.birnir@neuro.uu.se (B. Birnir), svante.winberg@neuro.uu.se (S. Winberg).
[1] Present address: Centre for Evolutionary Biology, The University of Western Australia, 35 Stirling hwy, Crawley, Western Australia 6009, Australia

Abbreviations: abat, 4-aminobutyrate aminotransferase; CCCs, chloride-cation co-transporters; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; GABA-T, GABA-α-ketoglutarate transaminase; GAD, glutamate decarboxylase; RT-qPCR, reverse transcription–quantitative polymerase chain reaction; SLC6, solute carrier 6; TAE, Tris–Acetate–EDTA; TBE, Tris–Borate–EDTA; *tbp*, TATA-box binding protein; *tuba1b*, tubulin α1b.

http://dx.doi.org/10.1016/j.neuroscience.2016.07.018
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GABA induces a conformational change to its ionotropic receptors, the type A GABA ($GABA_A$) receptors. Those are membrane-spanning homo- or heteropentamers (Connaughton et al., 2008; Olsen and Sieghart, 2008) and form a pore that allows the passage of anions (Bormann et al., 1987). A long extracellular amino-terminal, four transmembrane α-helices (M1, M2, M3, M4), and a short extracellular carboxyl-terminal domain are common features to the $GABA_A$ receptor subunits (Olsen and Sieghart, 2008; Sigel and Steinmann, 2012; Miller and Arcisescu, 2014). Zebrafish genome comprises 22 genes encoding for $GABA_A$ receptor subunits (α_1–α_{6b}, β_1–β_4, γ_1–γ_3, δ, π, ζ, ρ_1–ρ_{3a}) and seven subunit-like genes (α_2-like, α_3-like, two β_2-like, π-like, ρ_1-like, ρ_3-like). Experimental evidence has localized the α_1 subunit in the cerebellum (Delgado and Schmachtenberg, 2008) and α_1, α_3, ρ_1, ρ_1-like, ρ_{2a}, ρ_{2b} in the retina (Connaughton et al., 2008). $GABA_A$ receptors are selective ion channels mainly for chloride but they do allow the flow-through of other halides and small anions (e.g. bromide, bicarbonate) (Bormann et al., 1987). Chloride-cation co-transporters (CCCs) NKCCs and KCCs, which move Cl^- into and out of the cytoplasm, respectively, with regard to the extracellular space, contribute to setting the Cl^- electrochemical gradient of neurons (Payne et al., 2003). In the adult neuron this gradient moves Cl^- from the extracellular environment toward the cytoplasm, making GABA inhibitory.

GABA also exploits its inhibitory action through the heterodimeric, G-protein coupled type B GABA ($GABA_B$) receptors. The two subunits interact with a coiled-coil structure made by two α-helices, one per subunit (Burmakina et al., 2014). Activated $GABA_B$ receptors decrease adenylate cyclase activity (Wojcik and Neff, 1984) and divalent calcium membrane conductance, and increase potassium ions flow (Bowery et al., 2002). In zebrafish $GABA_B$ receptors have been found in the cerebellum (Delgado and Schmachtenberg, 2008). Of the three zebrafish genes for the $GABA_B$ receptor two, gabbr1a and gabbr1b, are homologous to the human gene for subunit B1 and a third one, gabbr2, to human B2.

Overall, there is little information on the GABA signaling system in the zebrafish, and specifically in the central nervous system and retinas. In this study the mRNA levels of the enzymatic machinery involved in GABA metabolism, as well as the receptor systems for this neurotransmitter, were measured. GABA signaling comprises other players, such as the trafficking $GABA_A$ receptor-associated protein (Chen and Olsen, 2007), gabarapa and gabarapb in zebrafish. Ion-dependent GABA removal from the extracellular environment (Chen et al., 2004) is mediated by GABA transporters. Those are transmembrane proteins that belong to the solute carrier 6 (SLC6) family, mediate secondary active transport via Na^+ and Cl^- gradients (Chen et al., 2004), and localize in both neurons and glial cells (Jin et al., 2013). In the present study, $GABA_A$ receptor-associated proteins, four GABA and four Cl^- transporters were also included, for the first time in zebrafish. The results start a mapping of the GABA system in the zebrafish brains, grouped brain regions, and retinas.

EXPERIMENTAL PROCEDURES

Experimental animals

All experimental handlings of the animals were performed according to ethical requirements in Sweden, and approved by Uppsala Ethics committee, permit Dnr. 55/13. Zebrafish belonging to the AB line were bred and eggs collected on 29th April 2014. They were put in an incubator at +28 °C (Termaks, Bergen, Norway) and checked every day; unfertilized eggs or non-lively embryos were removed. After 5 days the larvae (Kimmel et al., 1995) were placed in a 3 L Aquaneering (Aquaneering, Carlsbad, USA) tank with an aquarium heater and fed several times per day with ZM-000 (Zebrafish Management Ltd; Winchester, United Kingdom) fry food. After 15 days post fertilization they were moved into new 3 L tanks (K.H. Garpenstrand, personal communication) in an Aquaneering rack system with recirculating water, and fed with ZM-000 and ZM-100 food. The animals also started receiving brine shrimps of the *Artemia* genus (Platinum Grade Argentimia, Redmond, USA), which were hatched in the fish facility and are a protein source. When the juvenile stage was reached the fish were given flake food for tropical fish (Sera, Heisenberg, Germany) and brine shrimps. At the time of the experiments the fish were 1 year and 61 day old.

Sampling

Zebrafish were individually anesthetized in 1 L of water containing 5–10 mL of either 5% or 10% benzocaine (w/v, in ethanol) or 10% ethyl 3-aminobenzoate methanesulfonate, MS-222 (w/v, in water; both anesthetics from Sigma). The animal was pinned on a small polystyrene support and sacrificed by cutting the spinal cord. The organs of interest, brains and eyes, were soaked with RNAlater (Qiagen GmbH, Hilden, Germany, or Ambion, USA), removed by dissection and saved, again, in RNAlater. The same procedure was followed to sample cerebellum, brain stem, optic *tecta*, diencephalon and olfactory bulbs and telencephalon. Those brain areas are schematically depicted in Fig. 1. Brains were sampled for 14 fish, six females and eight males. Twenty-three fish, 12 females and 11 males, were used to sample the brain regions. For 12 of those animals, seven females and five males, the eyes were also collected. Average animal length and weight were 3.2 ± 0.29 cm and 0.37 ± 0.07 g, respectively. Extra brains and eyes were sampled to test reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primer pairs, but they were not included in the relative quantification experiment (see below). Sampling was carried out under a Wild M5A stereomicroscope (Wild Heerbrugg, Switzerland).

RNA extraction and cDNA synthesis

Brains and eyes were individually processed. Samples dedicated to primer test were processed with GenElute Mammalian Total RNA Miniprep Kit (Sigma), including instructions' optional steps. Brains and eyes for relative

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