Neurochemistry International 61 (2012) 207–218

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

# The alteration of 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors is involved in neuronal apoptosis of goldfish cerebellum following traumatic experience

Xueqing Hu [a,1], Yan Li [b,1], Zhiying Hu [c], John A. Rudd [d], Shucai Ling [b], Fangzhen Jiang [a], Henry Davies [e], Marong Fang [b,*]

[a] Division of Plastic and Reconstructive Surgery, The Second Affiliated Hospital, Medical College, Zhejiang University, Hangzhou, China
[b] Institute of Anatomy and Cell Biology, Medical College, Zhejiang University, Hangzhou, China
[c] Department of Obstetrics and Gynaecology, Hangzhou Red Cross Hospital, Hangzhou, China
[d] School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China
[e] Medical College, Zhejiang University, Hangzhou, China

ARTICLE INFO

*Article history:*
Received 4 December 2011
Received in revised form 20 April 2012
Accepted 25 April 2012
Available online 3 May 2012

*Keywords:*
Posttraumatic stress disorder
5-$HT_{2A}$ receptor
5-$HT_{2C}$ receptor
Apoptosis
Cerebellum
Anxiety
Doxepin
Fluoxetine

ABSTRACT

5-HT receptor changes remain controversial in posttraumatic stress disorder (PTSD) models. This study looks at the relationship between traumatic injuries and the alterations in 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors in the goldfish brain. The effect of treatment with doxepin and fluoxetine, known to be selective serotonin reuptake inhibitor (SSRI) antidepressants, on 5-HT receptor expression in goldfish with fin ablation was also investigated. We demonstrated that fin ablation induced anxiety-like behavioural alterations and significant up-regulation of c-fos expression in goldfish cerebellum. The behavioural alterations correlated well with an increased expression of 5-$HT_{2A}$ receptors in the cerebellum of the fish with traumatic injury. An increase in the number of apoptotic cells and a higher caspase-8 protein level was present in the brains of goldfish with fin ablation compared to the control. Our findings suggest that neuronal apoptosis occured in the cerebellum as a result of fin ablation and may be related to the alterations of 5-$HT_{2A}$ and 5-$HT_{2C}$ levels and that the beneficial clinical effects of doxepin/fluoxetine treatment are due to the down-regulation of 5-$HT_{2A}$ and up-regulation of 5-$HT_{2C}$ receptors in the brain.



## 1. Introduction

Exposure to a traumatic event may result in the development of acute stress disorder (ASD) and posttraumatic stress disorder (PTSD). PTSD, as a severe anxiety disorder, is a highly debilitating condition that develops in response to life-threatening stressors, e.g. a traumatic experience of a natural disaster, combat-related trauma, or even after orthopaedic trauma. There is clearly a need to focus on alleviating these problems as part of the rehabilitation process (Ebrahimzadeh and Rajabi, 2007; Jason et al., 2008; Ponsford et al., 2008). The neurobiological mechanisms underlying the PTSD are still poorly understood, and despite considerable efforts, the efficacy of current treatments is still unsatisfactory. Traumatic stressors are defined by the Diagnostic and Statistical Manual (DSM) as threats to life of self or significant others with intense fear, horror or helplessness (Bohnert and Breslau, 2011; Bremner, 2006; Jason et al., 2008). Traumatic amputations, such as war-related foot and ankle amputations, as well as the less traumatic ones done by orthopaedic surgeons, are all causes of acute stress disorder, so patients usually need psychiatric supportive treatment after traumatic amputations (Copuroglu et al., 2010; Mahan and Ressler, 2011). There has been less research done on the stresses caused by ablation of parts of the body. Whether such acute traumatic stressor leads to similar pathogenesis and symptoms as in acute stress disorder and PTSD remains unclear.

Goldfish (*Carassius auratus*) are excellent model organisms for understanding neuroendocrine signalling and the regulation of reproduction in vertebrates and numerous other fields (Jason et al., 2008). Despite this finding, little research has been carried out into the potential relationship between traumatic stresses, behaviour and the activation of specific neuropathological processes in the different brain regions of goldfish. An approach was developed to perform ablation of fins similar to those that might occur in nature. One distinct advantage of this type of wound for research purposes is that it entails comparatively low blood loss.

The serotonergic system is of particular interest since evidence suggests it plays a role in many aspects of human and animal behaviour (Harvey et al., 2004; Pritchard et al., 2008), and is consistently implicated in symptom mediation in PTSD (Briski and Gillen

* Corresponding author. Tel.: +86 571 88208160; fax: +86 571 88208094.
*E-mail addresses:* huxueqing@zju.edu.cn (X. Hu), yanlee20@126.com (Y. Li), huzhiyingf@yeah.net (Z. Hu), jar@cuhk.edu.hk (J.A. Rudd), lingshucai@zju.edu.cn (S. Ling), 9308260@qq.com (F. Jiang), henrydavies2002@yahoo.ie (H. Davies), fangmaro@zju.edu.cn (M. Fang).
[1] These authors contributed equally to this work.

0197-0186/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2012.04.022

2001; Hasegawa et al., 1998). Cortical 5-$HT_2$ receptors are anxiogenic and involved in the adaptation to stressors (Alves et al., 2004; Hasegawa et al., 1998). Studies have shown balance-stress relationships in goldfish following ablation of the fin, a vital organ to control balance and motion. These balance–stress correlations appear to involve the integrated activity of serotonergic network. 5-$HT_{2A}$ receptor expression in neurons may provide a basis for a coordinated effect on mediating postural adjustments, autonomic responses, and structures important in anxiety and conditioned fear (Balaban, 2002). Given that the postulated homology between the medial section G in teleosts and the amygdala, which is associated with emotion and behaviour in higher vertebrates (Butler, 2002; Norihcutt, 1995), we hypothesized that motor control and behaviours such as anxiety and hyperarousal may be associated with activation of 5-$HT_{2A}$ receptors in specific brain regions.

Apoptosis plays an important role in normal CNS development (Balaban, 2002; Butler, 2002; Jason et al., 2008; Mahan and Ressler, 2011; Pritchard et al., 2008) and in the pathogenesis of a number of neurodegenerative diseases (Bosch et al., 2001; Briski and Gillen, 2001; Contestabile et al., 1998; Hasegawa et al., 1998; Mahan and Ressler, 2011; Norihcutt, 1995; Rinaman, 2003). Chronic stress can lead to stress-induced development of major depression, and changes in neuronal function, including atrophy and functional impairments in the cerebral cortex (Bienvenu and Neufeld, 2011; Bosch et al., 2001; Haack et al., 2008; Riedel et al., 2005). Previous studies have also demonstrated neurodegeneration, enhanced neuronal apoptosis and decreased neuronal survival occur with chronic stress (Challet et al., 1997; Haack et al., 2008; Riedel et al., 2005). Therefore, we investigated whether surgical injury causes neuronal apoptosis in goldfish model. It has been well established that sequential activation of the caspases cascade plays a central role in the execution-phase of cell apoptosis. Caspase-8, an initiator caspase, is activated within the death-inducing signalling complexes by the extrinsic pathway. Once activated, caspase-8 cleaves and activates caspase-3 (Bosch et al., 2001; Hasegawa et al., 1998; Mahan and Ressler, 2011). BAD, a pro-apoptotic Bcl-2 family proteins, plays a pivotal role in determining cell death and survival (Fan et al., 2010). Herein, an investigation of the potential relationship between the apoptotic status and expression of BAD and caspase-8 was also carried out. Activated c-fos proteins, frequently documented for identifying activities in neurons or fiber tracts of the nervous systems of vertebrates upon stimulus or related activities (Bosch et al., 2001; Briski and Gillen, 2001; Rinaman, 2003), were used to evaluate the extent of injury. This may also be involved in apoptosis (Contestabile et al., 1998; Hasegawa et al., 1998).

Increased synaptic 5-HT levels following a stressful event may precipitate or worsen anxiety through activation of 5-$HT_{2A}$ receptors. Selective serotonin reuptake inhibitors (SSRI) attenuate many of the debilitating symptoms of PTSD, and are widely to treat psychiatric disorders. 5-HT is both anxiogenic and anxiolytic and the neurobiological mechanism responsible for its clinical effectiveness is only partly understood.

To gain a fuller understanding of the mechanism of PTSD pathogenesis, we performed fin ablations on goldfish and recorded the effect on 5-$HT_{2A}$ and 5-$HT_{2C}$ receptor expression, BAD expression, c-fos expression in the cerebellum and on behaviour. We also investigated the effect that doxepin and on fluoxetine had on 5-HT receptor expression.

## 2. Materials and methods

### 2.1. Animals, injuries, behavioural measurement, and drug treatment

A total of 126 adult goldfish (*Carassius auratus*), 8.3 ± 1.0 cm (mean ± S.D.) in body length, both sexes, were obtained from a local supplier. They were kept in an open fish aquaria (75 × 50 × 50 cm), at a temperature of 22–25 °C, fed with fresh bloodworms (Chironomus) once daily in the morning. The water was continuously aerated and changed every day. The fish were allowed to adapt to such an environment for a week before the experiments began. The animal care and experimental procedure was conducted in accordance with the policies of the National Institute of Health Guide for the Care and Use of Laboratory Animals, with approval from the Animal Ethics Committee of the Chinese University of Hong Kong. At the beginning of the experiment, the fish were randomly assigned to a normal control group and traumatic groups of 24, 48, and 72 h ($n$ = 6 for each treatment group at each time point). The first group without amputation of the fins was taken as the control ($n$ = 18), and the other four groups were submitted to ablation of dorsal fins, abdominal fins, dorsal and abdominal fins, and caudal fins ($n$ = 18, for each fin ablation group, six fish per group at each time point were sacrificed). Almost no bleeding resulted from these operations.

With regard to the drug treatment experiments, another 36 goldfish were obtained, and randomly assigned to six groups ($n$ = 6, for each group): the normal group without any lesion or drug treatment, the uninjured group but treated with doxepin, the uninjured group but treated with fluoxetine, the group with only ablation of dorsal and abdominal fins, the group with ablation of dorsal and abdominal fins and doxepin treatment alone, or the group with ablation of dorsal and abdominal fins but with fluoxetine treatment alone. For drug treatment, the fish were kept in water containing doxepin (5 mg/L) or fluoxetine (25 mg/L) for 3 days, during which the water was refreshed daily. The doses of doxepin and fluoxetine were deduced from preliminary experiments. Seventy-two hour after surgery and treatment, the cerebellum of each fish was harvested for Western blotting.

To estimate the immediate response after surgery and the adaptive reaction, the behavioural patterns of the goldfish were carefully recorded using a video recorder (DCR-SR46, SONY) at 30 min and 72 h after surgery. At 24, 48 and 72 h post injury, six fish for each time point were sacrificed by decapitation at the posterior border of the operculum, the brains were carefully dissected and cerebellums harvested. The specimens for histology and immunohistochemistry were fixed in 4% paraformaldehyde in teleost Ringer's solution (pH 7.0). Those for Western blotting were put into liquid nitrogen for instant freezing and then stored at −80 °C until analysis.

### 2.2. Immunohistochemistry

Five micrometer serial sections were transversely cut in a cryostat to caudal direction in the brain area comprised cerebellum. Two cross sections were chosen G and K (Giraldez-Perez et al., 2009.), section G contained cerebellar valvula and section K contained cerebellar body. Sections were deparaffinized with xylene, rehydrated with graded ethanol, and washed with phosphate buffered saline (PBS). Antigen retrieval was carried out by placing the samples in citrate buffer (pH 6.0) and heating for 10 min using an 850w microwave, followed by cooling for another 10 min. Sections were then incubated for 15 min with 3% $H_2O_2$ in methanol to block endogenous peroxidases, subsequently followed by three rinses in Ringer's solution (5 min each). After incubating for 1 h in 2% bovine serum albumin (BSA) in Ringer's solution containing 0.1% Triton X-100, sections were incubated overnight at 4 °C with a rabbit primary antibody (anti-5-$HT_{2A}$ antibody, AB 16028-100, Abcam; anti-caspase-8 antibody, SC-7890, Santa Cruz, respectively) diluted in 0.2% BSA (1:300, 1:200, respectively). Subsequently, sections were rinsed three times in Ringer's solution (5 min each) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody in 0.2% BSA for 2 h (1:500, Invitrogen). Sections were