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Food restriction does not relieve PTSD-like anxiety

Hendrikus Hendriksen*, Diewertje I. Bink, Dana L.E. Vergoossen, E. Suzet van Slobbe, Berend Olivier, Ronald S. Oosting

Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

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ABSTRACT

We used the inescapable foot shock paradigm (IFS) in rats as an animal model for post-traumatic stress disorder (PTSD). Previously we showed that exercise reversed the enhanced stress sensitivity induced by IFS. From literature it is known that food restriction has antidepressant and anxiolytic effects. Since both treatments influence energy expenditure, we questioned whether food restriction reduces anxiety in the IFS model *via* a comparable, NPY dependent mechanism as enrichment.

Anxiety of IFS-exposed animals was measured as change in locomotion and freezing after sudden silence in an open field test, before and after two weeks of food restriction. In addition a forced swim test (FST) was performed. Next, using qPCR, the expression of neuropeptide Y (NPY) and the neuropeptide Y_1 receptor (Y_1 receptor) was measured in the amygdala.

Food restriction increased locomotion and decreased freezing behavior both in control and IFS animals. These effects were small. IFS-induced anxiety was not abolished after two weeks of food restriction. IFS did not influence immobility or the duration of swimming in the FST of animals fed *ad libitum*. However, food restriction increased swimming and decreased the duration of immobility in IFS-exposed animals. Y_1 receptor expression in the basolateral amygdala decreased after both IFS and food restriction. Although food restriction seems to induce a general anxiolytic effect, it does not operate *via* enhanced Y_1 receptor expression and has no effect on the more pathogenic anxiety induced by IFS.

1. Introduction

Previously we showed that environmental enrichment with or without voluntary exercise increased behavioral recovery in rats exposed to inescapable foot shocks (Hendriksen et al., 2010). The lingering question is what is the molecular basis of the anxiolytic effects of exercise? Exercise leads to a shift in energy balance and to an increase in a number of hormones that stimulate food intake (Popovic and Duntas, 2005; Flores et al., 2006; El Elj et al., 2010; Stensel, 2010). Comparable molecular changes occur as a consequence of fasting (food/caloric restriction). Food restriction, just like exercise, causes a decrease in the amount of energy stored in an organism. As a consequence, a biological program is started aimed to restore the energy balance; basically stimulating an organism to eat (Lewis et al., 1993; Hanson and Dallman, 1995; Stensel, 2010).

In addition to the stimulation of food intake, both food restriction and exercise have beneficial effects on anxiety and depression-like behavior in rodents and humans (Newman and Motta, 2007; Duman et al., 2008; Lutter et al., 2008a, 2008b; Yamamoto et al., 2009).

In humans a high co-morbidity exists between eating disorders and anxiety disorders (Swinbourne and Touyz, 2007). Furthermore, some specific components that restore energy balance also activate mood-lifting and anxiety-reducing pathways. For instance, neuropeptide Y (NPY), ghrelin, orexin, leptin, and insulin-like growth factor-1 (IGF-1) are influenced by exercise and food restriction and exhibit antidepressive-like effects in addition to their role in food intake (Bi et al., 2003; Kotz, 2006; Lu et al., 2006; Lutter et al., 2008a, 2008b; Wang et al., 2008; Duman et al., 2009; Karacabey, 2009; Spasic et al., 2009).

Recovery from depression or anxiety disorders following antidepressant treatment is associated with an increase in neurogenesis in the hippocampus (Duman et al., 2001, 2008; Warner-Schmidt and Duman, 2006, 2007). Neurotrophic factors, such as insulin-like growth factor-1 (IGF-1), brain-derived nerve factor (BDNF) and vascular endothelial growth factor (VEGF), are thought to mediate the antidepressant-induced increase in neurogenesis (Duman, 2004; Warner-Schmidt and Duman, 2007, 2008; Duman et al., 2009). In the brain, IGF-1 is increased by exercise, while BDNF is increased after both food restriction and IGF-1 injection (Lee et al., 2000). Other factors that play a role in food intake, like leptin, NPY, and AMP-activated protein kinase (AMPK), also promote neuronal

* Correspondence to: Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands. Tel.: +31 681429599.
E-mail address: h.hendriksen@uu.nl (H. Hendriksen).

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survival and neurogenesis (Garza et al., 2008; Moon et al., 2009; Spasic et al., 2009; Decressac et al., 2010). In recent experiments we showed that NPY infusion in the basolateral amygdala normalized the anxiety of the animals subjected to inescapable footshock (IFS) (Hendriksen et al., 2012). Taken together, these data indicate that there is a possible link between genes involved in the regulation of food intake and depression/anxiety. Here we investigated whether food restriction has anxiolytic effects in the inescapable foot shock (IFS) paradigm, our model of post-traumatic stress disorder. Animals subjected to IFS show enhanced freezing following a sudden stressor and decreased locomotion in the open field test until 10 weeks after the traumatic event (van Dijken et al., 1992; Hendriksen et al., 2010).

2. Materials and methods

2.1. Ethics statement

All animal experiments were handled in strict accordance with the Dutch Experiments on Animals Act. All animal work was approved by the Ethical Committee for Animal Research of Utrecht University (DEC-ABC) DEC nr: 2007.I.01.010/vv-1-3 and 2009. I.12.108.

2.2. Animals

Forty-eight male Sprague Dawley rats (Harlan, The Netherlands) were used that weighed 175–199 g (46–50 days in age) upon arrival. Animals were housed in groups of four in macrolon IV cages. Animals were acclimated to their surroundings for one week. All animals were housed in a temperature (± 22.5 °C), humidity ($\pm 55\%$), and light controlled environment (lights on from 6 am to 6 pm). Food and water were provided *ad libitum* in the home cages during the first two weeks. The behavioral tests were carried out during the light phase of the day–night cycle between 9.00 am and 12.00 am. Upon completion of the experiments the animals were killed by decapitation under anesthesia.

2.3. Inescapable foot shock

Animals were placed in a dark shock box ($30 \times 24 \times 21$ cm^3) with a grid floor (5 mm separated by 1 cm) situated in a sound attenuated chamber. IFS animals were exposed to 10 inescapable foot shocks (1 mA, scrambled, and with a duration of 6 s) over the course of 15 min, with a variable inter-shock interval. Four animals were exposed to the IFS in parallel and the shock-boxes were cleaned thoroughly between sessions with a 70% ethanol solution.

2.4. Food restriction

Rats exposed to the food restriction were given 60% of the average daily food intake of the *ad libitum* (AL) groups around 2.00 pm. The 60% quantum was calculated from the weight of food that the *ad libitum* fed animals consumed one full day before. The food restriction animals received between 53 and 59 g of food per cage (4 animals) per day. At the time food restriction started the animals were around 300 g, approximately 10 weeks old and considered to be adults.

2.5. Open field and SOS test

Four black open field boxes ($72 \times 72 \times 45$ cm^3) were lit at 20 lx at floor level. Testing took place in the morning. At the start of the test each animal was placed in the center of the open field. The animals were tested twice. One week after IFS the animals were tested for 5 min with 85 dB background noise. After two weeks food restriction (three weeks after IFS) the animals were tested 5 min with 85 dB background noise and another 5 min after the sound was suddenly terminated (stress of sudden silence (SOS)). Animals were tracked using Noldus EthoVision® 3.1. The first two min of the sudden silence freezing was measured using Ethovision software with a mobility setting of 3%. These settings were validated by a skilled observer that assessed the freezing from recorded movies.

2.6. Forced swim test (FST)

Rats were placed in transparent, cylindrical plastic tanks (40 cm high, 18 cm diameter) filled with water of ± 25 °C to a height of 22 cm. The tanks were shielded from each other by black partitions. The FST-procedure was taken from Porsolt et al. (1978). On day 1 the rats were placed in the tank for the duration of 15 min and 24 h later again for 5 min. The behavior of the rats during both times in the tank was recorded on videotape and subsequently analyzed by two observers with the Observer software from Noldus, The Netherlands (version 5.0). Three types of behavior were quantified: immobility, swimming, and climbing. Immobility was defined as floating or no active movements made other than those necessary to keep the nose above the water. Swimming was defined as active motions throughout the swim tank. Climbing was defined as upward-directed movements of the forepaws against the wall. After the swim training and test, animals were dried with a towel and returned to the home cage.

2.7. Tissue collection, RNA isolation, and cDNA synthesis

One day after the SOS test animals were decapitated under deep anesthesia and their brains were recovered and stored at -80 °C. For the collection of the basolateral amygdala samples we consulted the micro dissection atlas from Palkovits for guidance (Palkovits and Brownstein, 1988) with modifications described by McBride et al. (2010). 500 μm thick sections from frozen brains were sliced in a cryostat at -10 °C and a punch needle of 0.8 mm diameter was used to collect the basolateral amygdala. All surfaces and tools in contact with the tissue were cleaned with RNaseZAp (Ambion). Total RNA was isolated first with Trizol (Invitrogen) followed by an RNA cleanup kit (Nucleospin® RNA cleanup XS Kit, Marcherey Nagel, Germany). Consequently, cDNA was synthesized using 300 ng of RNA and the iScript™ cDNA Synthesis Kit (Bio Rad, United States).

2.8. Quantitative PCR

qPCR reactions were performed using the iQ SYBR Green Supermix (Bio Rad, United States) and the Biorad CFX96 Real-Time system. For all PCR reactions, the following protocol was used: 3 min 95 °C, followed by 40 cycles consisting of 15 s at 95 °C, and 30 s at 55 °C. Levels of cyclophilin, a household gene, were used for normalization. Data are presented as fold difference compared to the control animals. Primers: NPY: 5′-CTCTGCGACACTACATCAATC-3′, 5′-CTCTTGCCATATCTCTGTCTG-3′, Y_1 receptor: 5′-ATGCTACTTCAAGATATACG-3′, 5′-TCCATCATGTTGTTTCTC-3′, cyclophilin: 5′-GAGCGTTTTGGGTCCAGGAAT-3′, 5′-AATGCCCGCAAGTCAAAGAAA-3′.

2.9. Study outline

Twenty-four animals received an inescapable foot shock procedure (IFS). Control animals were left undisturbed. One week after the IFS all animals performed an open field test to measure the effect of IFS on locomotion. Four days after the open field food restriction started (FR). After two weeks of food restriction a second open field test was performed. One day later the FST was

performed. Next the animals were sacrificed by decapitation and the brains were removed for further analysis. The four different groups used in this study are called: C-AL = control animals fed *ad libitum*, C-FR = control animals exposed to food restriction, IFS-AL = animals exposed to inescapable foot shock fed *ad libitum*, IFS-FR = animals exposed to inescapable foot shock on a restricted diet.

2.10. Statistical analysis

Data is expressed as mean ± S.E.M. Statistical analysis was performed using a independent sample *t*-test, or one-way or two-way ANOVAs, followed by post-hoc analysis with corrections for multiple comparisons (SPSS 16.0). Significant differences were assumed at a level of $P < 0.05$. For PCR data Dunnet's post hoc testing was performed using the C-AL group as a control.

3. Results

3.1. Weight changes

Animals in the food restriction groups had on average the same weight as the *ad libitum* fed groups before the start of the food restriction ($F[3,47] = 0.582$, $P > 0.05$). As expected, food restriction inhibited the growth of the animals (see Fig. 1). Repeated measures ANOVA for the effect of food restriction on body weight was significant ($F[3,44] = 93.98$, $P < 0.001$). There was no effect of IFS on body weight ($F[3,44] = 0.02$, $P > 0.05$). Although the food restriction animals stopped growing and even lost some weight, their brain development (Fig. 1B) continued normally as shown by their similar brain weights after two weeks of food restriction ($F[3,47] = 0.769$, $P > 0.05$).

3.2. Open field

The open field test was carried out twice. One week after IFS (but before the start of the food restriction) the shocked animals showed significantly less locomotion compared to the non-shocked control groups ($P < 0.05$). Analysis of the second open field test, three weeks after IFS, showed a significant effect of shock on locomotion ($F[3,47] = 15.99$, $P < 0.001$) and locomotion after SOS ($F[3,47] = 28.41$, $P < 0.001$) (Fig. 2A) and freezing ($F[3,47] = 28.53$, $P < 0.001$) (Fig. 2B). Two weeks of food restriction resulted in a significant improvement on locomotion ($F[3,47] = 4.98$, $P < 0.05$), locomotion after SOS ($F[3,47] = 5.19$, $P < 0.05$) (Fig. 2A) and freezing

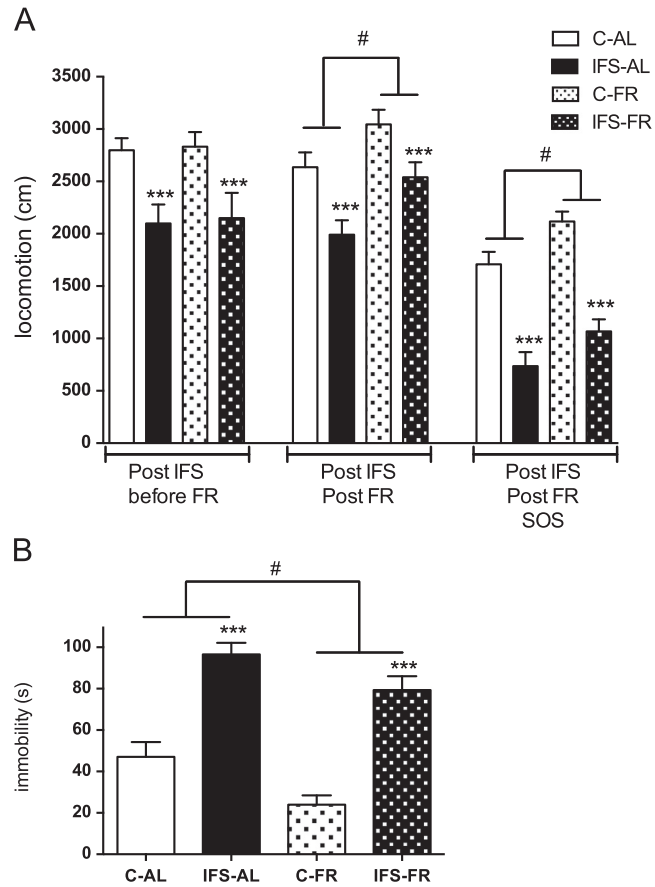

Fig. 2. Two weeks of food restriction increased locomotion and reduced freezing, but did not cause recovery of the IFS animals. (A) Locomotion in the open field one week after shock (before food restriction) and three weeks after IFS (two weeks after food restriction) with noise on and after SOS. (B) Freezing behavior in the first two min after SOS. Two-way ANOVA, effect of IFS $^{***}P < 0.01$, effect of FR $^{\#}P < 0.5$, $N = 12$/group. C = no treatment; IFS = inescapable foot shock treatment; AL = *Ad libitum*; FR = food restriction.

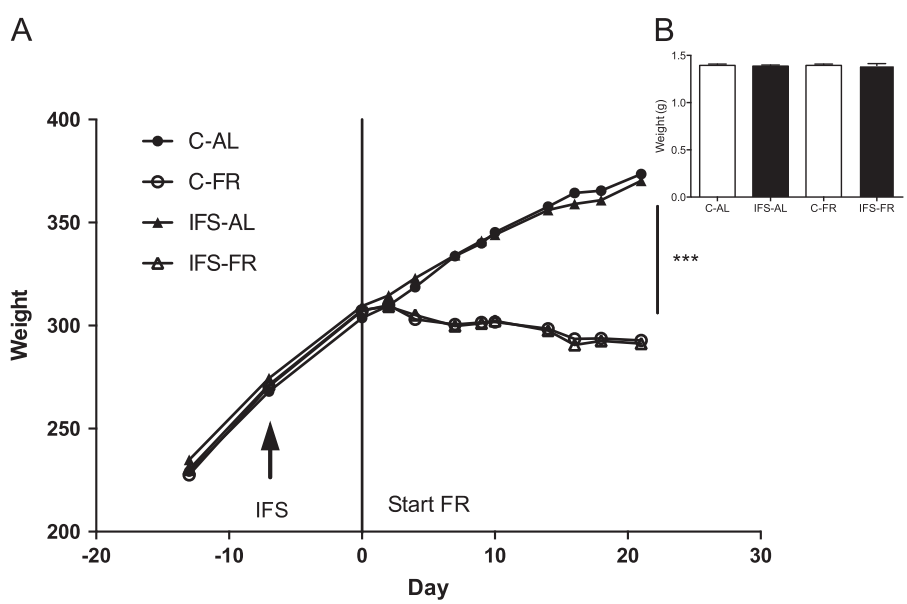

Fig. 1. Body and brain weight. No initial body weight changes were observed before food restriction. Body weight was not different between control and IFS animals. Inset shows no difference in brain weight between groups. *** repeated measure ANOVA, $P < 0.001$, $N = 12$/group. C = no treatment; IFS = inescapable foot shock treatment; AL = *Ad libitum*; FR = food restriction.

($F[3,47]=7.10$, $P<0.05$) (Fig. 2B). There was, however, no significant interaction between the effect of IFS and food restriction for these three parameters.

3.3. Forced swim test

We analyzed the forced swim test data with two-way ANOVA and found a significant effect of food restriction ($F[3,48]=5.3$, $P<0.05$), but no significant effect of IFS ($F[3,48]=0.37$, $P>0.05$) on swimming in the first swim test of 15 min (Fig. 3A). Immobility (Fig. 3B) was also significantly affected by food restriction ($F[3,48]=5.19$, $P<0.05$) and not by IFS ($F[3,48]=0.45$, $P>0.05$). The next day, in the second swim test, we found an interaction between IFS and food restriction on swimming ($F[3,41]=16.71$, $P<0.001$).

IFS-FR animals showed less immobility than all other groups (one-way ANOVA, $F[3,46]=6.663$, $P<0.05$, *post hoc*: $P<0.01$). This difference is mainly caused by an increase in swimming behavior compared to all other groups ($F[3,45]=12.275$, $P<0.05$, *post hoc*: $P<0.001$). There was no difference in climbing behavior between the groups in both swim sessions (data not shown).

3.4. NPY and Y_1 receptor expression in the basolateral amygdala

In order to investigate a possible involvement of the NPY system in the effects of IFS and food restriction, we measured mRNA levels of NPY, and Y_1 receptor in the basolateral amygdala. A two-way ANOVA revealed no difference in NPY expression due to diet ($F[1,26]=0.745$, $P>0.05$) or treatment ($F[1,26]=3.45$, $P>0.05$). The results for the Y_1 receptor expression in the basolateral amygdala can be found in Fig. 4. A main effect of shock treatment ($F[1,25]=1.375$ $P<0.05$) and an interaction effect between diet and treatment ($F[1,25]=0.979$, $P<0.05$) were found. The Dunnet *t*-test elucidated that all three groups showed significantly reduced expression levels of Y_1 receptor compared to the C-AL group (C-FR: $P<0.05$; IFS-AL: $P<0.01$; IFS-FR: $P<0.05$; see Fig. 4). The interaction effect suggests that food restriction reduces Y_1 receptor expression in de non-shock groups, but does not additionally change the Y_1 receptor expression in the IFS treatment groups.

4. Discussion

Endogenous proteins, like NPY and ghrelin, known to be involved in regulating food intake, may also have antidepressant and anxiolytic effects (Jahng et al., 2007; Lutter et al., 2008a, 2008b; Deng et al., 2009; Yamamoto et al., 2009). Previously we found that environmental enrichment attenuated anxiety and

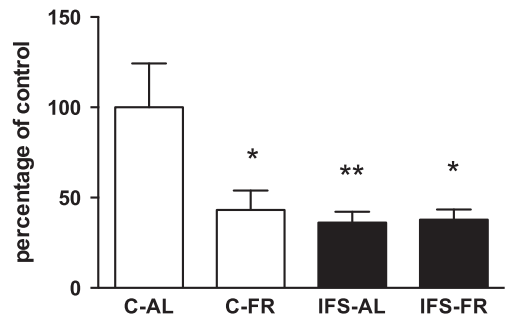

Fig. 4. Y_1 receptor expression in the basolateral amygdala. mRNA levels were determined by q-PCR. Values represent mean ± S.E.M.. Dunnet's post hoc testing was performed using the C AL group as a control. $^{*}P<0.05$, $^{**}P<0.01$. $N=7$/group. C = no treatment; IFS = inescapable foot shock treatment; AL = Ad libitum; FR = food restriction.

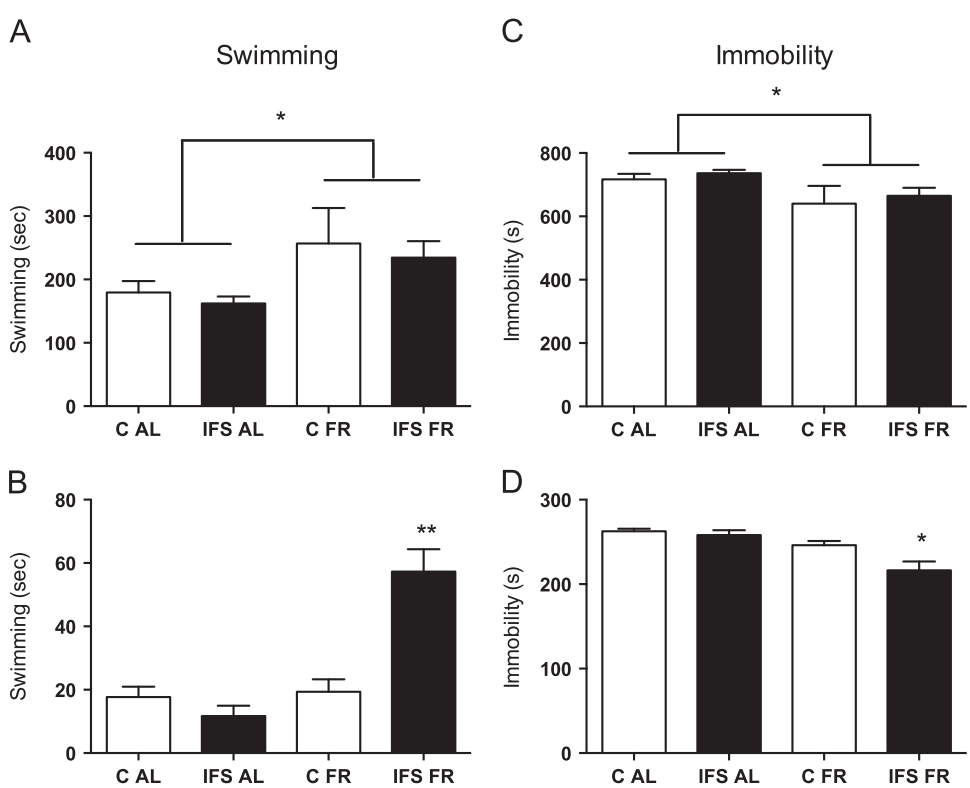

Fig. 3. Two weeks of food restriction caused an increase in swimming behavior and a decrease in immobility in FR animals during the first swim test. During the second swim test only the IFS-FR group showed increased swimming and decreased immobility. (A) The first swim test (15 min). (B) The second swim test (5 min). (C) Immobility during the first swim test. (D) Immobility during the second swim test. $^{*}P<0.05$, $^{**}P<0.01$, compared to control AL, $N=12$/group. C = no treatment; IFS = inescapable foot shock treatment; AL = *Ad Libitum*; FR = food restriction.

enhanced Y_1 expression in the amygdala. Moreover, an Y_1 receptor agonist was able to reduce IFS induced anxiety. This led to the idea that the Y_1 receptor plays a role in the anxiolytic effect of environmental enrichment (Hendriksen et al., 2012). With these observations in mind we hypothesized that, food restriction could also exert an anxiolytic effect in the IFS model of PTSD *via* changes in the expression of the NPY system in the amygdala.

In this experiment we showed that food restriction lowers anxiety of control and IFS animals, as is apparent from the increased locomotion (Fig. 2A) and reduced freezing following the stress of sudden silence (SOS) (Fig. 2B) in the open field test. Food restriction had no extra effect on IFS animals, leaving their locomotion and freezing in the SOS test still different from non-shocked controls. The increase in locomotion during the first 5 min may be the result of an overall small augmentation of the exploration of the food-restricted rats. However, the increased locomotion after the SOS and the decreased freezing indicate that food restriction leads to a general reduction in anxiety. A possible explanation for this could be that hunger makes animals less anxious and more explorative to enlarge the chance of finding food. However, as is clear from our results, this effect will not be enough to overcome the more pathological anxiety state of the IFS animals. Because we have shown before that the Y_1 receptor plays an important role in the environmental enrichment induced reduction of anxiety following IFS (Hendriksen et al., 2012) we were interested in the expression of Y_1 in the amygdala of the various experimental groups. As Fig. 4 shows, Y_1 receptor expression decreased in the IFS animals, but food restriction did not reverse this. On the contrary, food restriction also led to a decreased Y_1 receptor expression in de basolateral amygdala.

In other studies food restriction has been shown to reduce anxiety-like behavior (Levay et al., 2007; Lutter et al., 2008a, 2008b; Yamamoto et al., 2009). For instance, in the social defeat stress model, food restriction completely reverses the effects of the social defeat stress and provides the same additional benefits for defeated animals as for healthy animals (Lutter et al., 2008a). This raises the question why food restriction can reverse the effects of social defeat stress and not the effects of IFS. One difference between these two models of PTSD is that the IFS model is resistant to the effects of antidepressants, like the selective serotonin reuptake inhibitors (SSRIs) (Hendriksen et al., 2010), while these drugs do reverse the anxiety-like behavior following social defeat stress (Venzala et al., 2012). Another interesting observation in this regard is that food restriction increases fear extinction learning through a serotonin transporter (SERT) dependent mechanism (Riddle et al., 2013). Fear extinction, a process that is notably also hindered in PTSD patients, could be a mechanism through which food restriction has its beneficial effects. Taken together, the lack of an effect of food restriction on IFS animals may be because this model shows resistance to SERT modulation.

During the training phase of the forced swim test (FST) food restricted animals swam more than *ad libitum* fed animals. IFS animals fed *ad libitum* did not differ from control animals in immobility in the forced swim test (FST). The IFS-FR animals swam significantly more than all other groups. The question is now how to interpret these findings.

Classically, primarily based on the effects of selective serotonin reuptake inhibitors, the increase in swimming and the concomitant reduction in immobility on the second day are considered as anti-depressive effects (Porsolt et al., 1978; Finn et al., 2003; Cryan et al., 2005). If this hypothesis about the meaning of the FST is correct, we have to conclude that IFS-FR animals are the least depressed. Since this seems unlikely, we have to consider other explanations.

Another interpretation of the FST involves the role of memory formation during the training. In this view immobility is considered as a coping response that is learned during the training phase of the FST. During the first session the animal probably learns that floating/immobility costs less energy and that escape by swimming is impossible. A longer duration of immobility during the test on the second day may therefore represent improved cognitive and adaptive abilities to cope with psychologically stressful events (Collins et al., 2009). This hypothesis is supported by research showing that drugs that affect memory performance, but do not function as antidepressants, also increase mobility in the FST (Kitada et al., 1981; De Pablo et al., 1989, 1991). Based on this hypothesis, the FR-IFS animals show an impaired memory performance compared to the food restriction group. Interestingly, Collins et al. (2009) reported that 4 weeks of voluntary exercise increases immobility in the FST test. They suggest that this is due to enhanced cognitive capabilities of the exercised animals. The data by Collins et al. and our FST results make our initial assumption that exercise and food restriction have common underlying anxiolytic mechanisms less likely. Taken together, enhanced anxiety may explain the increased swimming of the rats of both food restriction groups during the first swim test best. The most likely explanation for the differences in the second swim test is that the IFS-FR animals do not remember the training session from the day before as well as the animals from the three other experimental groups. Whatever the meaning of the FST, food restriction does not seem to have a beneficial effect on IFS rats, but rather the opposite.

5. Conclusion

In this study a link between food restriction and a beneficial effect on anxiety, as shown by others (Jahng et al., 2007; Lutter et al., 2008a, 2008b; Deng et al., 2009; Yamamoto et al., 2009), is not that strong. Depending on the paradigm in which the IFS animals were tested, we have found some positive effects (SOS) or clear negative effects (FST). The positive effect was confined to the "adaptive" anxiety also seen in the control animals. Food restriction did not alleviate the IFS induced anxiety.

The idea behind this experiment was based upon similarities between certain biochemical changes following exercise and food restriction. The lack of clear anxiolytic effects of food restriction in IFS induced anxiety and the lack of food restriction to restore Y_1 expression in the basolateral amygdala indicate that our initial assumption that food restriction and exercise may have comparable mechanisms of action is incorrect. Our results do not support food restriction as a possible (add-on) therapy for the treatment of PTSD.

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