Unpredictable chronic stress decreases inhibitory avoidance learning in Tuebingen long-fin zebrafish: stronger effects in the resting phase than in the active phase

Remy Manuel1,*, Marnix Gorissen1, Jan Zethof1, Lars O. E. Ebbesson2, Hans van de Vis3, Gert Flik1 and Ruud van den Bos1

ABSTRACT
Zebrafish (Danio rerio Hamilton) are increasingly used as a model to study the effects of chronic stress on brain and behaviour. In rodents, unpredictable chronic stress (UCS) has a stronger effect on physiology and behaviour during the active phase than during the resting phase. Here, we applied UCS during the daytime (active phase) for 7 and 14 days or during the night-time (resting phase) for 7 nights in an in-house-reared Tuebingen long-fin (TLF) zebrafish strain. Following UCS, inhibitory avoidance learning was assessed using a 3 day protocol where fish learn to avoid swimming from a white to a black compartment where they will receive a 3 V shock. Latencies of entering the black compartment were recorded before training (day 1; first shock) and after training on day 2 (second shock) and day 3 (no shock, tissue sampling). Fish whole-body cortisol content and expression levels of genes related to stress, fear and anxiety in the telencephalon were quantified. Following 14 days of UCS during the day, inhibitory avoidance learning decreased (lower latencies on days 2 and 3); minor effects were found following 7 days of UCS. Following 7 nights of UCS, inhibitory avoidance learning decreased (lower latency on day 3). Whole-body cortisol levels showed a steady increase compared with controls (100%) from 7 days of UCS (139%), to 14 days of UCS (174%) to 7 nights of UCS (231%), suggestive of an increasing stress load. Only in the 7 nights of UCS group did expression levels of corticoid receptor genes (mr, gra, grβ) and of bdnf increase. These changes are discussed as adaptive mechanisms to maintain neuronal integrity and prevent overload, and as being indicative of a state of high stress load. Overall, our data suggest that stressors during the resting phase have a stronger impact than during the active phase. Our data warrant further studies on the effect of UCS on stress axis-related genes, especially grβ; in mammals this receptor has been implicated in glucocorticoid resistance and depression.

KEY WORDS: Danio rerio, Behaviour, Telencephalon, Gene expression, Diurnal effects

INTRODUCTION
An upcoming research area is the use of zebrafish as a model to study the effects of chronic stress on brain and behaviour in relation to depression, anxiety and other mood-related disorders (Chakravarty et al., 2013; Gerlai, 2010a; Gerlai, 2010b; Norton, 2013; Piao et al., 2011; Stewart et al., 2014). Zebrafish may be of interest for chronic stress research as they express two glucocorticoid receptors (GR), GRa and GRβ, as humans do but not rodents (Schaaf et al., 2009; Schoonheim et al., 2010). GRβ has been associated with glucocorticoid resistance in humans, which is relevant for a number of diseases including major depression (Carvalho et al., 2014; Pace and Miller, 2009; Schoonheim et al., 2010; Silverman and Sternberg, 2012; Webster et al., 2001; Zhou and Cidlowski, 2005). Unpredictable chronic stress (UCS) impedes inhibitory avoidance learning in a single-trial inhibitory avoidance paradigm in the AB zebrafish strain (Piao et al., 2011). In this paradigm, fish learn to avoid swimming from a white to a black compartment to avoid an electric shock (Blank et al., 2009; Ng et al., 2012). Recently, we studied the effects of different shock intensities on inhibitory avoidance learning in an in-house-reared Tuebingen long-fin (TLF) zebrafish strain and showed associated changes in the regulation of a number of genes involved in anxiety, fear, learning and memory when measured 2 h following the task, including gnr (Manuel et al., 2014). In rats it has been shown that UCS has a stronger impact on physiology and behaviour when given during the resting phase than during the active (or awake) phase (Aslani et al., 2014). Zebrafish are active during the daytime and rest during the night-time (Hurd et al., 1998). Disturbances during the night-time may negatively impact zebrafish behaviour (Löhr and Hammerschmidt, 2011; Singh et al., 2013). We therefore studied the effects of daytime and night-time UCS on inhibitory avoidance learning, whole-body cortisol content and gene expression levels, notably of grα and grβ, in TLF zebrafish to further assess the usefulness of zebrafish as an animal model in research related to chronic stress.

To induce UCS we modified an earlier published UCS protocol (Piao et al., 2011) to a milder regime to prevent exhaustion interfering with avoidance learning (Piao et al., 2011). We administered UCS for 7 or 14 days during the daytime (the 7 days/14 days UCS groups). We predicted that 14 days of UCS would reduce inhibitory avoidance learning, increase whole-body cortisol content and change the expression of associated genes (see next paragraph) more strongly than 7 days of UCS would. We also applied the UCS protocol during the night-time for 7 nights (the 7 nights UCS group). We predicted that the UCS protocol applied during the night-time would have a stronger impact compared with application during the active phase [based on studies on rats (Aslani et al., 2014)]. Further, we extended our inhibitory avoidance protocol (Manuel et al., 2014) with a second training session as multiple training sessions may improve the learning performance of zebrafish (Arthur and Levin, 2001; Williams et al., 2002). We therefore predicted that an additional training session would provide
a better resolution of the effects of UCS on inhibitory avoidance learning.

As in an earlier study (Manuel et al., 2014), we assessed an array of genes in the telencephalon. Genes were selected on the basis of their relation to the stress response, anxiety and fear, as well as fear conditioning (de Kloet et al., 1999; Liu et al., 2004; Maximino et al., 2013; Upadhya et al., 2013) and include: brain derived neurotrophic factor (bdnf), serotonin receptor 1ab (htr1ab), cocaine- and amphetamine-regulated transcript 4 (cart4), corticotropin-releasing factor (crf), crf-binding protein (crf-bp), cannabinoid receptor 1 (cnr1), gra, grβ and mineralocorticoid receptor (mr). The telencephalon was specifically chosen for its involvement in learning and memory as well as fear and fear conditioning (lateral and medial zone of the dorsal pallium (Broglio et al., 2005; Mueller et al., 2011)). As many stress-related genes change their expression levels differentially in different parts of the brain, measuring gene expression in the telencephalon specifically provides a higher resolution compared with whole-brain analysis regarding the effects of UCS on inhibitory avoidance learning.

RESULTS

General observations

No mortality was observed as a result of the UCS protocol. Fish appeared healthy, were active in their home tanks and accepted food directly.

There were no fish that did not enter the black compartment on day 1. We observed no behavioural differences between groups during exposure to the shock: all groups showed erratic movements, seeking to escape or jumping out of the water when the shock was applied (Manuel et al., 2014). In addition, we did not observe any abnormal behaviour, such as periods of freezing behaviour (Kalueff et al., 2013), on any of the test days.

Inhibitory avoidance learning

Daytime UCS

To assess inhibitory avoidance learning, latency times of fish were recorded before training (day 1) and after training (day 2 and day 3; Fig. 1A). All fish entered the black compartment within 60 s on day 1, except one fish in the control group (80 s). Median latencies of groups ranged from 6 to 8 s. Mann–Whitney tests revealed no significant differences between groups (all $P>0.16$).

Compared with day 1, the median latencies of all groups on day 2 significantly increased, but were notably stronger in the control group (all $P<0.01$). Furthermore, compared with the control group, the 7 days UCS group did not have a lower median latency to enter the black compartment ($U=288.5$, $P=0.09$), while the 14 days UCS group had a significantly lower median latency ($U=328.5$, $P=0.003$). There was no difference between the 7 days UCS group and 14 days UCS group ($U=193$, $P=0.33$).

On day 3, the second shock led to an increase in the median latency in all groups, but only in the 7 days UCS group (80 s). Median latencies of the 14 days UCS group remained significantly lower compared with the control group ($U=401.5$, $P=0.03$) but not compared with the 7 days UCS group ($U=162$, $P=0.08$).

Night-time UCS

To assess the effect of 7 nights of UCS on inhibitory avoidance learning, latency times of fish were recorded before training (day 1) and after training (day 2 and day 3; Fig. 1B). All fish entered the black compartment within 60 s on day 1, except for one fish in the control group (89 s) and one fish in the 7 nights UCS group (169 s). On day 1, the median latency of the control group was not significantly different from the median latency of the 7 nights UCS group ($U=153$, $P=0.18$).

Compared with day 1, the median latencies on day 2 were significantly increased in both the control group ($U=72.50$, $P=0.014$) and the 7 nights UCS group ($U=65.50$, $P<0.0001$). There was no
significant difference between the median latencies of the control group and the 7 nights UCS group ($U=203$, $P=0.99$) on day 2.

On day 3, the second shock did not lead to a significant increase in median latency of the control group and the 7 nights UCS group when compared with day 2 (all $P\geq 0.24$). However, the median latency of the 7 nights UCS group was significantly lower than the median latency of the control group ($U=130$, $P=0.04$).

When compared with the latencies of the 7 days UCS group, we found that the latencies of the 7 nights UCS group were also significantly lower ($U=140.5$, $P=0.05$), while they did not differ from the 14 days UCS group ($U=275.5$, $P=0.48$) on day 3 (comparing day 3 of Fig. 1A, B).

**Whole-body cortisol content**

Fig. 2 shows the whole-body cortisol content for each group. The data show a gradual increase in cortisol level from controls (100%) to 7 days UCS (139%), to 14 days UCS (174%) to 7 nights UCS (231%). *Post hoc* analysis, following a significant one-way ANOVA (see Fig. 2), revealed that the 7 nights UCS group had significantly higher levels of whole-body cortisol content compared with the control group and the 7 days UCS group, but not compared with the 14 days UCS group. We observed no further statistically significant differences.

**Gene expression analysis: daytime and night-time UCS**

Fig. 3 shows the relative normalised gene expression profiles and $mr$/$gra$ as well as $grβ$/$gra$ ratios of the different groups. The figures exclude the following outliers as detected by Grubbs’ test: one control subject for $cart4$ (normalised expression 1.55); one 7 nights UCS subject for $hir1αβ$ (normalised expression 2.69) and one 7 days UCS subject for $cnr1$ (normalised expression 1.48). For ease of reading, one-way ANOVA values and associated $P$-values are indicated in each panel. For $cart4$ (Fig. 3A), we found that the 7 days UCS group had significantly higher levels of expression compared with the 7 nights UCS group and the control group, but not compared with the 14 days UCS group. For $hir1αβ$ (Fig. 3B), the 7 days UCS group had significantly higher levels of expression compared with the control group, the 14 days UCS group and 7 nights UCS group. For $crf-hp$ (Fig. 3C) and $crf$ (Fig. 3D), there were no significant differences in expression levels between groups. For $bdnf$ (Fig. 3E) and $grβ$ (Fig. 3F), the 7 nights UCS group had significantly higher levels of expression compared with the control group, the 7 days UCS group and the 14 days UCS group. For $cnr1$ (Fig. 3G), following 14 days of UCS and 7 nights of UCS, we observed significantly higher levels of expression compared with the 7 days UCS group, but not compared with the control group. For $mr$ (Fig. 3H), the 7 nights UCS group had significantly higher levels of expression compared with the control group, the 7 days UCS group and the 14 days UCS group. Expression of $gra$ (Fig. 3I) was significantly higher in the 7 nights UCS group compared with the control group, the 7 days UCS group and the 14 days UCS group. There was no significant effect on the $mr$/$gra$ ratio (Fig. 3J). The $grβ$/$gra$ ratio (Fig. 3K) was significantly higher for the 7 nights UCS group compared with the control group, the 7 days UCS group and the 14 days UCS group.

**Principal component analysis**

We performed a principal component analysis (PCA) across the four different groups (control, 7 days UCS, 14 days UCS and 7 nights UCS). Although the Kayer–Meyer–Olkin (KMO) measure was moderate for this dataset (KMO=0.673), the Bartlett’s test of sphericity indicated that the correlations between items were sufficiently large for PCA ($χ^2=140.617$, d.f.=45, $P=0.0001$). We extracted three components, which together explain 69.78% of variance (Table 1): $cart4$, $hir1αβ$, $crf-hp$ and $crf$ loaded strongly on component 1; $bdnf$, $gr β$, $cnr1$ and $mr$ loaded strongly on component 2; $mr$, $gra$, $crf$ and whole-body cortisol content loaded strongly on component 3. To assess the overall effect of these components, we ran a one-way ANOVA for each of these components. This revealed a weak main effect for component 1 ($F_{3,32}=2.329$, $P=0.09$). For component 2, a significant main effect was observed ($F_{3,32}=10.060$, $P<0.001$); *post hoc* analysis (least square differences, LSD) revealed that the 7 nights UCS group had significantly higher scores compared with the other three groups. No further statistical differences were observed. For component 3, a weak main effect was observed ($F_{3,32}=2.808$, $P=0.06$).

**DISCUSSION**

We assessed the effects of daytime and night-time UCS on inhibitory avoidance learning, whole-body cortisol levels and gene expression in an in-house-reared TLF zebrafish strain. Both 14 days and 7 nights of UCS resulted in poorer inhibitory avoidance learning compared with the control group. Whole-body cortisol content was moderately (but not significantly) increased in the 14 days UCS group, it significantly increased in the 7 nights UCS group. Only in the 7 nights UCS group was the expression of $bdnf$, $gr β$, $mr$ and $gra$ increased compared with the controls. In both the 14 days UCS and 7 nights UCS group, $cnr1$ levels were increased compared with the 7 days UCS group. The 7 days of UCS treatment had only a small effect on learning and whole-body cortisol content, and this group showed increased expression levels of $cart4$ and $hir1αβ$ compared with controls or 14 days of UCS and 7 nights of UCS.

**Inhibitory avoidance learning**

All fish tolerated the UCS protocol well, as we did not see any abnormal behaviour or mortality. Independently of the UCS protocol, all fish quickly entered the dark compartment on day 1, as...
was their expected preference (Stephenson et al., 2011), showing that UCS had no effect on anxiety per se. The UCS protocol applied appeared to be effective as we observed a moderate (14 days) to strong (7 nights) increase in whole-body cortisol content, and poorer inhibitory avoidance learning following 14 days or 7 nights of UCS (Piato et al., 2011). Seven days of UCS only had minor effects on inhibitory avoidance learning and led to a small increase in whole-body cortisol content. These results are in line with our prediction that our UCS regime would have a strong effect after 14 days, but not after 7 days. Also in line with our prediction, the effects of UCS were stronger when given in the night-time than in the daytime: while we observed a minor effect in the 7 days UCS group on day 2, which disappeared on day 3, we observed a clear effect on day 3 in the 7 nights UCS group. Fish exposed to 7 days UCS significantly improved their performance on day 3, whereas those exposed to 7 nights UCS did not. Thus, extending the inhibitory avoidance protocol in time allowed for a more precise discrimination of UCS effects (Arthur and Levin, 2001; Williams et al., 2002).

Our data support earlier studies in zebrafish that showed that disturbances during the night negatively impact zebrafish behaviour (Löhr and Hammerschmidt, 2011; Singh et al., 2013). Furthermore, in rats, UCS during the resting phase has more impact than during the active phase (Aslani et al., 2014). Interestingly, in mice, repeated social defeat had a more pronounced negative outcome when applied during the active phase (Bartlang et al., 2012). Differences in inhibitory avoidance learning could be related to differences in responsiveness of the sleep–wake cycle (Löhr and Hammerschmidt, 2011; Singh et al., 2013). Furthermore, in rats, UCS during the resting phase has more impact than during the active phase (Aslani et al., 2014).

Fig. 3. Comparison of telencephalic gene expression in the control group, the 7 days UCS group, the 14 days UCS group and the 7 nights UCS group. Expression was normalised to that of elf1a/rpl13. Bars represent group means + s.e.m. Groups with different letters are significantly different from each other (LSD post hoc analysis following a significant one-way ANOVA; P<0.05). N=number of fish. Note that J and K display ratios and not actual gene expression levels.
While we only observed minor effects on the expression levels of crf (P=0.06), the overall expression appeared to increase in the UCS groups, most notably in the 7 days UCS group (significant to control: P=0.01). This weak effect of UCS on crf expression seems at variance with the literature as crf expression has been shown to strongly increase following UCS (Chakravarty et al., 2013; Piato et al., 2011). The reasons underlying these differences may be related to sampling procedures: while we sampled the telencephalon specifically, others sampled the whole brain (Chakravarty et al., 2013; Piato et al., 2011).

Expression of crf can be found in many different areas outside the telencephalon (Alderman and Bernier, 2007), areas that may differently respond to chronic stress. It has been shown in rats that in both the amygdala and hippocampus, two forebrain areas, ERK1/2 activity (an effector molecule in the actions of CRF) is decreased after 2 weeks of UCS but not after 4 weeks (Castro et al., 2012), a temporally differential effect akin to what we observed in our present study (7 days versus 14 days UCS). Thus, in the telencephalon, a short duration or lower stress load (7 days UCS) may lead to different adaptive changes than a longer duration or higher stress load (14 days UCS and 7 nights UCS) at the level of crf expression. As we took samples following the task, while others sampled independent of the task, our data may also reflect an interaction between the UCS protocol and the task, rather than effects of UCS per se. Thus, it is possible that the changes in crf expression which we observed are not solely related to UCS. At least for the 7 days UCS group, the crf increase observed here may be related to learning and memory (Radulovic et al., 1999) as there was a significant improvement in performance observed here which may be related to learning and memory (Radulovic et al., 1999) as there was a significant improvement in performance observed here.

Follow 7 nights of UCS, we found increased expression levels of grf, mr, bdnf and cnr1. Interestingly, grf, mr, bdnf and cnr1 (which probably follows the expression pattern of crf) loaded onto the same component in the PCA. In our earlier experiments, concerning the effects on inhibitory avoidance learning of different shock intensities, grf and bdnf also loaded onto the same component (Manuel et al., 2014), supporting the present data. The loading of these genes onto one single component, and their similar changes following 7 nights of UCS suggest that they may be associated in the context of a high stress load as deduced from the strong increase in whole-body cortisol following 7 nights of UCS. While most studies in rodents show that chronic stress decreases expression of bdnf (Suri and Vaidya, 2013), an increase in bdnf following UCS is not unprecedented for zebrafish (Chakravarty et al., 2013). Interestingly, it has been suggested that activation of MR or GRα may have different effects on BDNF: while MR activation stimulates bdnf expression, GRα activation decreases bdnf expression (Suri and Vaidya, 2013). Accordingly, the increase in bdnf expression in our present study may be a result of a stronger increase in mr than grα expression, which is supported by the increased mr/grα ratio following 7 nights of UCS (P=0.02, versus control). Under basal conditions, telencephalic MR is fully occupied with maintaining basal neuronal activity (Reul and de Kloet, 1985); an increased level of mr and grα, and the accompanying increase in bdnf expression following 7 nights of UCS may be a means to maintain neuronal integrity under chronic stress. It has indeed been suggested that an increase in bdnf is a compensatory mechanism that allows the development of an inhibitory phenotype by contributing to the upregulation of inhibitory mechanisms (McMillan et al., 2004). The increased expression of grf also seems to be in line with these protective changes as it has been suggested that GRβ exerts a dominant-negative activity over GRα (Schoonheim et al., 2010). Increased expression levels of grf could therefore be a mechanism to protect the brain against chronic elevated glucocorticoid exposure. This hypothesis is supported by the observed increase in the grfβ/grα ratio in the 7 nights UCS group, which is indicative of a stronger increase in the expression of grf compared with grα. However, this increase in grf is suggested to be mediated by pro-inflammatory cytokines (Webster et al., 2001) and may lead to glucocorticoid resistance associated with several diseases, among them major depression (Carvalho et al., 2014; Zhou and Cidlowski, 2005). Thus, the present data raise the question of the extent to which the observed effects are indicative for liability of disease in our 7 nights UCS subjects. It is clear that this warrants further studies.

For cnr1, we observed an increased expression in the 14 days and 7 nights UCS groups when compared with the 7 days UCS group. Studies in rodents have shown the involvement of the endocannabinoid system in the regulation of the stress response during chronic stress and recovery (Gorzalka et al., 2008; Hill et al., 2011; Lee and Hill, 2013). For example, CB1 (CNR1 in zebrafish) was down-regulated in the rat hippocampus during chronic stress, while it was up-regulated during the recovery period (Lee and Hill, 2013). As we measured gene expression 3 days after terminating the UCS protocol, the effects we observed may be due to up-regulation during the recovery period. The lack of increase in the 7 days UCS group may reflect that these fish have already recovered as suggested by their avoidance learning. Further studies focusing on chronic stress and CNR1 in zebrafish seem warranted. Studies on mammals have also shown that CB1 is associated with pain and pain perception, where activation of CB1 reduces pain sensitivity (Fine and Rosenfeld, 2013; Guindon and Hohmann, 2009; Wilson and Nicoll, 2002). Higher levels of CNR1 could contribute to poorer inhibitory avoidance learning as the paradigm is based on a potentially noxious stimulus (i.e. electric shock), although, as indicated, we did not see any differences in the behavioural response to receiving the shock.

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<tr>
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<td>0.008</td>
<td>0.869</td>
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<tr>
<td>crf</td>
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<td>0.781</td>
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<tr>
<td>Cortisol</td>
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<td>0.350</td>
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Variables loaded onto components by a principal component analysis (PCA):

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We observed that *mr*, *gra*, *crf* and whole-body cortisol content loaded onto the same component in the PCA. Previously, we also found that *mr* and *gra* loaded onto the same component (Manuel et al., 2014) strengthening our current observation. MR is involved in the early stages of stress-related changes, while GRα is key in long-term changes and normalisation following stress (de Kloet et al., 2005; Joëls et al., 2007). Still, our data suggest a relationship between these receptors in our experiments [without stress (Manuel et al., 2014) and with stress (reported here)]. Thus, this relationship may also be the result of an interaction of the UCS with the task, as both MR and GR are involved in learning and memory (Cerqueira et al., 2005; ter Horst et al., 2012; Lupien and McEwen, 1997; Zhou et al., 2010). The relative balance between these receptors affects memory in adults and changes in the *mr/grα* ratio have been associated with decreased learning and memory (de Kloet et al., 1999; Lupien and Lepage, 2001). Previous studies have shown that chronic stress in zebrafish increases the *mr/grα* ratio (Pavlidis et al., 2011). Although we did not find a strong overall effect on the *mr/grα* ratio here, we did see a steady increase in this ratio with increasing stress load (reflected by whole-body cortisol content). This may have contributed to the decrease in avoidance learning, which is supported by the fact that we did see a significantly higher *mr/grα* ratio when comparing the 7 nights UCS group with the control group specifically (*P*=0.02). These results warrant further studies focusing on the *mr/grα* ratio and how this is involved in learning and memory in zebrafish.

**Putative mechanisms**

Several mechanisms may be proposed to underlie the effects of chronic stress on avoidance learning. First, chronic stress may change the appraisal of the shock. In rodents, chronic stress has been shown to increase anxiety and fear conditioning, accompanied by changes in synaptic activity in the amygdala (Roozendael et al., 2009), which find their homologue in the medial zone of the dorsal pallium of the telencephalon (Mueller et al., 2011). We did not observe differences between groups on day 1 (latency to enter the dark compartment; anxiety) nor differences between groups concerning their behavioural response to receiving the shock. Moreover, we observed decreased rather than increased avoidance learning, making chronic stress-induced hypersensitivity in sensory processing or appraisal unlikely in our study. Second, increased levels of cortisol may lead to atrophy, decreased proliferation and changes in synaptic meta-plasticity in the dorsal zone of the lateral pallium (the telencephalic homologue of the hippocampus) as has been described for the rat hippocampus (de Kloet et al., 1999; Krugers et al., 2010; Lynch, 2004; Magariños and McEwen, 1995; McEwen, 2005; Yan et al., 2011). The hippocampus plays an important role in the formation and retrieval of memory related to contextual fear learning (Wiltgen et al., 2006), but not cue learning (Olvera-Cortés et al., 2013; Wahlsten et al., 2006) as well as rearing conditions within and between laboratories (Mahabir et al., 2013; Parker et al., 2012; Pavlidis et al., 2013) may affect the outcome of experiments. Our data strongly suggest that at a behavioural and physiological level the UCS protocol is robust and reproducible in decreasing inhibitory learning and elevating whole-body cortisol content across strains of zebrafish, i.e. the TLF strain used in our study and the AB strain in the study of Piato and colleagues (Piato et al., 2011), which is critical for advancing the zebrafish as a model for brain–behaviour studies associated with depression, anxiety and other mood-related disorders (Chakravarty et al., 2013; Gerlai, 2010a; Gerlai, 2010b; Norton, 2013; Piato et al., 2011). In addition, our data reveal that the reduction in inhibitory avoidance learning relates to increasing levels of stress load. However, only after a strong stress load (i.e. 7 nights of UCS) did we observe clear changes in telencephalic gene expression. These observations offer a selection of candidate genes (e.g. *bdnf*, *mr*, *grβ* and *cnr1*) to be studied in more detail in future work.

**MATERIALS AND METHODS**

**Ethical approval**

Experimental procedures were approved by the ethical committee of Wageningen UR (DEC: 2012010.1.b) and were conducted in accordance with Dutch laws (national legislation Wet op de Dierproeven 1996) and European regulations (Directive 86/609/ EEC).

**Animals and housing**

The fish used for this experiment were an in-house-reared TLF zebrafish strain. Fish were a mix of offspring from two parental couples and hatched within the same week. At the age of 12 months, 132 animals (a mix of males and females) were pooled and randomly assigned to 33 aquarium (21 volume, four fish per tank). Three animals died during the acclimation period (8 weeks), resulting in 129 animals in the experiment. Fish used for the 7 nights UCS treatment were acclimated to the new photoperiod for 8 weeks prior to the start of the stress protocol. Each aquarium received an artificial floating plant and was provided with independent water in- and out-flow (Nijmegen tapwater: 26°C, pH 8.0, 400 µS cm⁻¹); inflow water had passed through a biological filter (300 l volume). The daytime UCS group was kept under a photoperiod of 12 h light:12 h dark (lights on from 07:00 h to 19:00 h) with feeding at 09:00 h (artemia) and 15:00 h (TetraMin, Tetra, Melle, Germany). The night-time UCS group was kept on an adjusted photoperiod of 12 h light:12 h dark (lights on from 12:00 h to 24:00 h) with feeding at 13:00 h (artemia) and 18:00 h (TetraMin).
**Experimental groups**

Six experimental groups were used in the experiment. For practical reasons, the night-time control and UCS fish were kept on different photoperiods. The daytime group consisted of four experimental groups: two daytime control groups (N=22 each), one 7 days UCS group (N=18) and one 14 days UCS group (N=26). To control for the occurrence of changes in learning behaviour over the course of the experiment and to replicate the data of the (first) control group, we included a second control group at the end of the experiment. These groups did not show statistical differences in avoidance behaviour and, for this specific parameter, data were therefore pooled (N=44). Only the first group was used for whole-body cortisol content and gene expression analysis. The night-time group consisted of two experimental groups: a night-time control group (N=17) and a 7 nights UCS group (N=24). The night-time controls served to control for possible differences caused by the shift in photoperiod and potential effects related to the time of testing, i.e. inhibitory avoidance learning was assessed at 11:00 h for the daytime groups (4 h after lights on) and at 15:00 h for the night-time groups (3 h after lights on).

**UCS protocol**

To induce UCS, a previously published protocol was modified to a milder regime in order to prevent exhaustion, which confounds avoidance learning (Pinato et al., 2011). Briefly, for a total of 7 days, 14 days or 7 nights (Fig. 4A), fish were subjected twice daily, at random times to a chosen challenge. All fish were given the same stressor at the same moment. Stressors included: rapidly (within 1 min) heating the tank water up to 33°C for 30 min; rapidly (within 1 min) cooling the tank water down to 23°C for 30 min; crowding of 20 fish for 60 min in a 500 ml glass beaker; netting stress for 30 min; lowered water levels (dorsal part of the body was air-exposed) for 15 min; three consecutive (biofiltered) water replacements with fish in their tank (i.e. fish and water were poured into a net, fish were returned to the aquarium and water was replenished); chasing fish for 2×5 min with a net (15 min rest in between); air-exposure for 3×1 min (5 min rest in between); and fasting for 24 h. Following some of the stressors (e.g. crowding), fish were randomly divided into new groups of four, giving an additional, social, stressor.

**Inhibitory avoidance protocol**

For inhibitory avoidance learning, a previously described protocol was used (Blank et al., 2009; Manuel et al., 2014) with one modification: not one shock but two were given (see Fig. 4B). In short, an aquarium (60×30×30 cm; 10 cm water level) was split into two equal compartments, separated by a manually operated sliding door. The surfaces of one compartment were white while the surfaces of the other compartment were black. Compartments were not covered by a lid and light was measured at 350 lx at the surface of the water. The black compartment contained two metal plates (covered with black sound box mesh to prevent light reflection by the metal) that covered two opposite walls completely. Both plates were wired to a power source, which allowed 3 V AC to be put between the plates in the water (measured at the middle of the tank, across a distance of 15 cm between electrodes). Inhibitory avoidance learning was assessed for each fish individually (i.e. not as a group).

On day 1, a fish was placed in the white compartment with the sliding door closed. After 60 s of acclimation, the sliding door was lifted, giving the fish free access to the black compartment. As is their natural tendency, zebrfish will readily enter the black compartment when given this option (Stephenson et al., 2011). Once the fish had completely entered the black compartment, the sliding door was closed and an electric shock (3 V AC) was given for 5 s, after which the fish was returned to its home tank. Fish that would not enter the black compartment within 180 s were excluded from further experimentation. For each fish, the latency time to enter the black compartment was recorded. Higher latencies indicate that animals are less anxious in the white compartment.

On day 2, the procedure was repeated. After 60 s, the sliding door was opened and the fish was given 180 s to make a choice to enter the black compartment. If a fish entered the black compartment within 180 s, it was placed back into the white compartment. If a fish did not enter the black compartment within 180 s, it was given a second shock and entered the black compartment within 180 s, it was placed back into the white compartment. If a fish did not enter the black compartment within 180 s, it was given a second shock and entered the black compartment within 180 s, it was placed back into the white compartment. The 2 h post-inhibitory avoidance test was repeated.

**Fig. 4. Schematic overview of the housing photoperiods and the inhibitory avoidance learning protocol.**

(A) The two different photoperiods used for the daytime UCS and night-time UCS. Indicated are the phase in which UCS was applied and the times for feeding and inhibitory avoidance learning. (B) On day 1, fish were trained in the inhibitory avoidance protocol by receiving a shock upon entering the dark compartment. On day 2, fish were assessed for inhibitory avoidance learning. Fish were selected on the basis of their behaviour: non-avoider (<60 s latency) and avoider (>180 s latency). The 2 h post-inhibitory avoidance fish were sampled for assessment of telencephalic (indicated in red) gene expression (brain derived neurotrophic factor, bdnf; glucocorticoid receptor α and β, grα and grβ; mineralocorticoid receptor, mr; cocaine and amphetamine regulated transcript, cart; serotonin receptor 1ab, htr1ab; corticotropin-releasing factor, crf, and crf-binding protein, crf-bp) and whole-body cortisol content.
compartment or not. When the fish entered the black compartment within this time window, a second shock was given. Fish that did not enter within 180 s were carefully driven into the black compartment with a small net and then received the second shock. This was done to give all fish the same number of shocks. For each fish, the latency to enter the black compartment was recorded. Higher latencies are indicative of increased avoidance learning.

On day 3, fish were again placed within the white compartment. After 60 s, the sliding door was opened and the fish were given 180 s to choose to enter the black compartment or not. This time, when the fish entered the black compartment, no shock was given and fish that did not enter within 180 s were not driven into the black compartment. Then, fish were taken out of the experiment and killed 2 h after the test (see ‘Tissue collection and preparation’). For each subject, the latency to enter the black compartment was recorded. Higher latencies are indicative for increased avoidance learning.

Operation of the sliding door was done manually and visual observations were made in real-time and recorded on-site. Recording of the latency time was done by hand using a stopwatch. One experimenter (R.M.) recorded all latencies. All procedures were carried out in a manner that caused the least possible disturbance to fish during the experiment.

Tissue collection and preparation
As indicated above (see ‘Inhibitory avoidance protocol’), on the third day of the inhibitory avoidance protocol, tissues were collected 2 h after the test (Manuel et al., 2014). Not all fish were used for whole-body cortisol content and gene expression analysis, as some were used for other purposes (e.g. in situ hybridisation; not reported here). For each group, 8–10 fish were randomly taken. Time of sampling was chosen based on a study by Morsink and colleagues (Morsink et al., 2006), showing that between 1 and 3 h post-challenge a sufficient number of non-immediate early genes respond. Anaesthesia was given by placing fish from each group in water containing 2-phenoxethanol (0.1% v/v). Once deeply anaesthetised (within 30 s), the telencephalon was dissected out, snap-frozen in liquid nitrogen and stored at −80°C until whole-body cortisol content analysis (see ‘Whole-body cortisol content’).

Whole-body cortisol content
To evaluate stress levels as a result of the UCS protocol followed by the inhibitory avoidance paradigm, we assessed whole-body cortisol content. Zebrafish (previously stored at −80°C; see ‘Tissue collection and preparation’) were thawed on ice and homogenised individually in 1 ml PBS (80 mmol l−1 Na2HPO4, 20 mmol l−1 NaH2PO4, 100 mmol l−1 NaCl, pH 7.4) with a microblender. The homogenate was then mixed with 4 ml methanol (J. T. Baker, Deventer, The Netherlands) and left at 4°C for 1 h. Subsequently, the mixture was centrifuged (4°C, 3000 g, 5 min) and the supernatant was collected. The pellet was resuspended in 4 ml methanol and left at 4°C for 30 min followed by centrifugation and collection of the supernatant (this step was repeated twice). The collected methanol (16 ml) was evaporated under N2 gas, leaving a residue film containing steroids, which was reconstituted in 200 μl radioimmunoassay buffer (100 mmol l−1 Tris, 0.9% NaCl, 0.1% 8-aminolo-1-naphthalensulphonic acid, 0.02% NaN3).

Cortisol was measured as previously described (Gorissen et al., 2012). Briefly, 96-well microtitre plates were coated with mouse monoclonal (XM210) cortisol-antibodies (Abcam, Cambridge, MA, USA) in coating buffer. Plates were cleared of coating buffer and washed before blocking possible non-specific binding sites with blocking buffer. Wells were cleared of blocking buffer and 10 μl of standard or sample, together with 90 μl of 3H-cortisol tracer, was added to designated wells. Non-specifics received assay buffer (10 μl) and tracer only. After the incubation period, wells were cleared and washed before scintillation liquid was added and radioactivity measured with a β-counter (detection limit: 4 ng ml−1; inter-assay VC: 12.5% and intra-assay VC: 2.5%). Cross-reactivity with other relevant steroids was considered insignificant (less than 1% at 50% cortisol saturation).

Gene expression analysis
Genes analysed were babf, htr1ab, cart4, crf, crf-bp, cnr1, gra, grβ and mr. Two internal standards [ribosomal protein L13 (rpl13) and elongation factor 1α (elf1α)] were used for normalisation. Primer sequences are shown in Table 2.

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, including an additional precipitation step with ethanol and sodium acetate. RNA concentration and purity were measured by nanodrop spectrophotometry. Genomic DNA was removed by treatment with DNase I (Invitrogen) as follows: 1 μl of DNase I (1 μ l−1) and 1 μ l DNase I reaction buffer (10 μ l) were added to 8 μ l total RNA (500 ng) and incubated in a total volume of 10 μ l at room temperature for 15 min. Subsequently, DNase was inactivated by addition of 1 μ l EDTA (25 mmol l−1) and incubation for 10 min at 65°C. Next, each sample received 1 μ l random primers (250 ng μ l−1), 4 μ l First Strand buffer 5x, 1 μ l dNTP mix (10 mmol l−1), 1 μ l DTT (0.1 mol l−1), 1 μ l RNaseOUT (10 U) and 0.5 μ l Superscript II Reverse Transcriptase (100 U μ l−1) (all from Invitrogen) and incubated at 25°C for 10 min, followed by 50 μ l at 42°C and finally 15 min at 70°C. Afterwards, cDNA was diluted 5 times with ultra-pure water and stored at −20°C until analysis. Relative gene expression was assessed by real-time qPCR. Briefly, 4 μ l of cDNA was used as a template in a reaction with 10 μ l IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.8 μ l forward and reverse primer each (10 μmol l−1; Table 2) and 4.4 μl ultra-pure water. qPCR (3 min 95°C, 40 cycles of 1 s at 95°C and 1 min at 60°C) was carried out on a CFX 96 (Bio-Rad) qPCR machine. Data were analysed with the ΔΔCT-method, corrected for primer efficiency and normalised for two house-keeping genes (see Vandesompele et al., 2002).

As the ratio of mr/grα has been used as an indicator for the stress load of subjects (de Kloet et al., 1999; Lupien and Lepage, 2001), we calculated this ratio from the gene expression data using the normalised (i.e. non-relative) expression levels. We also calculated the grβ/grα ratio as this may also indicate the extent to which the expression of the different isoforms of gr changes as a function of stress load (Stolte et al., 2008).

Table 2. Nucleotide sequences of the forward and reverse primers used for qPCR

<p>| Table 2. Nucleotide sequences of the forward and reverse primers used for qPCR |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>elf1α</td>
<td>AY422992</td>
<td>CTTGAGGCACGCATCAGAACAT</td>
<td>TCAAGAAGAGTACGGCATGGACATTAC</td>
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<tr>
<td>rpl13</td>
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<td>bdnf</td>
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<td>GTGGAATCTTACGTCAGTCG</td>
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<tr>
<td>gra</td>
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<td>GATTGCGGAACCACAC</td>
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<td>grβ</td>
<td>EF436285.1</td>
<td>GATGAACTACGAATGTCTTA</td>
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<tr>
<td>nr</td>
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<tr>
<td>cart4</td>
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<tr>
<td>htr1ab</td>
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<tr>
<td>cnr1</td>
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<td>crf</td>
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<td>crf-bp</td>
<td>NM_001003459</td>
<td>ACAATGATCTCAAGAGGTCCAT</td>
<td>CCACACAGAGTCAACAAAA</td>
</tr>
</tbody>
</table>

Elongation factor 1α (elf1α) and ribosomal protein L13 (rpl13) were used as housekeeping genes. Genes of interest were brain derived neurotrophic factor (bdnf), glucocorticoid receptors α (gra) and β (grβ), mineralocorticoid receptor (nr), cocaine and amphetamine regulated transcript 4 (cart4), serotonin receptor 1α (htr1ab), corticotropin-releasing factor (crf) and crf-binding protein (crf-bp).
Statistical analysis
Statistical analyses were performed with IBM SPSS Statistics 21 for Mac (IBM, Armonk, NY, USA). Data were plotted with GraphPad Prism 5.0 for Mac (GraphPad Software Inc., La Jolla, CA, USA).

As we introduced a cut-off point of 180 s, differences in latency times between groups were analysed with non-parametric tests: a Kruskal–Wallis test (followed by pair-wise comparisons) and a Mann–Whitney test for within-group analysis between day 1 and day 2, and day 2 and day 3, as we could not individually label the fish, which prevented pair-wise analyses.

Data for whole-body cortisol content and gene expression were subjected to Grubbs’ test for outliers (extreme studentised deviate) using a stringent criterion (\(\alpha=0.01\)). Changes in whole-body cortisol content and telencephalic gene expression were analysed using a one-way ANOVA. Post hoc testing following significance was done by a least square differences (LSD) post hoc test (Ferguson, 1981).

To assess inter-relations between gene expression levels and whole-body cortisol content, we conducted a PCA with orthogonal rotation (varimax with Kaiser normalisation). In the case of missing samples, data were excluded list-wise. The number of components to retain was based on their eigenvalue (>1) and a visual inspection of the scree plot. Additionally, the KMO measure of sampling adequacy and Bartlett’s test of sphericity were run to ensure that the data obeyed analysis criteria. Component scores were saved and used for further analysis. The component loading cut-off point was =0.400 or 0.400 (Ferguson, 1981).

In all cases, significance was accepted when \(P\leq0.05\) (two-tailed) unless otherwise stated (i.e. adjusted \(\alpha\) in the case of multiple comparisons).

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Competing interests
The authors declare no competing financial interests.

Author contributions
R.M., M.G., L.O.E.E., H.v.d.V. and G.F. critically revised the manuscript; M.G., J.Z., L.O.E.E., H.v.d.V. and G.F. critically revised the manuscript; R.M., M.G. and J.Z. carried out the experiments and data acquisition; R.M., M.G. and R.v.d.B. analysed and interpreted the data; R.M. and R.v.d.B. drafted the manuscript; M.G., J.Z., L.O.E.E., H.v.d.V. and G.F. critically revised the manuscript.

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