Progress in Neuro-Psychopharmacology & Biological Psychiatry 55 (2014) 40–49

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

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

Anxiety, hyperactivity and stereotypy in a zebrafish model of fragile X syndrome and autism spectrum disorder

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ARTICLE INFO

Available online 27 March 2014

Keywords:
Anxiety
Autism spectrum disorder
Fragile X syndrome
Hyperactivity
Stereotypy
Zebrafish

ABSTRACT

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and is caused by a loss of function of the fragile X mental retardation (*fmr1*) gene. Animal *fmr1*-knockout (KO) models are not only of interest for the study of FXS, but have also important implications for our understanding of autism spectrum disorder (ASD). Here we report the behavioral changes in *fmr1*-knockout zebrafish in an open field with two white and two transparent walls. The neophobic responses that in wild-type (WT) zebrafish normally occur during the first 5–10 min in an unfamiliar environment (such as freezing, hypo-activity and preferences for the bottom and opaque walls of the tank), were weakened in *fmr1* mutants, suggesting a reduction of novelty-induced anxiety. The *fmr1*-KO zebrafish showed somewhat increased vertical activity beyond the 'neophobic phase', but no overall hyperactivity. The mutants demonstrated a clear habituation-independent preference for the transparent walls. Whether this was attributable to altered spatial information processing or to reduced avoidance of open spaces is discussed. Finally, since restrictive repetitive (or stereotypical) behaviors are frequently present in FXS and ASD patients, we analyzed relative turning angles, directional and preferential turning ratios and performed frequency-domain analysis. However, no indications of abnormal movement patterning were detected. The possible reasons for the absence of stereotypical behaviors are discussed in terms of behavioral endpoint selection and of eliciting conditions. Overall, our findings are consistent with those reported in *fmr*1-KO mice and suggest that further analysis of the *fmr1*-KO zebrafish model has potential to deepen our understanding of FXS and ASD.

1. Introduction

Fragile X syndrome is the most common genetic cause of inherited intellectual disability. In males, it has a prevalence of about 1:4000, in females 1:6000 (Turner et al., 1996). FXS is caused by an expansion of a CGG repeat (normally consisting of about 30 units) in the *fmr1* gene (located on the X chromosome) which leads to silencing of the gene when the repeat exceeds 200 units (Verheij et al., 1993; Verkerk et al., 1991). Common behavioral characteristics include learning and cognitive deficits, such as impaired working memory and visuospatial cognition, hyperactivity and attention deficit, anxiety, shyness, simple stereotypic behaviors (such as hand flapping and echolalia), impaired motor coordination and sensory processing (Hagerman, 2002; Baranek et al., 2005; Reiss and Hall, 2007; Bailey et al., 2008). Certain similarities with autism spectrum disorder (ASD) exist (Bernardet and Crusio, 2006; Budimirovic and Kaufmann, 2011) and disruption of the FMR1 pathway has been proposed to play a central role in ASD (Iossifov et al., 2012). An important objective is to understand how the distinct impairments associated with these disorders are the result of an initial deficit (Cornish et al., 2004). For instance, what are the connections between stereotypical behaviors, hyperactivity and anxiety in FXS? Furthermore, the list of clinical symptoms does not specify the aspects and characteristics of their involvement. For instance, non-social and social anxieties, unconditioned and conditioned fears can be differentially affected by the mutation. Animal models of the disease can contribute to answering those questions by way of experimental isolation of conditions. Importantly, animal models also provide a basis for testing novel therapeutic approaches.

The most commonly studied *fmr1*-KO animal model is the mouse. Many of the behavioral deficits in FXS and ASD patients have been studied in this model with sometimes seemingly mixed results (Bernardet and Crusio, 2006). For instance, non-social anxiety seems to be decreased according to one study (Dansie et al., 2013) as measured by the time spent in the open arms on the elevated-plus maze and the

Abbreviations: ASD, autism spectrum disorder; fmr1, fragile X mental retardation; fps, frames per second; FXS, fragile X syndrome; IACUC, institutional animal care and use committee; KO, knockout; LTD, long-term depression; LTP, long-term potentiation; PCR, polymerase chain reaction; WT, wild-type.

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http://dx.doi.org/10.1016/j.pnpbp.2014.03.007
0278-5846/

time spent in the center of an open field, which seems to contradict the role of anxiety in FXS. Hyperactivity is a possible confounding factor (Dansie et al., 2013). Hyperactivity of *fmr1*-KO mice in the open field test has been reported (Spencer et al., 2005). Moreover, Zhao et al. (2005) reported that trace fear memory was impaired in *fmr1*-KO mice, but that unconditioned anxiety-like behaviors in the open field and the elevated-plus maze were not affected by the mutation. Trace fear conditioning is susceptible to attention deficit. This task is dependent on the anterior cingulate cortex (Han et al., 2003).

Interestingly, Zhao et al. (2005) found that long-term potentiation (LTP) was completely abolished in the anterior cingulate cortex of *fmr1*-KO mice. LTP was also decreased in the lateral amygdala (Zhao et al., 2005) and long-term depression (LTD) was increased in the hippocampus (Huber et al., 2002). The amygdala plays an important role in emotional conditioning (Hermans et al., 2014) and, under certain circumstances, the hippocampus has a role in trace conditioning (Chan et al., 2014). Thus, in *fmr1*-KO mice impairments of certain forms of fear conditioning are to be expected, leaving other forms of fear or anxiety intact.

Furthermore, Kramvis et al. (2013) have studied the interaction between hyperactivity, behavioral perseveration and novelty during attentional rule acquisition as well as the characteristics of open field behavior in dependency of novelty. One interesting finding was that in *fmr1*-KO mice hyperactivity was observed only in an unfamiliar open field and during initial rule learning and rule reversal learning. In this connection, it is also interesting to note that the often-reported perseverative behaviors in these mice (e.g. McNaughton et al., 2008; Dansie et al., 2013) were normalized after familiarization with the environment or experimental procedures (Kramvis et al., 2013). Together, these finding suggest that in case of novelty-induced responses, cognitive inflexibility or resistance to change might be a decisive factor in the behavioral changes seen in the mutant mice (Moon et al., 2006). Other behavioral changes in *fmr1*-KO mice have been reported that resemble findings in FXS patients: sensory responses, such as conditioned eye blink reflex (Koekkoek et al., 2005) and auditory startle response (Nielsen et al., 2002), were altered and certain aspects of social behaviors were affected (Spencer et al., 2005; Mineur et al., 2006; McNaughton et al., 2008).

Zebrafish models of FXS, ASD and other neurological or psychiatric disorders are of major interest because they provide a powerful substrate for high-throughput drug-screening and rescue studies (Tropepe and Sive, 2003; Mathur and Guo, 2010; Rihel et al., 2010; Stewart et al., 2011, 2012, 2014; Kalueff et al., in press). Furthermore, some behavioral assays for complex social interactions are easier to perform in zebrafish. This is for instance true for social interaction tests involving groups since zebrafish readily form shoals (Miller and Gerlai, 2011, 2012; Maaswinkel et al., 2013b; Mahabir et al., 2013; Vital and Martins, 2013).

Zebrafish *fmr1* protein has strong similarity with the human protein (van 't Padje et al., 2005). An *fmr1*-KO zebrafish line has been generated (den Broeder et al., 2009). Ng et al. (2013) found that long-term potentiation (LTP) was reduced and long-term depression (LTD) was enhanced in telencephalic slices of *fmr1*-KO zebrafish, which is reminiscent of the findings in *fmr1*-KO mice (see above). The behavior of *fmr1*-KO zebrafish has only been investigated in one study (Ng et al., 2013), showing that locomotor activity was increased in an open field (alluding to hyperactivity), white aversion was decreased in a light/dark box (alluding to decreased unconditioned fear) and emotional learning was impaired (alluding to decreased conditioned fear). Based on the studies with *fmr1*-KO mice, we wanted to further explore whether in the zebrafish model the occurrences of anxiety-like responses and hyperactivity are also dependent on the novelty of the environment. In zebrafish, neophobic responses are well-studied and are fast-habituating (Bencan et al., 2009; Wong et al., 2010; Rosemberg et al., 2011; Maaswinkel et al., 2012, 2013a; Stewart et al., 2012), thus allowing us to investigate the behaviors of the *fmr1*-KO zebrafish during and after the 'neophobic phase' (i.e. first 5–10 min). Furthermore, we assessed stereotypical behaviors which are among the most characteristic features of FXS and ASD patients and were also present in the mouse model (in the form of perseverations of responses).

2. Materials and methods

2.1. fmr1-knockout zebrafish

fmr1^{hu2787}-zebrafish embryos (AB background) were purchased from the Zebrafish International Resource Center. One prolific breeder pair was selected to generate zebrafish, which were raised and genotyped at an age of about 3 months. Only homozygous zebrafish (n = 53) and WT siblings (n = 47) were used for behavioral testing. For maintenance, genotyping and behavioral testing of the zebrafish the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of xyZfish and Stony Brook University were followed. After genotyping, the zebrafish were maintained in eight-liter tanks, each containing 10–15 zebrafish. Water temperature was approximately 78 °C. Light regimen: 14 h lights on (6:00–20:00), 10 h lights off. Zebrafish were fed three times a day: 8:00 TetraMin tropical flakes; 12:00 live brine shrimp larvae; 15:00 TetraMin tropical flakes. On the days of the experiments, the zebrafish were fed only at 8:00. Thirty percent of the zebrafish were females, fifty-three percent were males and seventeen percent could not be clearly sexed. There was no significant difference of sex ratios for WT and KO zebrafish.

2.2. Polymerase Chain Reaction (PCR)

Adult fishes were genotyped by PCR (Roche Exo- Faststart PCR kit). A mutation was created in one of the PCR primers which led to a new restriction endonuclease cutting site. The new cutting site only exists in either wild type or mutated allele. To genotype the hu2787 allele, a mismatch (G) was introduced into the forward primer ZFMARRSA5: 5'- gaa tat gca gcc tgt gat gcc acc cta aat gaa atc gtc aca tta gag agg Gta-3'. During PCR, this mismatch creates an RsaI restriction enzyme site in the amplified product derived from the WT DNA template. The RsaI site is not present in the PCR product containing the hu2787 mutation. The reverse primer was ZFMR2 : 5'- ttg gcc aaa ctc cat gac atc ctg c -3'. A 259-bp PCR product was generated after 40 cycles of PCR reaction. The PCR reaction conditions began with a denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. After amplification, the PCR product was digested by RsaI restriction enzyme in 1 X restriction enzyme buffer. Finally, digested PCR products were separated by electrophoresis in 2% agarose gel. The PCR products derived from the WT template were cleaved to 206 bp and 53 bp DNA fragments. The PCR product derived from hu2787 allele is 259 bp.

2.3. Open field apparatus

The open-field recording chamber (Fig. 1) has been described before (Maaswinkel et al., 2012, 2013a-c). In short, it consists of stackable chambers (91 × 46 × 56 cm, l × w × h) closed by a dark green curtain. The observation container (25 × 25 × 18 cm, l × w × h; filled with water with a depth of 13.5 cm) is placed close to one side on the long axis of the chamber, the camera (Bumblebee 2; Point Grey Research Inc, Vancouver, Canada) close to the other side. The bottom and two walls of the observation container (one wall facing the inner chamber wall opposite to the camera, the other wall situated to the right of the camera) are painted white in order to increase contrast between zebrafish and background for better recording. The other two walls are transparent. Although both white and transparent walls were selected for technical reasons, it turned out that they provide an additional dimension to the test (see Maaswinkel et al., 2013a; compare also Blaser and Rosemberg, 2012). The camera records the front view and the top view (via a mirror suspended over the tank) simultaneously. The