Social defeat during adolescence and adulthood differentially induce BDNF-regulated immediate early genes

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INTRODUCTION
Stressful life events generally enhance the vulnerability for the development of human psychopathologies such as anxiety disorders and depression. The incidence rates of adult mental disorders steeply rise during adolescence in parallel with a structural and functional reorganization of the neural circuitry underlying stress reactivity. However, the mechanisms underlying susceptibility to stress and manifestation of mental disorders during adolescence are little understood. We hypothesized that heightened sensitivity to stress during adolescence reflects age-dependent differences in the expression of activity-dependent genes involved in synaptic plasticity. Therefore, we compared the effect of social stress during adolescence with social stress in adulthood on the expression of a panel of genes linked to induction of long-term potentiation (LTP) and brain-derived neurotrophic factor (BDNF) signaling. We show that social defeat during adolescence and adulthood differentially regulates expression of the immediate early genes BDNF, Arc, Carp, and Tieg1, as measured by qPCR in tissue lysates from prefrontal cortex, nucleus accumbens, and hippocampus. In the hippocampus, mRNA levels for all four genes were robustly elevated following social defeat in adolescence, whereas none were induced by defeat in adulthood. The relationship to coping style was also examined using adult reactive and proactive coping rats. Gene expression levels of reactive and proactive animals were similar in the prefrontal cortex and hippocampus. However, a trend toward a differential expression of BDNF and Arc mRNA in the nucleus accumbens was detected. BDNF mRNA was increased in the nucleus accumbens of proactive defeated animals, whereas the expression level in reactive defeated animals was comparable to control animals. The results demonstrate striking differences in immediate early gene expression in response to social defeat in adolescent and adult rats.

Keywords: social defeat, stress, adolescence, BDNF, synaptic plasticity, hippocampus, mesocorticolimbic system, rat
of the BDNF gene is associated with heightened aggression to conspecifics, anxiety, and learning deficits, without affecting depression-like behavior in standard tests (Duman and Monteggia, 2006; Feder et al., 2009; Ito et al., 2011). Treatment with antidepressant drugs triggers enhanced BDNF gene expression and signaling that is required for the restorative behavioral effects in animal models of depression (Alme et al., 2007; Rantamaki et al., 2007; Adachi et al., 2008).

In the dentate gyrus, expression of the immediate early gene Arc (activity-regulated cytoskeleton associated protein) is required for formation of stable, transcription-dependent long-term potentiation (LTP) induced by brief intrahippocampal infusion of BDNF or by brief high-frequency stimulation (HFS) of the perforant pathway (Messoudi et al., 2007; Bramham et al., 2010). Wibrand et al. (2006) further identified a panel of genes that are robustly co-up-regulated with Arc in dentate granule cells during both forms of LTP. These genes were subsequently found to be differentially regulated in brain region-specific manner following chronic antidepressant treatment in rats (Alme et al., 2007).

The social environment is an important source of stress (conflicts and tension) in everyday life of humans. To induce social stress in rats, we used the social defeat paradigm. Björkqvist (2001) suggested that the social defeat paradigm is an ecologically valid model to study the consequences of social stress, victimization, and social subjugation. Social defeat has been shown to induce long-lasting behavioral, physiological, and neurobiological changes. These include changes in social anxiety, heart rate, body temperature, activity as well as structural and functional changes in various brain neurocircuitries of rats (Meerlo et al., 1999; Buwalda et al., 2005). Therefore, we used social defeat on two consecutive days as a stressor in our experimental animals, which has been shown to induce reliable long-lasting effects on behavior and physiology (Meerlo et al., 1996).

We hypothesized that age-dependent differences in stress reactivity could reflect differences in the induction of BDNF-regulated and synaptic plasticity-linked immediate early genes. Directly after exposure to social defeat on two consecutive days, mRNA expression of BDNF (exon IV), Arc, Carp, and Tieg1 was determined by qPCR in tissue lysates obtained from prefrontal cortex, hippocampus, and nucleus accumbens. The BDNF, Arc, Carp, and Tieg1 genes are strongly up-regulated during BDNF–LTP in the dentate gyrus of the hippocampus (Wibrand et al., 2006). Gene expression of BDNF exon IV (i.e., exon III prior to Aid et al., 2007) was determined, since this exon is regulated as an immediate early gene and shows a peak level 1 h after stimulation (Lauterborn et al., 1996). Carp (calcium/calmodulin dependent protein kinase (CaMK)-related peptide), also known as ANIA-4 (Berke et al., 1998) is an alternative splice variant of the doublecortin-like kinase (DCLK) gene. Tieg1 (transforming growth factor-β-inducible early gene) is a member of the specificity protein/Kruppel-like factor (SP/KLF) family of zinc finger transcription factors (Subramaniam et al., 1995; Suske et al., 2005) and functions in enhancement of TGF-β-dependent gene expression (Johnsen et al., 2002).

The prefrontal cortex was chosen because of its major structural reorganization during adolescence (Kalsbeek et al., 1988) and its important role in emotional regulation (Quirk and Beer, 2006) and aggressive behavior (Blair, 2004; Siever, 2008). The prefrontal cortex is significantly involved in modulation of social behavior and in control of mood and motivational drive, functions that are important components of the personality of an individual (Miller, 2000; Miller and Cohen, 2001). Social defeat has been shown to have a major impact on hippocampal structure and functioning (Buwalda et al., 2005; Artola et al., 2006). In addition, we selected the nucleus accumbens because the ventral tegmental area–nucleus accumbens (VTA–NAcc) pathway has been shown to play a major role in the difference in resilience to social defeat (Berton et al., 2006; Krishnan et al., 2007).

In addition to age-dependent vulnerability to stress, humans exhibit large individual variations in vulnerability to stress-induced disorders and variability in measures of temperament and personality may largely predict this disease risk. Accordingly, variability in behaviorally relevant brain circuit functions due to differences in activity-dependent gene expression and synaptic plasticity may represent one of the causal factors determining the vulnerability to disease. In animals, behavioral differentiation in terms of coping style (personality) reflect trait characteristics that are stable over time (Koolhaas et al., 1999) and these characteristics are strongly correlated with a differentiation in the underlying neurobiological mechanisms (Veennema and Neumann, 2007; Koolhaas et al., 2010). For example, high levels of aggressive behavior are generally associated with low levels of brain serotonin and its metabolite 5-HIAA (De Boer et al., 2010; Koolhaas et al., 2010). Recent evidence suggests differences in molecular mechanisms of synaptic plasticity as well. For instance, non-aggressive, reactive coping male mice show a higher structural neuronal plasticity (Veennema and Neumann, 2007) and a higher hippocampal and prefrontal cortex expression of neuronal plasticity-related genes (Feldker et al., 2003).

To examine a possible link between individual differences in coping to social stress and expression of plasticity-linked genes, adult male wild-type Groningen (WTG) rats were individually characterized for their coping style using their displayed aggressiveness in a resident-intruder paradigm. Rats of this strain differ widely in the level of offensive aggression expressed toward an unfamiliar intruder male, ranging from no aggression at all (reactive coping) to very high levels of intense aggressive behavior (proactive coping). It has been shown that this broad individual variation in aggressiveness can be considered more generally as a variation in actively coping with environmental challenges (De Boer et al., 2003).

MATERIALS AND METHODS

ANIMALS

Adolescent and adult male (WTG rats (Rattus Norvegicus; originally wild-trapped animals and bred under laboratory conditions for over 50 generations in our own facilities) were used. Animals were weaned at postnatal day 28 and housed in groups until the start of the experiment.

All animals were housed in temperature-controlled rooms (21 ± 2°C) under a 12-h light-dark cycle (lights off at 1 p.m.) with food and water available ad libitum. Experiments were approved by the Groningen University Committee on Animal Experiments.

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characterization of adult animals

Adult animals were screened for the level of offensive aggressive behavior in a standard resident-intruder paradigm at an age of approximately 120 days. Animals were housed in large observation cages (80 cm × 55 cm × 40 cm) with a sterilized female (oviduct-ligated) for 1 week to avoid social isolation and facilitate territorial behavior. After 1 week, the baseline level of aggressive behavior was tested in the resident-intruder test in the first half of the dark phase.

Before testing, the female was removed from the cage. During the first three tests an unfamiliar male conspecific (intruder) was introduced into the cage and the attack latency (time between introduction of the intruder and first attack) was scored. The intruder was removed after the first attack. If no attack occurred within 10 min the intruder was removed.

During the fourth test the full range of behaviors was scored during 10 min. The frequency and duration of behavioral elements were scored. A total of 12 behavioral acts and postures were scored and grouped in five behavioral categories: (1) **Offense** (lateral threat, clinching, keep down, chasing, upright posture); (2) **Social exploration** (moving toward, nosing, investigating opponent, ano-genital sniffing, crawl over, attempted mount, social groom); (3) **Non-social exploration** (ambulation, rearing, sniffing, scanning, digging); (4) **Inactivity** (sitting, lying, immobile, freezing); (5) **Grooming** (washing, shaking, scratching).

The behavioral data of the last test and the four attack latencies were used to classify the offensive behavior of animals. Adult animals were divided in two groups: proactive (<15% time spent on offensive behavior) and reactive (>65% time spent on offensive behavior). All adult animals were solitary housed after aggression screening.

social defeat

The experimental setup is illustrated in Figure 1. Half of the adolescent and adult (proactive and reactive) animals were subjected to social defeat on two consecutive days for 1 h. Adolescent animals were defeated at postnatal day (pnd) 45 and 46, whereas adult animals were defeated at approximately pnd 140. The resident rats were also WTG rats and were housed in a separate room in large cages (80 cm × 55 cm × 40 cm) with a female to stimulate territorial aggression. Prior to the experimental procedure, females were removed from the resident’s cage. Residents were trained to attack a naïve intruder, only residents that attacked within 2 min were used for the experiment. By using animals with a more or less similar readiness to attack we tried to avoid variation in attack intensity.

Experimental animals were moved to the room of the residents. Animals in the defeat groups were introduced in the cage of the resident and were attacked for 15 min. Thereafter animals were placed in a wire mesh cage (30 cm × 15 cm × 15 cm) in the cage of the resident for 45 min. In this way, animals were protected from further attacks and injury, but remained in visual, auditory, and olfactory contact with the resident. This period of psychosocial stress is known to be highly stressful (Tornatzky and Miczek, 1994).

On the second day of the defeats, animals were directly exposed to another aggressive resident for 5 min and placed in the wire mesh cage for 55 min. Defeats took place in the first half of the dark phase. Brain material was collected immediately after the end of the second defeat. The control animals were directly taken from the home cage and were sacrificed at the same time as defeat animals.

Collection of brain material

Directly after the end of the second defeat, rats were anesthetized by CO2, decapitated, and the brain was removed. The
prefrontal cortex, hippocampus, and nucleus accumbens were rapidly dissected on ice. The tissue was immediately frozen in N₂ and stored at −80°C. The 1 h time point after the start of the second defeat was chosen because previous studies indicated that the mRNAs under study are all induced within 1 h after BDNF infusion into the dentate gyrus (Dagestad et al., 2006; Wibrand et al., 2006).

RNA ISOLATION AND cDNA PREPARATION
Total RNA was isolated using the mirVana™PARIS™miRNA isolation kit (Ambion, AM1556) according to the manual. DNase treatment was carried out to remove genomic DNA contamination prior to cDNA synthesis (Ambion, EN0521). The yield and quality of the RNA were determined by measuring the absorbance at 260/280 nm. Single-stranded cDNA was synthesized from 2 μg of total RNA according to the MMLV reverse transcriptase kit instructions (Ambion, AM2043).

REAL-TIME QUANTITATIVE PCR
Real-time quantitative PCR was performed on a Roche LightCycler® 480 II (Roche Applied Science) using cDNA from individual animals. cDNA corresponding to 10 ng total RNA was analyzed in 25 μl reactions using 2× TaqMan PCR mix (Applied Biosystems). PCR quantification was performed in triplicate and the relative standard curve method to determine gene expression levels was used for each animal using the Roche LightCycler® 480 Software (release-1.5.0 SP4).

Three housekeeping genes were analyzed (hrpt1, ubiquitin B, and cyclophilin A) and gene expression levels for Arc, BDNF, Carp, and Tieg1 were determined. Commercially designed TaqMan® Gene expression assays were as follows (genes in parentheses): Rn00571208_g1 (Arc), Rn01484927_m1 (BDNF exon IV), Rn00572049_m1 (Carp), Rn00579697_m1 (Tieg1), Rn00690933_m1 (cyclophilin A), Rn03062801_g1 (ubiquitin B), and Rn01527840_m1 (Hrpt1). Hrpt1 expression was used as endogenous reference for the adolescent samples and adult gene expression was normalized to ubiquitin expression. The relative gene expression levels are presented as fold change based on the average group gene expression level of adolescent control and adult (reactive) control animals.

STATISTICS
Results are presented as mean ± SEM. Statistical analysis was performed using SPSS (version 16). Data of the adolescent social defeat were analyzed using a Student’s t-test. Adult data were analyzed using a two-way ANOVA with coping style and defeat as between subject factors. A p-value less than 0.05 was considered to be statistically significant.

RESULTS
SOCIAL DEFEAT DURING ADOLESCENCE LEADS TO BRAIN REGION-SPECIFIC UPREGULATION OF BDNF–LTP RELATED IMMEDIATE EARLY GENES
Quantitative real-time PCR was used to determine changes in the level of mRNA expression of BDNF–LTP related genes in the prefrontal cortex, hippocampus, and nucleus accumbens after adolescent social defeat. In the prefrontal cortex (Figure 2A), the level of Arc (p = 0.04) and Tieg1 (p = 0.04) were significantly up-regulated by social defeat compared to control animals, whereas the levels of BDNF and Carp did not differ significantly between control and defeat animals. In the hippocampus (Figure 2B), the levels of Arc (p = 0.02), BDNF (p < 0.001), Carp (p = 0.03), and Tieg1 (p = 0.04) were all increased in social defeat animals. In the nucleus accumbens (Figure 2C), the level of Arc
was up-regulated three-fold in defeat animals (p = 0.001), BDNF, Carp, and Tieg1 levels did not differ between groups.

**SOCIAL DEFEAT DURING ADULTHOOD AND EFFECT OF COPING STYLE**

Proactive and reactive animals were divided in control and defeat groups. A justification of the composition of the four experimental groups and the behavioral profile is given in Table 1. The data represent the time spent on five different behavioral categories and the attack latency during the 10-min offensive aggression test. There are no statistically significant differences between the various control and defeat groups.

The level of mRNA expression of BDNF–LTP related genes in adult animals is depicted in Figure 3. The level of Arc mRNA expression was significantly elevated in the prefrontal cortex (F1,19 = 9.29, p = 0.007; Figure 3A) and nucleus accumbens (F1,18 = 7.26, p = 0.015; Figure 3C) of defeated animals, whereas Arc mRNA levels were not affected in the hippocampus (Figure 3B). BDNF mRNA was significantly increased in the nucleus accumbens of defeat rats (F1,19 = 5.00, p = 0.04). A trend toward a significant interaction between the coping style of animals and defeat was seen in the level of BDNF mRNA in the nucleus accumbens (F1,19 = 3.23, p = 0.09) was found. Tieg1 mRNA expression levels were increased only in the nucleus accumbens (F1,19 = 5.07, p = 0.04). The level of Carp was not affected by social defeat in any of the examined brain regions. In the hippocampus, none of immediate early genes examined were induced in adult proactive and reactive rats, whereas all were induced following social defeat in adolescent rats.

**DISCUSSION**

This study shows that adolescent and adult social stress leads to brain region-specific upregulation of genes associated with BDNF-induced LTP. There is a major age-dependent effect of social defeat on gene expression in the hippocampus. Arc, BDNF, Carp, and Tieg1 were all up-regulated by social stress in the hippocampus of adolescent animals, whereas in adults none of these genes were induced. Similar age-dependent effects have been demonstrated by Toth et al. (2008). In young rats, BDNF protein levels in the hippocampus were increased after chronic mild stress, whereas decreased levels were found in the hippocampus of adult rats (Toth et al., 2008). In addition, in young, but not adult, rats hippocampal BDNF protein induces prolonged elevations in corticosterone secretion (Talaz et al., 2011).

In the prefrontal cortex of adolescent animals Arc and Tieg1 were up-regulated after defeat. In contrast to the hippocampus, the prefrontal cortex appears to be more affected by social defeat in adult animals. Gene expression levels of Arc are almost two-fold higher in adult defeated animals compared to adolescent defeated animals. Several studies reported an induction of Arc mRNA in the prefrontal cortex from both acute and chronic stress paradigms as well (Ons et al., 2004, 2010; Mikkelsen and Larsen, 2006).

In the nucleus accumbens, Arc gene expression was markedly increased after social defeat both in adolescent and in adult animals. BDNF was significantly up-regulated only in adult defeated animals. This age related difference corresponds with reports on late developmental changes in neural systems in the nucleus accumbens (Tarazi et al., 1998, 1999). However these findings are not unanimous. For example, Leslie et al. (1991) did not show a change during adolescence in dopamine receptor density (Leslie et al., 1991).

The results demonstrate region-specific and age-dependent effects of social stress on immediately early genes linked to LTP and BDNF signaling. In the hippocampus, the striking age-dependent effects suggest that social defeat mobilizes a strong immediate early gene response that is absent in adult animals. This raises the possibility that hippocampal long-term synaptic plasticity is selectively engaged in social stress during adolescence. The function of the gene expression in the hippocampus is not known; it could reflect a transient adaptive response (resilience) or a step toward the experience-dependent maturation of the hippocampal response to social defeat.

It is often assumed that adolescents are more vulnerable to social stress, since the prefrontal cortex and the hippocampus are still undergoing structural reorganization during this developmental period (Kalsbeek et al., 1988; Andersen et al., 2000; Andersen, 2003). Based on the current data, we can indeed conclude that adolescent social stress is qualitatively different from adult social stress in the expression of synaptic plasticity-related genes.

We expected baseline differences in the level of genes related to BDNF–LTP in adult animals with different coping strategies. It is known that reactive coping male mice show a higher expression of several genes coding for cytoskeletal and signal transduction proteins in the hippocampus compared to proactive coping mice (Feldker et al., 2003). In addition, the intra- and infra-pyramidal mossy fibers terminal fields in the hippocampus of reactive coping mice are larger (Slyuter et al., 1994). In the rat however, these observations are not supported by a difference in baseline gene expression profiles.

No difference was found in the level of gene expression between proactive and reactive coping after social defeat. However, a trend

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**Table 1 | Behavioral profile (attack latency in seconds and percentage time spent on the six distinct behavioral categories during a 10-min resident-intruder aggression test) of the four different groups used for the study.**

<table>
<thead>
<tr>
<th></th>
<th>Attack latency (s)</th>
<th>Offense</th>
<th>Social explore</th>
<th>Social interaction</th>
<th>Non-social explore</th>
<th>Inactivity</th>
<th>Grooming</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control reactive</td>
<td>375 ± 104***</td>
<td>3 ± 1***</td>
<td>15 ± 1***</td>
<td>18 ± 2***</td>
<td>43 ± 6***</td>
<td>38 ± 6***</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Defeat reactive</td>
<td>545 ± 56***</td>
<td>3 ± 2***</td>
<td>17 ± 3***</td>
<td>19 ± 5***</td>
<td>57 ± 7***</td>
<td>22 ± 8***</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Control proactive</td>
<td>33 ± 15</td>
<td>83 ± 5</td>
<td>5 ± 3</td>
<td>88 ± 4</td>
<td>9 ± 3</td>
<td>1 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Defeat proactive</td>
<td>28 ± 9</td>
<td>79 ± 5</td>
<td>4 ± 2</td>
<td>83 ± 5</td>
<td>13 ± 3</td>
<td>1 ± 1</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SEM. ***p < 0.001 two-way ANOVA.*
toward a differential effect of defeat on proactive and reactive coping animals was found in BDNF mRNA in the nucleus accumbens. Reactive animals show increased levels of BDNF mRNA after defeat, whereas proactive BDNF mRNA levels are comparable to levels of control animals.

The differential BDNF mRNA expression after defeat in reactive and proactive animals might be associated with resilience to social defeat. Krishnan et al. (2007) showed that BDNF mRNA levels in the nucleus accumbens of mice subjected to social defeat are equal to control animals. However, in a group of susceptible mice BDNF protein levels are increased, whereas BDNF levels in unsusceptible mice are unaffected (Krishnan et al., 2007). Manipulation of BDNF gene expression levels in the mesolimbic dopamine pathway by local deletion of the BDNF gene reduces the long-term neural and behavioral response to social defeat stress similar to effects produced by antidepressant treatment (Berton et al., 2006).

Arc is a key effector protein for BDNF-induced LTP, but Arc is multifunctional protein required for other forms of synaptic plasticity such as long-term depression and homeostatic scaling (Rial Verde et al., 2006; Shepherd et al., 2006; Bramham et al., 2008; Waung et al., 2008) as well. Homeostatic plasticity may compensate LTP and LTD by scaling neuronal output without changing the relative strength of individual synapses (Shepherd et al., 2006). Synaptic plasticity in the developing visual cortex is an example of this homeostatic plasticity. Arc appears to be required for the experience-dependent processes that normally establish and modify synaptic connections in the visual cortex (McCurry et al., 2010). Arc induction after social defeat might induce a similar process of homeostatic plasticity.

It is unknown what mechanism determines whether Arc is selectively engaged in homeostatic plasticity, LTP or LTD. Social defeat not only reduces LTP in rats, but also enhances long-term depression (LTD) 7–9 months after repeated defeat experience (Kole et al., 2004; Artola et al., 2006). Therefore, the increased level of Arc found after social defeat in the current study might be functionally involved in the process of homeostatic plasticity.

One confounding factor may be that the current social defeat procedure includes individual housing after social defeat. Social isolation appears to be an important factor in the long-term effects of defeat since social housing has been shown to reduce the impact of social defeat (Ruis et al., 1999; De Jong et al., 2005). Solitary housing in itself affects LTP and LTD; LTP is higher in animals that are housed in an enriched environment compared to individually housed animals (Artola et al., 2006).

Other possible confounding factors are false-negative results due to the temporal dynamics of gene expression after social defeat. The fact that all genes were up-regulated in the hippocampus of adolescent rats makes it unlikely that there are false-negatives in the gene expression levels of other brain areas of adolescent and adult animals.

The temporal dynamics of mRNA expression following BDNF–LTP and HFS LTP has been studied by Wibrand et al. (2006) using in situ hybridization. They showed that the kinetics of mRNA was rapid (40 min following post-HFS) and sustained (3 h post BDNF) in the dentate gyrus granule cells (Wibrand et al., 2006). Based on this time course of gene expression it is unlikely that the chosen time point resulted in false-negative effects of social defeat.

We did not dissociate between sub-regions of the hippocampus, prefrontal cortex, and nucleus accumbens. However, there may be a difference in gene expression levels in different regions of the hippocampus. For example, Gronli et al. (2006) showed that chronic mild stress inhibits BDNF protein expression in the dentate gyrus, but not in the hippocampus proper and...
immobilization stress in rats is associated with greater impairments in BDNF mRNA expression in the dentate gyrus compared to the cornus ammonis (CA) region (Smith et al., 1995). Similarly, the effects of social defeat stress on gene expression may differ between subdivisions of the prefrontal cortex and nucleus accumbens. Whether subregional differences in gene expression in response to social defeat stress exist needs to be determined in future experiments using in situ hybridization.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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