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ORIGINAL ARTICLE

Zinc deficiency induces enhanced depression-like behaviour and altered limbic activation reversed by antidepressant treatment in mice

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Abstract A relationship between zinc (Zn)-deficiency and mood disorders has been suspected. Here we examined for the first time whether experimentally-induced Zn-deficiency in mice would alter depression- and anxiety-related behaviour assessed in established tests and whether these alterations would be sensitive to antidepressant treatment. Mice receiving a Zn-deficient diet (40% of daily requirement) had similar homecage and open field activity compared to normally fed mice, but displayed enhanced depression-like behaviour in both the forced swim and tail suspension tests which was reversed by chronic desipramine treatment. An anxiogenic effect of Zn-deficiency prevented by chronic desipramine and *Hypericum perforatum* treatment was observed in the novelty suppressed feeding test, but not in other anxiety tests performed. Zn-deficient mice showed exaggerated stress-evoked immediate-early gene expression in the amygdala which was normalised following DMI treatment. Taken together these data support the link between low Zn levels and depression-like behaviour and suggest experimentally-induced Zn deficiency as a putative model of depression in mice.

Keywords Brain zinc · Immediate-early gene Zif268 · Depression · NMDA · Elevated plus maze · Light/dark test

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Introduction

Zinc (Zn) ions are essential for life as they regulate the function of numerous structural, transcriptional, and enzymatic proteins (Brown and Dyck 2004; Frederickson et al. 2000). The CNS contains a large amount of Zn. A substantial fraction of it is located inside synaptic vesicles of glutamatergic terminals in chelatable forms and released with intense neuronal activity in a calcium-dependent manner (Ahn et al. 1998). In addition to a role in basic cellular functioning, Zn is co-released with either glutamate (Vogt et al. 2000) or γ-aminobutyric acid (GABA, Ruiz et al. 2004) and modulates *N*-methyl-D-aspartate (NMDA), $GABA_A$ (see below) and glycine (Park et al. 2008) receptors. Zn has been shown to inhibit NMDA receptor-activated channel currents via two distinct sites; one outside the membrane field affecting opening frequency, and the other inside the channel interfering directly with the passage of ions (Christine and Choi 1990; Chen et al. 1997; Choi and Lipton 1999; Williams 1996). Zn also inhibits $GABA_A$ receptors and reduces inhibitory postsynaptic currents (Ruiz at al. 2004; Westbrook and Mayer 1987) via three discrete binding sites; one located in the ion channel and two situated on the external amino (N)-terminal interface between α and β subunits (Hosie et al. 2003). Zn is not evenly distributed throughout the brain and intriguingly Zn-containing neurons are found in areas known to be important in depression and anxiety including cerebral cortical regions, hippocampus, most amygdaloid nuclei, and the lateral septum (Brown and Dyck 2004).

It has been suspected that alteration in Zn homeostasis is associated with clinical depression as reduced Zn plasma levels have been observed in patients with major depression (Maes et al. 1994; Wojcik et al. 2006). Interestingly

there appears to be a negative correlation between the severity of depression and serum Zn concentration (Maes et al. 1994). On the other hand, depressed patients who respond to antidepressant treatment also show increases in Zn plasma levels (McLoughlin and Hodge 1990) whereas no such increases are observed in treatment resistant patients (Hansen et al. 1983). Increases in Zn concentration following antidepressant treatment are also observed in rodents following chronic treatment with antidepressants (imipramine or citalopram) as well as electroconvulsive shock (as reviewed in Nowak et al. 2005). Furthermore, Zn supplementation is active in the modulation of depression-like behaviour in mice in that it reinforces the effect of classical antidepressants. For example, it was shown that an ineffective dose of Zn given jointly with an ineffective dose of antidepressant (imipramine or citalopram) induces an antidepressant effect in the forced swim test (Szewczyk et al. 2002; Rosa et al. 2003; Kroczka et al. 2001; Kroczka et al. 2000).

However, the effect of experimentally-induced Zn-deficiency on emotional behaviour has not been examined so far. Human Zn deficiency can be translated into rodents via reducing the dietary intake of Zn. We therefore examined whether graded Zn-deficiency by feeding mice with a low Zn-containing diet would lead to alteration in depression- or anxiety-like behaviour in established animal tests. To further validate this model we tested whether the behavioural effect of Zn-deficiency could be influenced by a clinically established antidepressant; desipramine (DMI) (Parker 2001) and *Hypericum perforatum* extract LI160 (*Hyp*) which is suggested to be effective in atypical depression spectrum disorders (Murck 2003). Finally, to identify neuronal substrates associated with a potential behavioural effect of Zn-deficiency we used expression of the immediate early gene Zif268 as a marker of neuronal activation. This method has been used to label activated neurons with high spatial resolution in widespread regions and pathways of the brain (Amin et al. 2006; Hefner et al. 2008; Schulte et al. 2006; Jenkins et al. 2006).

Materials and methods

Mice

Male C57BL/6N mice were obtained at 8 weeks of age from Charles River Germany (Sulzfeld, Germany) and housed (4–5/cage) side-by-side in a temperature- (22–24°C) and humidity- (50–60%) controlled vivarium under a 12 h light/dark cycle (lights on 07:00 h). All experimental procedures were approved by the local Ethical Committee on Animal Care and Use (Bundesministerium für Wissenschaft und Verkehr, Kommission für Tierversuchsangelegenheiten, Austria) and are in compliance with international laws and policies.

Zinc-deficiency and drug treatment

Mice were assigned to one of four different groups; control ($n = 8$–10), Zn-deficient ($n = 8$–10) and Zn-deficient mice chronically treated with DMI ($n = 6$–10) or *Hyp* (LI160/Lichtwer, Germany, $n = 8$–10). Mice assigned to the control group were fed a standard diet containing 65 mg/kg Zn (EF R/M control experimental diet, Ssniff Spezialdiäten, Soest, Germany). Food pellets containing low Zn (12.3 mg/kg Zn) or low Zn and *Hyp* (2 g/kg food, 13.6 mg/kg Zn) were commercially prepared (Ssniff Spezialdiäten, Soest, Germany) and based upon the EF R/M diet used in the control group. The slight difference in Zn concentration between Zn-deficient and *Hyp* containing Zn-deficient food was due to the fact that there is Zn contained within the *Hyp* extract. The daily dose of *Hyp* was based on a mean food intake of 4 g/day per mouse, evaluated in preliminary experiments. Corrected by the factual daily food intake and the weight of the mice, daily intake of 275 mg/kg *Hyp* resulted. However, this figure is based on the mean food consumption per cage and it has to be stated as a caveat that the individual doses obtained may vary due to variation in food consumption. A group of Zn-deficient mice were chronically treated with DMI (30 mg/kg per day) via the drinking water. This dose was based on the mean water consumption per cage and individual doses obtained may vary due to variation in individual water consumption.

Behaviour experiments

Mice were left undisturbed in their home cages for 3 weeks from the commencement of diet and drug treatment till the start of behavioural testing. Prior to behavioural testing (carried out between 9 am and 5 pm), mice were allowed to habituate to the testing room for at least 24 h.

Home cage activity

On day 21 and 28 days after the start of treatment (see above), control and Zn-deficient mice were individually placed into a mouse cage (36 × 20 × 15 cm) and assessed for spontaneous locomotor activity in their home cage as previously described (Singewald et al. 2004). Measurement was started at the beginning of the dark cycle (19:00) after 5 h of habituation. Locomotion was recorded in 1 min intervals for 60 h including three dark and two light cycles by an automated system (Inframot, TSE, Bad Homburg, Germany). The system monitored the activity of the mice by sensing the body heat image, i.e., infra-red radiation,

and its spatial displacement over time. No movements were monitored when mice were sleeping, inactive, or during moderate self-grooming. Data of 1 min bins were pooled to 1 h intervals.

Open field test

On the 25th and 26th days of the experiment mice were subjected to the open field test as previously described (Tschenett et al. 2003). The open field consisted of a plastic box (41 × 41 × 41 cm) equipped with an automated activity monitoring system (Tru Scan, Coulbourn Instruments, Allentown, USA). Illumination at floor level was 150 lux. Mice were individually placed into the periphery of the open field and their behaviour was tracked for 10 min. The overall distance travelled by the mice during the test session was quantified.

Light/dark test

On the 33rd and 34th days of the experiment mice were subjected to the light/dark test as previously described (Singewald et al. 2004). The fully automated light/dark test apparatus consisted of a top-open square box separated into a brightly illuminated white (20.5 × 41 × 41 cm high, 400 lux) compartment and a covered black compartment (20.5 × 41 × 41 cm high, 10 lux) (Tru Scan, Coulbourn Instruments, Allentown, USA). The compartments were connected by a small opening (7 × 7 cm wide) located in the centre of the partition at floor level. Animals were individually placed into the dark compartment facing away from the opening and allowed to freely explore the apparatus for 10 min. Behaviour of each mouse was tracked by the computer-assisted scanning system. The following parameters were quantified: (1) latency to the first entry into the lit compartment, (2) time spent in the lit compartment, (3) number of shuttle crossings between the two compartments (entries into the lit arena), (4) number of rearings and (5) the overall distance travelled by the mice.

Elevated plus maze

On the 36th day of the experiment mice were subjected to the elevated plus maze as previously described (Tschenett et al. 2003) with minor modifications. The device consisted of a central part (5 × 5 cm), two opposing open arms (30 × 5 cm) and two opposing closed arms (same size) surrounded by 14 cm high non-transparent walls. The maze was elevated 73 cm above the floor and exposed to a light intensity of 10 lux. At the beginning of each trial, mice were randomly placed onto the central platform facing a closed open arm. During the 5 min testing period the behaviour was tracked and quantified by an automated system (VideoMot, TSE Systems, Bad Homburg, Germany). The following parameters were quantified: (1) percentage of time spent on the open arms, (2) entries into both open and closed arms, and (3) the overall distance travelled by the mice. Arm entry was defined when the mouse placed its two front paws in that arm.

Novelty suppressed feeding

On the 45th day of the experiment mice were subjected to the novelty suppressed feeding test as previously described (Bodnoff et al. 1989). Mice were food-deprived for 24 h and placed into the open field arena (see above, lit at 40 lux) with a small amount of crushed oat flakes in the centre. Animals were allowed to freely explore the arena for 10 min. The latency to feed, i.e., when the animal approached and took its first bite of food, was recorded in minutes.

Tail suspension test

On the 52nd day of the experiment mice were subjected to the tail suspension test as previously described (Steru et al. 1985). Mice were securely fastened with medical adhesive tape by the tip (c.a. 1.0–1.5 cm) of the tail to a flat metallic surface and suspended for 6 min approximately 30 cm above the surface. The illumination was set at 100 lux. The activity of mice was videotaped over the entire testing period. The total time of immobility was measured during the entire 6 min of testing session by an observer blinded to the treatments. Immobility, defined as when mice passively hung without limb movement, was scored manually.

Forced swim test

On the 54th day of the experiment mice were subjected to the forced swim test as previously described (Singewald et al. 2004). Mice were individually placed in an open cylinder (diameter 12 cm, height 20 cm) containing 16 cm deep fresh tap water maintained at 23°C. Their activity was videotaped over a period of 6 min. The illumination was set at 100 lux. The total time of immobility was measured during the last 4 min of testing by an observer blinded to the treatments. Mice were considered immobile when floating passively in the water, performing only those movements required for keeping their heads above the water level.

Zif268 immunohistochemistry

Two hours following the onset of the forced swim test, mice were deeply anesthetized with an overdose of sodium pentobarbital (200 mg/kg) and transcardially perfused with

20 mL of 0.9% saline followed by 20 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffered solution (PBS, pH 7.4). Brains were then removed and post-fixed at 4°C overnight in 4% paraformaldehyde in PBS. Coronal sections (50 µm) containing the amygdala were cut with a vibratome (Ted-Pella, Redding, California) and collected in immunobuffer. The sections were processed for Zif268-like immunoreactivity as described previously (Hefner et al. 2008) via incubation with a polyclonal primary antibody (1:5,000; sc-189, Santa Cruz Biotechnology, Santa Cruz, California) and a biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, California) and were visualised by a DAB/H_2O_2 procedure. Cells containing a nuclear brown-black reaction product were considered as Zif268-positive cells. The anatomical localisation of Zif268-positive cells was aided by using the illustrations in a stereotaxic atlas (Paxinos and Franklin 2001). All Zif268-positive cells that were distinguishable from background staining were bilaterally counted in each region of interest within a defined area (0.01 mm^2) averaging counts from 2–4 sections per mouse depending on the subregion of the amygdala under investigation.

Statistics

All data are expressed as mean ± standard error of the mean (SEM) and were analysed for distribution of replicates using Levene's test. A repeated measure ANOVA was used for analysing home cage activity. One-way ANOVA was used to analyse behaviour and number of Zif268 positive cells per brain area followed by Bonferroni post hoc testing when required. Correlations between immobility time and number of Zif268 cells were performed using the Spearman's rank coefficient test. The threshold for statistical significance was set at $P < 0.05$ (statistical results above this threshold are not described).

Results

From the onset to the end of the experiment (day 54) animals of all experimental groups looked healthy as indicated by a shiny lustre on their fur and a normal increase in body weight. Nevertheless, the body weight gain differed between groups [$F(3,26) = 3.558$, $P = 0.028$]. Specifically, Zn-deficient mice had a higher gain in body weight than control mice ($P = 0.017$, Fig. 1) within the experimental time period. Body weight gain was reduced ($P = 0.011$) in Zn-deficient mice receiving DMI via the drinking water compared to untreated Zn-deficient mice, but was comparable to that of control mice. No statistically significant differences in body weight gain were observed between control ($P = 0.656$) or Zn-deficient and Zn-deficient mice chronically treated with *Hyp* ($P = 0.406$).

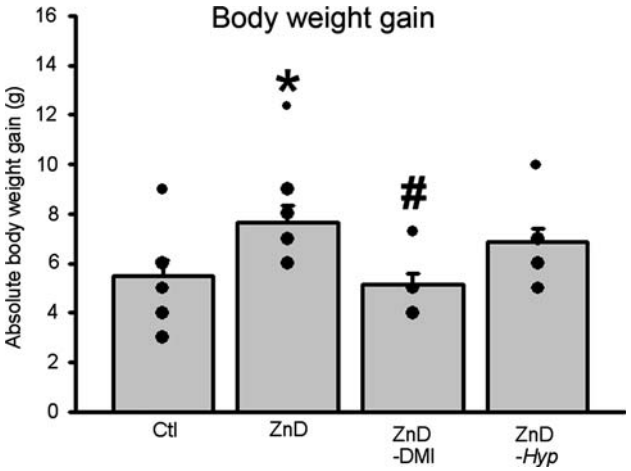

Fig. 1 Absolute gain in body weight during the course of the experiment of normally fed control (Ctl, $n = 8$) and Zn-deficient (ZnD, $n = 8$) mice receiving chronic DMI (ZnD-DMI, $n = 6$) or *Hyp* (ZnD-*Hyp*, $n = 8$) treatment. Values are expressed as mean ± SEM. Within-group scatter plot of individual weight gain is overlaid (*filled circles*). $^{*}P < 0.05$ ZnD versus Ctl, $^{\#}P < 0.05$ ZnD versus ZnD-DMI

Effect of Zn-deficiency on behaviour: influence of chronic antidepressant treatment

Locomotor activity

Measures of homecage activities revealed that Zn-deficiency did not affect spontaneous locomotor activity in mice [$F(61, 854) = 0.862$, $P = 0.764$, Fig. 2a]. As expected, there was a significant effect of circadian phases on spontaneous locomotor activity in both experimental groups, control [$F(61,427) = 9.816$, $P \leq 0.001$] and Zn-deficient [$F(61,427) = 9.822$, $P \leq 0.001$] with a pronounced elevation in the dark phase. During the light phase spontaneous locomotor activity was generally low in both groups. Furthermore no alteration in locomotor activity was observed in the open field test [$F(3,36) = 0.061$, $P = 0.980$, Fig. 2b].

Anxiety-related behaviour

In the novelty suppressed feeding test Zn-deficient mice displayed enhanced latencies to eat compared to control mice pointing towards an increased anxiety-related behaviour induced by Zn-deficiency (Table 1; Fig. 3). Chronic treatment with either DMI or *Hyp* prevented this anxiogenic effect as the latencies to eat were reduced in Zn-deficient mice chronically treated with DMI and ZnD-*Hyp* compared to Zn-deficient mice and did no longer

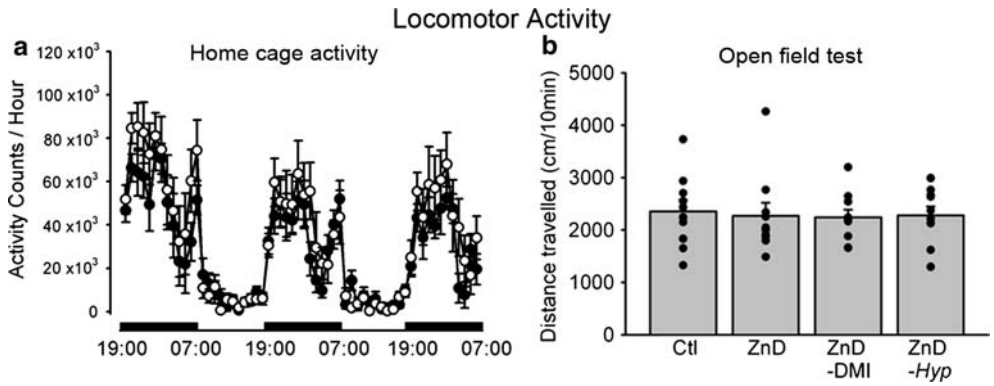

Fig. 2 **a** Hourly time course of home cage activity in Zn-deficient ($n = 8$, *open circles*) and control mice ($n = 8$, *filled circles*) recorded over three consecutive dark and two light cycles. *Black bars* indicate dark periods. **b** Locomotor activity in the open field test in control (Ctl, $n = 10$) or Zn-deficient (ZnD, $n = 10$) mice treated with DMI (ZnD-DMI, $n = 10$) or *Hyp* (ZnD-*Hyp*, $n = 10$). Values are expressed as mean ± SEM. Within-group scatter plot of individual distance travelled during the test session is overlaid (*filled circles*)

Table 1 Behavioural parameters quantified in anxiety and depression tests in control, Zn-deficient and Zn-deficient mice chronically treated with DMI or *Hyp*

	Ctl	ZnD	ZnD-DMI	ZnD-*Hyp*	Statistics
Anxiety phenotype					
Light/dark test					
Latency to enter the lit arena (s)	231.0 ± 19.3	271.2 ± 46.1	191.3 ± 37.3	228.5 ± 34.0	$F(3,36) = 0.846$, $P = 0.478$
Time spent in lit arena (s)	103.4 ± 15.7	90.4 ± 19.5	132.9 ± 19.3	126.3 ± 14.6	$F(3,36) = 1.294$, $P = 0.291$
Entries into the lit arena (number)	9.4 ± 1.3	8.4 ± 1.5	11.2 ± 1.4	11.9 ± 1.3	$F(3,36) = 1.376$, $P = 0.266$
Rearings (number)	14.2 ± 2.5	11.2 ± 2.1	11.3 ± 1.4	14.5 ± 2.3	$F(3,36) = 0.739$, $P = 0.536$
Distance travelled (cm)	2,602.4 ± 234.3	2,481.4 ± 210.4	2,419.4 ± 134.8	2,494.6 ± 154.9	$F(3,36) = 0.164$, $P = 0.920$
Elevated plus maze					
Open arm time (%)	6.0 ± 1.3	2.5 ± 0.3	4.1 ± 0.6	4.6 ± 1.1	$F(3,34) = 2.407$, $P = 0.087$
Open arm entries (%)	30.6 ± 3.6	19.6 ± 3.0	26.4 ± 3.0	26.5 ± 4.0	$F(3,34) = 1.973$, $P = 0.139$
Total arm entries (number)	18.8 ± 3.0	16.3 ± 1.6	16.1 ± 2.1	17.8 ± 1.7	$F(3,34) = 0.310$, $P = 0.818$
Distance travelled (cm)	890.2 ± 102.4	830.8 ± 94.4	866.6 ± 83.2	856.7 ± 60.3	$F(3,34) = 0.081$, $P = 0.970$
Novelty suppressed feeding					
Latency to eat (s)	116.3 ± 11.3	224.3 ± 34.2§	101.3 ± 26.3	116.6 ± 20.1	**$F(3,34) = 5.416$, $P = 0.004$**
Depression phenotype					
Tail suspension test					
Immobility time (s)	115.3 ± 9.5	152.0 ± 7.4*	74.2 ± 9.3$^{\#\#\#,\$\$}$	128.2 ± 6.1†††	**$F(3,34) = 15.777$, $P \leq 0.001$**
Forced swim test					
Immobility time (s)	84.6 ± 11.8	163.4 ± 7.2***	39.5 ± 10.7$^{\#\#\#,\$}$	141.5 ± 4.5†††,‡‡	**$F(3,26) = 29.971$, $P \leq 0.001$**

Control (Ctl, $n = 8$), Zn-deficient (ZnD, $n = 8$), and ZnD chronically treated with DMI (ZnD-DMI, $n = 6$) or *Hyp* (ZnD-*Hyp*, $n = 8$). Data are presented as mean ± SEM

*$P < 0.05$, ***$P < 0.001$ ZnD versus Ctl, $^{\#\#\#}P < 0.001$ ZnD versus ZnD-DMI, $^{\dagger\dagger\dagger}P < 0.001$ ZnD-*Hyp* versus ZnD-DMI, $^{\ddagger\ddagger}P < 0.01$, Ctl versus ZnD-*Hyp*, $^{\$}P < 0.05$ $^{\$\$}P < 0.01$ Ctl versus ZnD-DMI, $^{\S}P < 0.05$ ZnD versus Ctl, ZnD-DMI and ZnD-*Hyp*

differ from control mice. In the elevated plus maze the four experimental groups tended to differ in the percentage of time spent on the open arm but did not differ in the entries into the open arm indicating no effect of Zn-deficiency and *Hyp* or DMI treatment on anxiety-related behaviour in this test (Table 1). Locomotor activity, as revealed by total arm entries and distance travelled, was unaffected by any of the treatment regimens. We did not observe any influence of Zn-deficiency on anxiety-related measures revealed in the light/dark test including the latency to enter, entries into

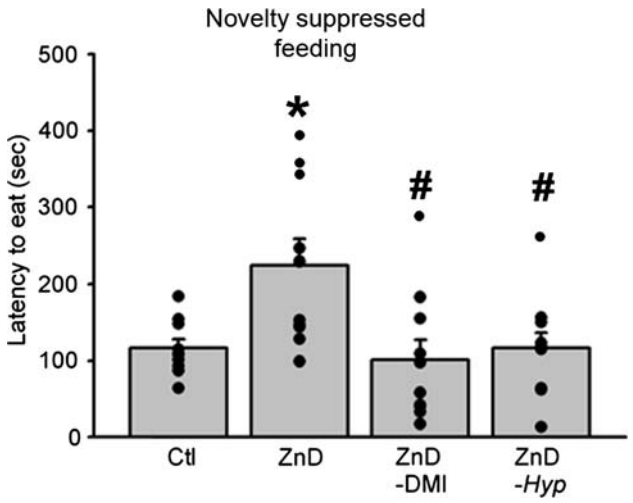

Fig. 3 Latency to eat as accessed in the novelty suppressed feeding test in control (Ctl, $n = 10$), Zn-deficient (ZnD, $n = 10$) mice and Zn-deficient mice chronically treated with DMI (ZnD-DMI, $n = 8$) or *Hyp* (ZnD-*Hyp*, $n = 10$). Values are expressed as mean ± SEM. Within-group scatter plot of individual latency to eat is overlaid (*filled circles*). $^{*}P < 0.05$ ZnD versus Ctl, $^{\#}P < 0.05$ ZnD versus ZnD-DMI and ZnD-*Hyp*

and time spent in the brightly lit arena, the distance travelled and number of rearings (Table 1). In the Zn-deficient group chronic treatment with either *Hyp* or DMI also did not alter parameters quantified in this test.

Depression-related behaviour

Zinc-deficient mice displayed a highly increased time spent in immobile postures compared to control mice in both the forced swim (Fig. 4a) and tail suspension (Fig. 4b) tests (Table 1), indicating that Zn-deficiency enhanced depression-like behaviour in the mice. Chronic treatment with DMI normalised these increased immobility times in the forced swim and tail suspension tests indicating an antidepressant treatment response in Zn-deficient mice. *Hyp* treatment had no effect on the enhanced depression-related behaviour of Zn-deficient mice.

Zinc-deficiency-related modulation of stress-induced immediate-early gene expression in the amygdala: influence of antidepressant treatment

Zif268-positive cells were quantified in several subregions of the amygdala including the central, medial, lateral and basolateral nuclei (Table 2). One-way ANOVA revealed statistically significant differences in forced swim-induced Zif268 expression in the basolateral (BA) and central lateral (CeL) amygdaloid nuclei (Table 2), but not in any other subregion. This suggests that the effect of Zn-deficiency on immediate-early gene expression is specific to particular brain regions. In more detail, in the BA we observed increased Zif268-induction in Zn-deficient mice compared to control mice ($P < 0.001$) following forced swim stress (Fig. 5a, b). Compared to untreated Zn-deficient mice, Zn-deficient mice chronically receiving DMI via the drinking water showed a reduced ($P < 0.001$) Zif268 response to forced swimming in this brain region. This indicates normalisation of the Zn-deficiency-induced hyperactivation by the treatment. In contrast, chronic *Hyp* treatment did not alter the Zif268 response in the BA of Zn-deficient mice ($P = 1.000$). For demonstrating a direct link between behaviour and brain region-specific Zif268 induction and, thus, strengthening the outcome of the present results, we performed correlation analysis. Indeed, a significant positive correlation between immobility time displayed during the forced swim test and the number of Zif268 positive cells was found in the BA ($R = 0.739$, $P < 0.001$, Fig. 5c). Zn-deficiency did not modulate stress-induced Zif268 expression in the CeA, however the

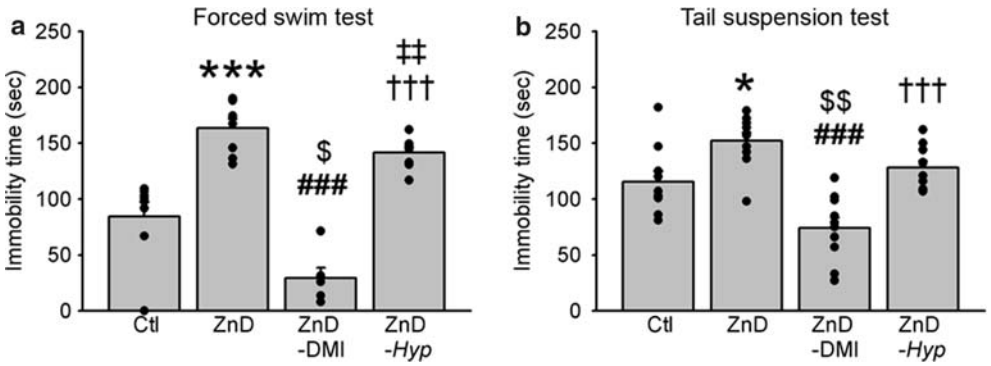

Fig. 4 Immobility times in the **a** forced swim test and **b** tail suspension test between control (Ctl, $n = 8$–10), Zn-deficient (ZnD, $n = 8$–10) mice and Zn-deficient mice chronically treated with DMI (ZnD-DMI, $n = 6$–8) or *Hyp* (ZnD-*Hyp*, $n = 8$–10). Values are expressed as mean ± SEM. Within-group scatter plot of individual immobility time is overlaid (*filled circles*). $^{*}P < 0.05$, $^{***}P < 0.001$ ZnD versus Ctl, $^{\#\#\#}P < 0.001$ ZnD versus ZnD-DMI, $^{\dagger\dagger\dagger}P < 0.001$ ZnD-*Hyp* versus ZnD-DMI, $^{\ddagger\ddagger}P < 0.01$, Ctl versus ZnD-*Hyp*, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ Ctl versus ZnD-DMI

Table 2 Zif268 expression following forced swim stress in control, Zn-deficient and Zn-deficient mice receiving chronic DMI or *Hyp*

Brain regions	Ctl	ZnD	ZnD-DMI	ZnD-*Hyp*	Statistics
Amygdala (Bregma -1.46 mm)					
Central, Medial (CeM)	11.2 ± 1.3	11.1 ± 0.4	12.8 ± 1.0	10.9 ± 1.3	$F(3,26) = 0.364$, $P = 0.779$
CeL	12.7 ± 0.6	11.9 ± 0.7	19.0 ± 1.2§	11.5 ± 0.9	**$F(3,26) = 9.266$, $P = 0.002$**
Central, Capsular (CeC)	13.0 ± 0.9	11.2 ± 1.6	16.1 ± 2.2	11.3 ± 1.7	$F(3,26) = 2.353$, $P = 0.096$
Lateral (LA)	30.9 ± 2.5	30.8 ± 1.5	29.2 ± 1.5	26.6 ± 2.7	$F(3,26) = 1.330$, $P = 0.310$
BA	10.8 ± 0.3	15.0 ± 1.5***	9.4 ± 0.5$^{\#\#\#}$	14.3 ± 0.4$^{\dagger\dagger\dagger, \ddagger}$	**$F(3,26) = 6.220$, $P = 0.009$**
Basomedial (BMA)	6.5 ± 0.8	7.2 ± 0.6	8.6 ± 0.5	8.7 ± 0.8	$F(3,26) = 0.220$, $P = 0.881$
Medial, posterodorsal part (MePD)	14.2 ± 1.2	14.7 ± 1.9	13.7 ± 0.5	11.9 ± 1.1	$F(3,26) = 0.586$, $P = 0.636$
Medial, posteroventral part (MePV)	19.6 ± 2.1	21.9 ± 0.4	20.3 ± 0.9	21.3 ± 0.8	$F(3,26) = 1.013$, $P = 0.420$
Anterior cortical (ACo)	24.3 ± 2.5	25.3 ± 2.4	23.8 ± 1.2	23.2 ± 2.5	$F(3,26) = 0.315$, $P = 0.814$
Posterolateral cortical (PLCo)	20.1 ± 1.7	25.6 ± 2.3	17.8 ± 1.8	17.3 ± 1.8	$F(3,26) = 2.849$, $P = 0.082$

Control (Ctl, $n = 8$), Zn-deficient (ZnD, $n = 8$), and ZnD chronically treated with DMI (ZnD-DMI, $n = 6$) or *Hypericum* (ZnD-*Hyp*, $n = 8$). Data are presented as mean ± SEM number of Zif268 positive cells/0.01 mm^2. In brackets is the brain level according to Bregma. $n = 6$–8/experimental group

***$P < 0.001$ ZnD versus Ctl, $^{\#\#\#}P < 0.001$ ZnD versus ZnD-DMI, $^{\dagger\dagger}P < 0.01$ ZnD-*Hyp* versus ZnD-DMI, $^{\ddagger}P < 0.05$, Ctl versus ZnD-*Hyp*, $^{\S}P < 0.001$ ZnD-DMI vs Ctl, ZnD and ZnD-*Hyp*

combination of Zn-deficiency and chronic DMI treatment resulted in an elevated number of Zif268-positive cells in the lateral subdivision of the central amygdaloid nucleus (CeL) compared to control ($P < 0.001$), Zn-deficient mice ($P < 0.001$) and Zn-deficient mice chronically treated with *Hyp* ($P < 0.001$) (Table 1). No effects of Zn-deficiency or drug treatment on immediate-early gene expression were noted in the other regions investigated (Table 1).

Discussion

In the present study we show that dietary Zn-deficiency in mice, to 40% of the recommended daily intake requirement (Reeves et al. 1993), caused a pro-depressive phenotype which was reversed by chronic DMI treatment. In addition, an anxiogenic effect was observed in Zn-deficient mice in the novelty suppressed feeding test, but not other anxiety tests performed. This effect was reversed by both chronic DMI and *Hyp* treatments. Zn-deficient mice showed exaggerated stress-evoked expression of the immediate-early gene Zif268 in the BA which was normalised following DMI treatment. These data support the association of low Zn levels with depression-like behaviour observed in humans and suggest experimentally-induced Zn-deficiency as a putative model of depression in mice.

Effect of Zn-deficiency in mice

Zn-deficiency induced a pro-depressive phenotype in both the forced swim and tail suspension tests, indicated by increased immobility scores in these tests. This was not due to an unspecific locomotor effect elicited by Zn-deficiency, since no alteration in locomotor activity in Zn-deficient mice was observed, either in the home cage or under more stressful conditions in the open field test. Concerning the mechanism of this behavioural effect, only speculations are possible at this moment. Given the antagonistic effect of Zn on NMDA receptor function (see "Introduction"), it is an attractive proposition that the pro-depressive phenotype associated with Zn-deficiency is mediated via enhanced NMDA receptor function. NMDA-mediated hyperactivity has been proposed to be involved in the pathophysiology of depression and a block of NMDA receptors by antagonists is shown to elicit an anti-depressive effect (see for example: Dhir and Kulkarni 2008; Poleszak et al. 2007; Padovan and Guimaraes 2004; Stewart and Reid 2002; Layer et al. 1995; Trullas and Skolnick 1990, for review Millan 2006). Interestingly, when Zn-deficiency is present already during neonatal development reduced NMDA receptor expression is observed which continues throughout life (Chowanadisai et al. 2005).

Zn-deficiency induced an anxiogenic effect in the novelty suppressed feeding test, indicated by increased latency to feed. The novelty suppressed feeding test is most commonly used as an anxiety-based test but is also attractive in depression research due to its ability to differentiate between subchronic and chronic effects of SSRI treatment in rodents (Gordon and Hen 2004). In this test hunger becomes the primary drive rather than exploration (Bodnoff et al. 1988). Zn-deficiency was associated with reduced appetite and reduced weight gain in rats fed a diet containing only 10% of the recommended Zn (Jing et al. 2008; Kwun et al. 2007) opening the possibility that reduced appetite rather than enhanced anxiety is the driving force behind the increased latency to eat in the

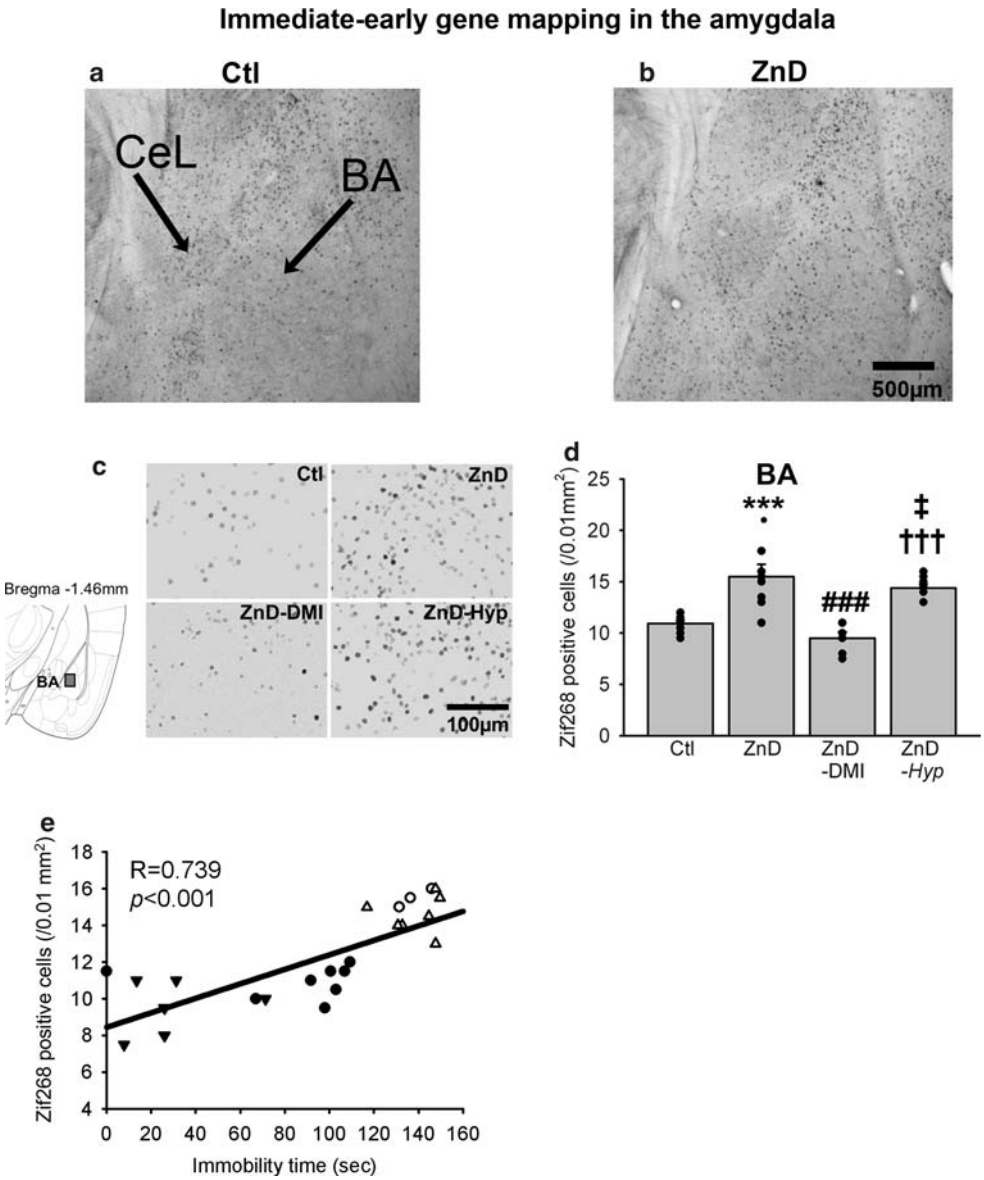

Fig. 5 Forced swim test-induced Zif268 expression in the amygdala. Representative photomicrographs showing an overview of the basolateral (BA) and central lateral (CeL) amygdala in control (Ctl, **a**) and Zn-deficient (ZnD, **b**) mice. **c** Representative photomicrographs showing Zif268 expression within the BA following forced swim stress in control (Ctl, $n = 8$), Zn-deficient (ZnD, $n = 8$) and Zn-deficient mice receiving chronic DMI (ZnD-DMI, $n = 6$) or *Hyp* (ZnD-*Hyp*, $n = 8$). **d** Zn-deficient mice displayed increased numbers of Zif268 positive cells in the BA. Chronic DMI, but not *Hyp*, reduced the number of Zif268 positive cells in Zn-deficient mice. Data are presented as mean ± SEM. Within-group scatter plot of individual number of Zif268 positive cells is overlaid (*filled circles*). **e** Immobility time in the forced swim test was positively correlated with the number of Zif268 positive cells in the BA. Control (*filled circles*), Zn-deficient (*open circles*), Zn-deficient mice chronically treated with either DMI (*filled triangles*) or *Hyp* (*open triangles*). $^{***}P < 0.001$ ZnD versus Ctl, $^{\#\#\#}P < 0.001$ ZnD versus ZnD-DMI, $^{\dagger\dagger}P < 0.01$ ZnD-*Hyp* versus ZnD-DMI, $^{\ddagger}P < 0.05$ Ctl versus ZnD-*Hyp*

novelty suppressed feeding test. However, under our much milder conditions of 40% recommended Zn containing diet we observed no evidence of reduced appetite, as even increased weight gain was noted in Zn-deficient mice compared to control mice. Alternatively, reduction in locomotor activity may contribute to the enhanced latency to eat in Zn-deficient mice. However, this can be excluded, as we observed no alteration in locomotor (see above) in these animals. Hence these data indicate an anxiogenic effect induced by Zn-deficiency. Interestingly, we observed a tendency for an anxiogenic effect of Zn-deficiency in the elevated plus maze, indicated by a reduction in open arm time, which however failed to reach statistical significance. No effect of Zn-deficiency on anxiety-related parameters was observed in the light/dark test. A potential reason explaining the lack of an anxiogenic effect in the light/dark test and elevated plus maze may be different sensitivity of tests or different aspects of anxiety/level of anxiety induced by novelty suppressed feeding versus elevated plus maze and light/dark test, respectively. This remains to be tested. On the other hand, since we used a battery of behavioural testing (Cryan and Holmes 2005) to be able to reduce the number of animals needed, the effect of previous test experience influencing subsequent testing should be considered. It is shown that the elevated plus maze test, but not the light/dark or forced swim tests, is particularly sensitive to previous testing experience (see e.g. Voikar et al. 2004). Hence, it is for example conceivable that the trend for an anxiogenic effect of Zn-deficiency seen in the elevated plus maze, would reach statistical significance if test naive animals are used.

Effect of desipramine in Zn-deficient mice

The pro-depressive phenotype induced by Zn-deficiency was reversed following chronic DMI treatment. Tricyclic antidepressants including DMI and imipramine have previously been shown to be active when administered via the drinking water in C57BL/6 mice (Caldarone et al. 2003; Goodwin et al. 1984; Singewald et al. 2004). The exact mechanism of action by which DMI abolished the enhanced depression-like behaviour induced by Zn-deficiency is not clear at present. Generally, tricyclic antidepressants inhibit the reuptake of both serotonin and noradrenaline, leading, after chronic treatment, to adaptive changes in monoamine receptor function, as well as modulation of various signalling pathways including those involved in neuronal plasticity and survival (for reviews, see D'Sa and Duman 2002; Leonard 1997; Millan 2006). Given the mentioned interaction between Zn and NMDA receptors, it is interesting to note that DMI has been shown to attenuate NMDA receptor function (Szasz et al. 2007; Watanabe et al. 1993; White et al. 1990; Sernagor et al. 1989). Thus, potentially enhanced NMDA receptor activity in Zn-deficient mice may be normalised by chronic DMI treatment leading to the attenuation of the enhanced depression-like behaviour observed in Zn-deficient mice.

Chronic DMI treatment also reversed the anxiogenic phenotype of Zn deficient mice in the novelty suppressed feeding test, which has been shown to be sensitive to chronic (but not acute) antidepressant treatment (reviewed in Gordon and Hen 2004). This finding supports the conclusion that the effect noted in this test was indeed related to anxiety and not some unspecific (e.g. locomotor) effects.

Effect of *Hypericum* in Zn-deficient mice

Chronic *Hyp* treatment did not alter the pro-depressive phenotype in Zn-deficient mice. This is an interesting dissociation as chronic *Hyp* treatment was effective in normalising the anxiogenic phenotype in the novelty suppressed feeding test (see below). Since the average daily intake of *Hyp* was 275 mg/kg per day per mouse and it was shown that 380 mg/kg per day per mouse of *Hyp* normalises a pro-depressive phenotype following magnesium-depletion (Singewald et al. 2004), one potential reason for the lack of effect of *Hyp* in the depression tests could be an insufficient dose used in the present study. However, in rats, antidepressant effects of *Hyp* are observed at 50, 150 and 300 mg/kg per day following daily administration for 3 consecutive days (Bhattacharya et al. 1998) rendering this explanation rather unlikely. Thus it seems that *Hyp* treatment is not sufficiently effective to reverse the pro-depressive phenotype induced by Zn deficiency. Inconsistent results have also been reported in clinical trials showing that *Hyp* is either superior (Papakostas et al. 2007; Fava et al. 2005; Lecrubier et al. 2002) or not superior (Shelton et al. 2001; Hypericum Depression Trial Study Group 2002; Moreno et al. 2006) to placebo in the treatment of mild to moderate depression. While a beneficial effect of *Hyp* treatment has been suggested in atypical depression spectrum disorders (Murck 2003), it failed to be effective in severe major depression (Fava et al. 2005). Hence it may be speculated that Zn-deficient models severe major depression, although this suggestion has to be further tested.

Similar to the effects of DMI, chronic *Hyp* treatment also attenuated the Zn-deficiency-induced anxiogenic phenotype in the novelty suppressed feeding test. In line with observations in the Mg-depletion model (Singewald et al. 2004), the anxiolytic effect of *Hyp* was seen when anxiety was induced in the novelty suppressed feeding test, but not the elevated plus maze or light/dark test which failed to detect an increase in anxiety by Zn-deficiency. Anxiolytic-like effects of *Hyp* treatment have been noted previously in different models (e.g. Vandenbogaerde et al. 2000; Butterweck et al. 2001; Kumar et al. 2001; Flausino et al. 2002). Despite considerable research effort, the active constituents of *Hyp* are still unclear and the mechanism(s) of action are not completely understood (for review, see Greeson et al. 2001; Mennini and Gobbi 2004). It has been proposed that *Hyp* inhibits the reuptake of transmitters including noradrenaline, dopamine, serotonin, glutamate and GABA in an unspecific manner (Kaehler et al. 1999; Wonnemann et al. 2000, for review, see Mennini and Gobbi 2004) and modulates neuronal excitability via glutamatergic and GABAergic mechanisms (Vandenbogaerde et al. 2000; Langosch et al. 2002). Interestingly, Hyperforin, a constituent of *Hyp*, is found to inhibit NMDA-induced calcium influx into cortical neurons in vitro (Kumar et al. 2006), potentially indicating that antagonism of NMDA receptor function is involved in the anxiolytic effect of *Hyp* in Zn-deficient mice.

Immediate-early gene mapping in the amygdala

The amygdala is well known to be implemented in the processing of emotion and mood in animals and humans (Anand and Shekhar 2003; Drevets 2003). It has been shown that stress-induced amygdala hyperactivation in depressed patients is normalised following successful antidepressant treatment (Davidson et al. 2003; Fu et al. 2004; Kalin et al. 1997; Surguladze et al. 2005). Thus we tested the specific hypothesis that amygdala hyperactivation would be observed in Zn-deficient mice which should be normalised following behaviourally active antidepressant treatment. Indeed, we found that neuronal populations

within the BA were hyperactivated in Zn-deficient mice compared to control mice. This hyeractivation was normalised following DMI treatment. This effect was specific for the BA since no alterations in immediate-early gene expression were noted in any of the remaining amygdala subregions quantified. Interestingly, immobility time was positively correlated with the number of Zif268 positive cells in the BA indicating that increased depression-like behaviour was associated with increased neuronal activity in this area. Interestingly, increases in depression-like behaviour and BA activation both normalised following oestrogen treatment has been observed in ovariectomised rats (Rachman et al. 1998). In high anxiety-related behaviour rats with comorbid depression (Landgraf and Wigger 2002) which also show signs of amygdala hyperexcitability (for review see Singewald 2007), chronic treatment with the SSRI paroxetine reduces the pro-depressive effect of these rodents. This treatment effect was associated with a reduction of stress-induced c-Fos response in the central amygdala, and a (not statistically significant) trend of reduction in the BA (Muigg et al. 2007). Taken together, attenuation of the neuronal hyperexcitability in the BA seems to play a role in the successful treatment response of DMI on the enhanced depression-like behaviour evoked by Zn-deficiency.

Interestingly, using a metallographic staining method, Zn levels have been shown to be particularly high in the BA, while for example the central amygdala shows very weak staining (Brown and Dyck 2004). Hence, it may be suggested that the observed BA hyperactivation in Zn-deficient mice is at least in part due to reduced inhibitory Zn action leading to enhanced NMDA receptor function in this area. Indeed, it is known that Zif268 activation is mediated (amongst other mechanisms) by NMDA receptor activation (reviewed in Knapska and Kaczmarek 2004). As mentioned above, part of the DMI effect may be related to reduction of NMDA receptor function. Hence the reversal of exaggerated stress-induced Zif268 expression in Zn-deficient mice by DMI may be in part mediated via this mechanism. Along these lines NMDA receptor antagonism has been shown to attenuate Zif268 activation in the BA (Milton et al. 2008).

Conclusions

These data demonstrate that dietary-induced Zn-deficiency leads to a robust pro-depressive phenotype and a specific anxiogenic phenotype in the novelty suppressed feeding test. Hence, we provide pre-clinical evidence for an association between reduced Zn intake and elevated depression. Furthermore, it was shown that chronic DMI treatment normalised the pro-depressive phenotype induced by Zn-deficiency. Both, chronic DMI and *Hyp* treatment normalised the enhanced anxiety observed in Zn-deficient mice in the novelty suppressed feeding test. Supporting the behavioural data, hyperactivation of the BA and subsequent normalisation by chronic DMI treatment was correlated with reversal of the depressive phenotype in Zn-deficient mice. Taken together these findings provide evidence that impairment of neuronal processing within the amygdala, a key region involved in the modulation of depression-like behaviour, contributes to the observed pro-depressive phenotype induced by Zn deficiency. Further, these data indicate that experimentally induced Zn-deficiency might be a useful rodent depression model for the screening of clinically active antidepressant substances.

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