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# Differential long-term effects of social stress during adolescence on anxiety in Wistar and wild-type rats

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## ABSTRACT

Severe and chronic stress may interfere with adolescent neuronal plasticity that turns the juvenile brain into an adult brain increasing the vulnerability to develop anxiety disorders. It is well-known from adult stress research that there is a large individual differentiation in stress vulnerability. The current study is aimed at the individual resilience and vulnerability to adolescent social stress. Two strains of rats that differ in social behavioral skills were subjected to social stress during adolescence. In three experiments we studied short and long term effects of adolescent social stress using a water conflict test in different contexts. Wistar rats which had been socially defeated on postnatal days 45 and 46 showed, following water deprivation, a strong decrease in the total amount of water consumed and time spent drinking when tested 2 days and 3 weeks later in the context where they received the defeat experience. Also a strong increase in drinking latency was noticed in the context of the previous defeat. No differences in these parameters were found between defeated and non-defeated wild-type rats. The results of the water conflict test in an environment where no association with the previous defeat experience was present showed that the adolescent social stress did not induce a generalized anxiety.

In conclusion, the water conflict test is a useful tool to measure the influence of social defeat on the motivation to obtain resources under conditions with different stimulus properties. In addition, our data suggest the importance of the strain used in adolescent stress experiments. The fact that Wistar rats showed a strong association with the context at adulthood whereas no effect was observed in the wild-type rats shows that victim characteristics are important determining factors for the long term effects of adolescent social stress.

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## 1. Introduction

Adolescence is considered to be an important period for neurobiological and behavioral development (Spear, 2000). This period is characterized by changes in the densities of monoamine transporters, monoamine levels and dopamine receptors in several areas of the brain (Knoll et al., 2000; Moll et al., 2000; Tarazi et al., 1998a,b). Other neurobiological changes during that period include extensive pruning of synapses in different brain areas (see Spear, 2000) and a higher rate of neurogenesis in comparison to adulthood (He and Crews, 2007). At the behavioral level, adolescence is characterized by risk taking behaviors, increase in exploratory behaviors in new environments (Laviola et al., 2003), and the development of different cogni-

tive and social competences required for adult life (see Spear, 2000).

It has been shown that social experiences at that time may be relevant to mould the way we will deal with social situations in the future. Several studies have suggested that stress induced by adverse social experiences may lead to lasting changes at the level of behavior, neurobiology and neuroendocrinology (Buwalda et al., 2005; Blanchard et al., 2001; Huhman, 2006; McCormick et al., 2010; Koolhaas et al., 1997). During the past decade, there has been an increasing interest in the relationship between social stress during critical periods of life and the development of psychopathologies (Romeo and McEwen, 2006). For instance, human studies have shown that victims of bullying during adolescence are more vulnerable to suffer from certain psychopathologies such as depression, anxiety disorders in adulthood (Gladstone et al., 2006). Bullying has been defined as a type of social stress in which a person is insulted physically or/and psychologically by one or more people (Sullivan, 2000; Nansel et al., 2004). A cross-national study performed in 25 different countries showed that from 5 to 20% of students reported being victims of bullying, and this experience is

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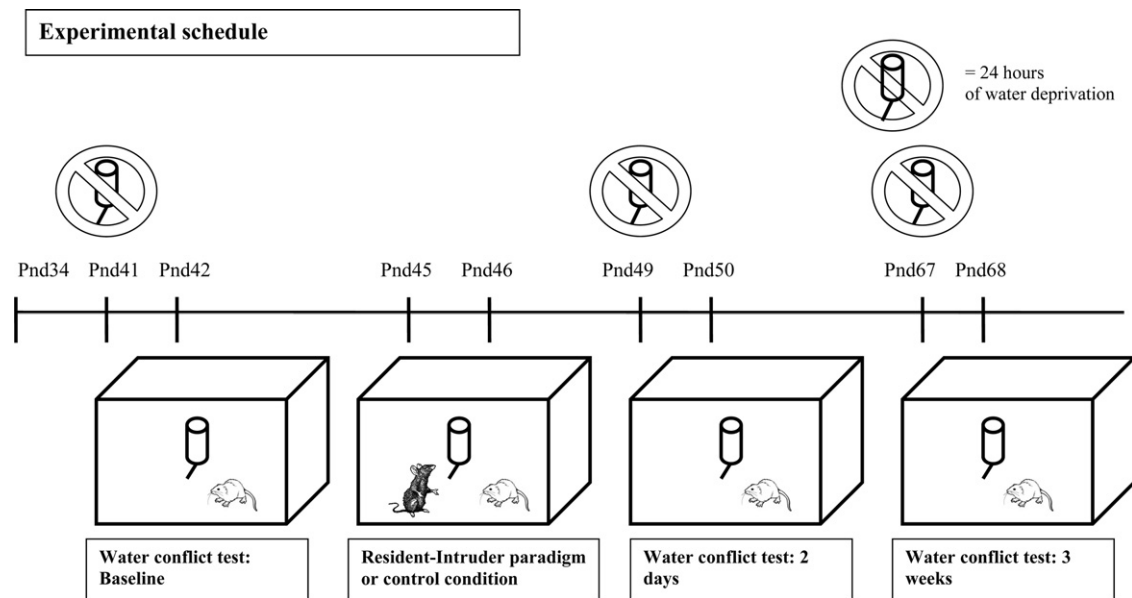


Fig. 1. Experimental procedure in experiment 1.

related with a reduced social psychological adjustment (Nansel et al., 2004).

Kumpulainen (2008) has noted that individual behavioral characteristics may determine the vulnerability to these social stressors. Indeed, studies have shown that there are two types of victims, one type of victims reported not suffering any negative emotional consequences after experiencing bullying while other type of victims reported negative emotions (Ortega et al., 2009), suggesting that some individuals are more vulnerable to the negative emotional consequences of bullying than other individuals who seem to be resilient. Additionally, Fox and Boulton (2005) showed that victims of bullying are perceived by teachers and peers to have poor social skills (Fox and Boulton, 2005). Some authors have emphasized the importance of studying individual differences in resilience and vulnerability to social stress (McCormick et al., 2010). However, to answer the question of individual vulnerability, it is essential to have baseline measures also known as pre-bullying measures. Most studies do not include pre-bullying measures (Bjorkqvist, 2001) because this type of data is difficult to obtain in human studies.

The present study is aimed at the further development of an animal model that allows the analysis of individual vulnerability and resilience to adolescent social stress. Based on previous studies showing that male wild-type rats differ in social behavior from male Wistar rat (de Boer et al., 2003), we used these two different strain rats to study individual resilience and vulnerability to adolescent social stress.

Bjorkqvist (2001) suggested that the resident-intruder paradigm may be an ecological valid animal model to study the consequences of being victim of bullying (Bjorkqvist, 2001). In the current experiment we will use this model with the objective to induce social stress in adolescent male rats.

Most of the studies involve the effects of social stress during adolescence on anxiety-like behaviors using paradigms such as the elevated plus-maze, social approach-avoidance tests and the open field (McCormick et al., 2010, 2008, 2005; Vidal et al., 2007; Watt et al., 2009). These studies show that social stress during adolescence has long-term consequences on anxiety-like behaviors. For example, Wistar rats which were socially defeated decrease the time spent in social exploration in a social approach-avoidance test when exposed to unfamiliar conspecific (Vidal et al., 2007). Furthermore, Sprague-Dawley rats which were socially defeated during adolescence showed an increase in the time spent in open

arms in the elevated plus-maze compared to controls and showed more locomotion in the open field when tested as adults, suggesting that these animals were more risk-taking than controls (Watt et al., 2009). In addition, studies using hamsters showed that social subjugated animals are more aggressive at adulthood (Delville et al., 1998; Ferris et al., 2005). Although these paradigms give important information about the behavioral consequences of victimization in terms of anxiety-like behaviors an important issue remains open. Most studies do not provide much information about the role of context in the development of fear and the degree in which fear generalizes over different contexts with similar characteristics. To study the capacity of subjects to control their levels of anxiety in different contexts, we will use a variation of the Vogel conflict test (Millan, 2003; Vogel et al., 1971) by exploring contextual fear against the background of the need to drink induced by water deprivation for 24 h.

In summary in the present study, two objectives were pursued. First, it will be determined to what extent the experience of being victim of social stress enhances the level of anxiety beyond the contextual condition. Second, the study is aimed at individual vulnerability, using Wistar rats and wild-type rats.

## 2. Experiment 1: water conflict test in the defeat context (same cage as where defeats took place)

### 2.1. Materials and methods

#### 2.1.1. Animals

16 male Wistar rats (Harlan, NL) and 16 wild-type Groningen (WTG) were used. The Wistar rats arrived at postnatal day 34 (PND34) and the wild-type rats were obtained from our own colony (PND34). Animals were habituated for one week to their own cages before the start of the experimental procedures. Rats were individually housed in standard clear Plexiglas cages (42 cm × 26 cm × 15 cm). Food and water was given ad libitum except during deprivation days (PND41, PND49, and PND67) where water was not available for the animals. The rats were maintained under standard conditions with a 12 h reverse light/dark cycle (lights on at 20:00 h) at constant temperature of 22 °C. All the procedures were approved by the Groningen University Committee on Animal Experiments (DEC 5153B).

### 2.1.2. Experimental procedure

All experimental procedures were conducted during the dark phase. Rats were randomly distributed in social defeat and control groups leading to four experimental groups: control-Wistar ( $n=8$ ), social defeat-Wistar ( $n=8$ ), control-wild-type ( $n=8$ ) and social defeat-wild-type ( $n=8$ ). The experimental procedure is shown in Fig. 1.

**2.1.2.1. Water conflict test.** To test the anxiety level and its context dependence, drinking behavior was recorded during 10 min in various conditions in 24 h water deprived rats. The water deprivation consisted of removing the water bottle from the home cage 24 h before the test. Within each of four different conditions three tests were performed: a baseline test at PND42, a test at PND50 measuring short-term effect of social defeat stress on drinking behavior (2 days after the defeat) and a test at PND68 measuring long-term effects on drinking behavior (3 weeks after the last social defeat experience). In experiment 1 (Fig. 1), water deprived animals were transferred to the resident–intruder room and tested in a resident–intruder cage (84 cm  $\times$  56.5 cm  $\times$  40 cm) without the resident of the cage being present (resident and its female removed 15 min before the test). The test lasted for a period of 10 min and during that time they had access to a water bottle and were allowed to freely explore the cage and drink as much water as needed. At PND42, PND50 and PND68 experimental rats were tested in the same cage as where they were previously defeated or received a control exposure. After the all tests the animals had ad libitum access to water. During all tests, behavior was recorded using a Sony video camera. In the water conflict test, the following parameters were registered: drinking latency, total water intake and time spent drinking. The amount of the water intake in the test was calculated by the differences between the weight of the bottle previous the exposition of the test and the amount drunk after the test. For the drinking latency, 600 s were given to all the animals that did not approach the bottle during the test.

**2.1.2.2. Social stress: resident–intruder paradigm.** Animals from the social stress groups (social stress-Wistar and social stress-wild-type) were exposed to social defeat, 2 days after the baseline water conflict test. For this social defeat procedure, the resident–intruder paradigm was used. The residents (wild-type Groningen rat) were housed in large cages (84 cm  $\times$  56.5 cm  $\times$  40 cm) each with a female wild-type rat. Residents were trained with the objective to elicit reliable levels of aggressive behavior. The attack latency was measured in every training session and only the residents with attack latency under 120 s on the last day of training were selected for the experiments. Females were removed approximately 15 min before the social defeat. Animals were exposed during two consecutive days (PND45 and PND46) to the residents for 10 min allowing direct physical contact with the resident. After 10 min each animal was returned to its own cage. The control animals were similarly transferred to the resident–intruder room, but placed in a cage (84 cm  $\times$  56.5 cm  $\times$  40 cm) without the presence of a resident. After the social stress and control procedure animals were returned to their own cages.

### 2.2. Statistics

Statistical analysis was performed using Statistica 8.0. Behavioral parameters from the water test such as drinking latency, total amount of water intake, time spent drinking were analyzed using repeated measures ANOVA with two between-subject factors; Stress (Social stress vs Control) and strain (Wistar vs Wildtype) and test session (Baseline vs 2 days vs 3 weeks) as a within factor. LSD test was calculated when required. Significance was set at  $p < 0.05$  for all analysis and values are expressed as mean  $\pm$  s.e.m.

### 2.3. Results

**Amount of water drunk (grams):** ANOVA showed a significant main effect of Stress ( $F(1,27)=15.40$ ;  $p < 0.001$ ). Moreover, the significant interaction effects between the factors Stress, Strain and Session ( $F(2,54)=6.36$ ;  $p < 0.01$ , Stress  $\times$  Strain ( $F(1,27)=13.68$ ;  $p < 0.001$ ) and a significant main effect of Strain ( $F(1,27)=10.26$ ;  $p < 0.01$ ) shows the differential response of the two strains to the social stress. LSD post hoc test indicated that Wistar rats that were socially defeated drank less water in comparison with control Wistars tested 2 days ( $p < 0.001$ ) and 3 weeks ( $p < 0.001$ ) after the defeat (see Fig. 2A). No differences were found between the wild-type defeated rats in comparison with wild-type control rats in baseline, 2 days and 3 weeks after defeat. The interaction Stress  $\times$  Session was significant ( $F(2,54)=13.68$ ;  $p < 0.0001$ ). In general, animals that were socially defeated consumed less water in comparison with control animals in the two post defeat test sessions.

**Time spent drinking (seconds):** Stress significantly reduced the time spent drinking (main effect of Stress  $F(1,28)=11.67$ ;  $p < 0.01$ ). In addition to a main effect of Strain, ANOVA showed a significant interaction between the factors Stress, Strain and Session ( $F(2,56)=4.12$ ;  $p < 0.025$ ), and Strain  $\times$  Session ( $F(2,56)=5.34$ ;  $p < 0.01$ ), indicating that the two strains reacted differently to the adolescent social stress. LSD post hoc test indicated that Wistar rats that were socially defeated showed less time drinking in comparison with controls Wistar 2 days ( $p < 0.0001$ ) and 3 weeks ( $p < 0.01$ ) after the defeat (see Fig. 2B). In the wild-type strain, no differences were found between the defeated rats in comparison with control rats in baseline, 2 days and 3 weeks after the defeat.

**Drinking latency (seconds):** ANOVA revealed a significant interaction between Stress, Strain and Session ( $F(2,56)=4.91$ ;  $p < 0.025$ ), indicating that the two strains differ in their response to social defeat during the water conflict task. LSD test post hoc analysis showed that socially defeated Wistar rats had a significantly higher drinking latency in the context of the previous defeat in comparison with control Wistar rats (see Fig. 2C). No differences were found between defeated wild-type rats in the drinking latency in comparison with control wild-types. Moreover, Strain  $\times$  Session interaction was significant ( $F(2,56)=6.23$ ;  $p < 0.01$ ). In general, social stress significantly enhanced drinking latency (main effect of stress  $F(1,56)=12.58$ ;  $p < 0.01$ ) when exposed to the water conflict test in the context whereas controls decreased their latency time.

## 3. Experiment 2: water conflict test in an unfamiliar context (cage without olfactory cues from residents)

### 3.1. Materials and methods

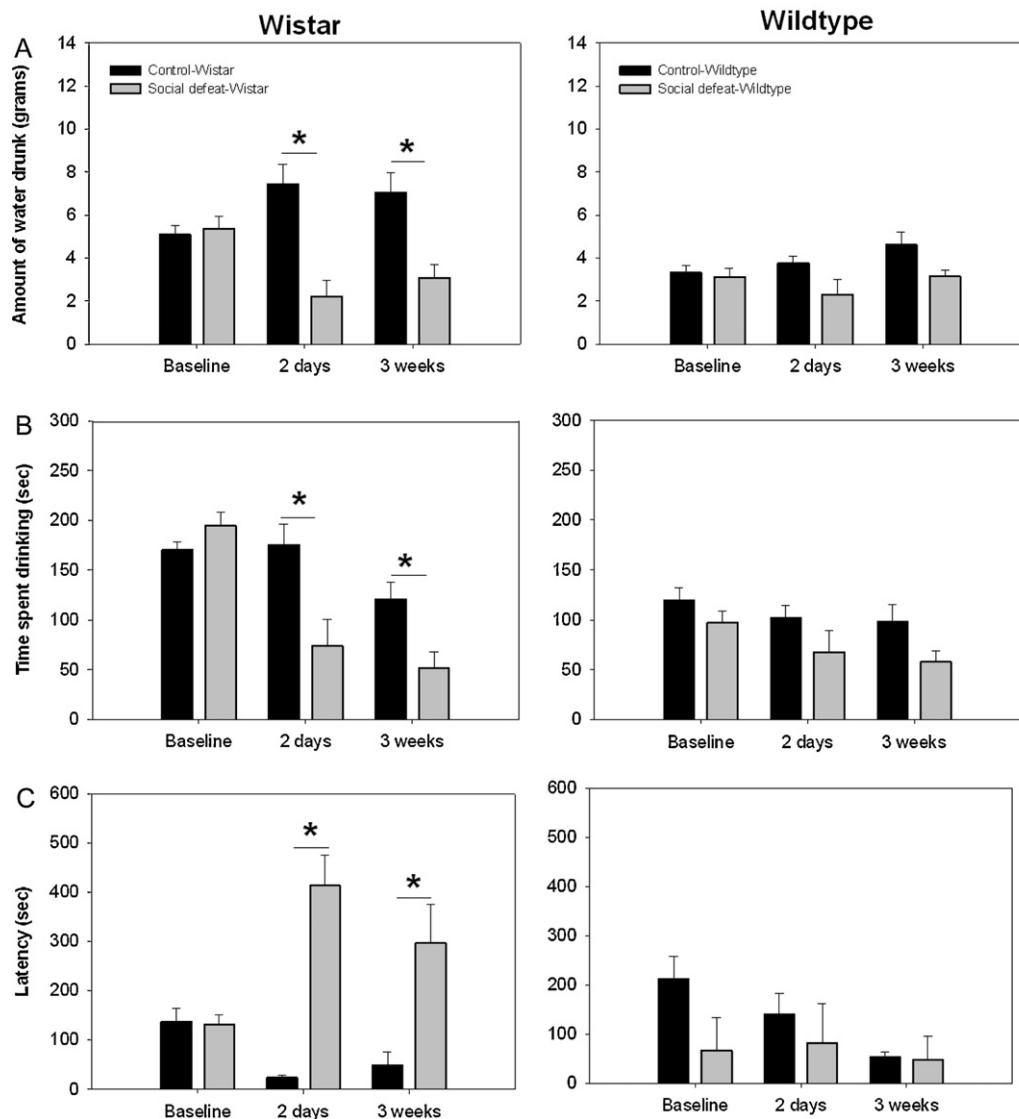
#### 3.1.1. Animals

16 male Wistar rats (Harlan, NL) and 12 wild-type Groningen (WTG) were used. The housing conditions and the animals' characteristics were the same in experiment 1 and in the experiment 2 (see experiment 1 for more details).

#### 3.1.2. Experimental procedure

All experimental procedures were conducted during the dark phase. Rats were randomly distributed in social defeat and control groups leading to four experimental groups: control-Wistar ( $n=8$ ), social defeat-Wistar ( $n=8$ ), control-wild-type ( $n=6$ ) and social defeat-wild-type ( $n=6$ ).

**3.1.2.1. Water conflict test.** In experiment 2, animals were tested at the same postnatal days as in experiment 1. For more details about the schedule and the procedure of the water deprivation see experiment 1. In experiment 2, each deprived animal was transferred to an unfamiliar test room and was tested in a clean cage with the same



**Fig. 2.** Effects of social stress on water conflict test performance in the context of previous defeat: (A) total amount of water intake during the test, (B) time spent drinking, and (C) drinking latency (\* $p < 0.05$ ).

dimensions as a resident–intruder cage (84 cm × 56.5 cm × 40 cm). Similar to experiment 1, the test lasted for a period of 10 min during which animals had access to a water bottle. Baseline, short- and long-term retesting was also performed in a clean cage. The behaviors analyzed were the same in all the experiments (see experiment 1).

**3.1.2.2. Social stress: resident–intruder paradigm.** The resident–intruder paradigm procedure was the same in all three experiments. For more detailed explanation about the procedure for the resident–intruder paradigm see experiment 1.

### 3.2. Statistics

The same data analysis techniques were used in the experiments 1–3 (for more details see experiment 1).

### 3.3. Results

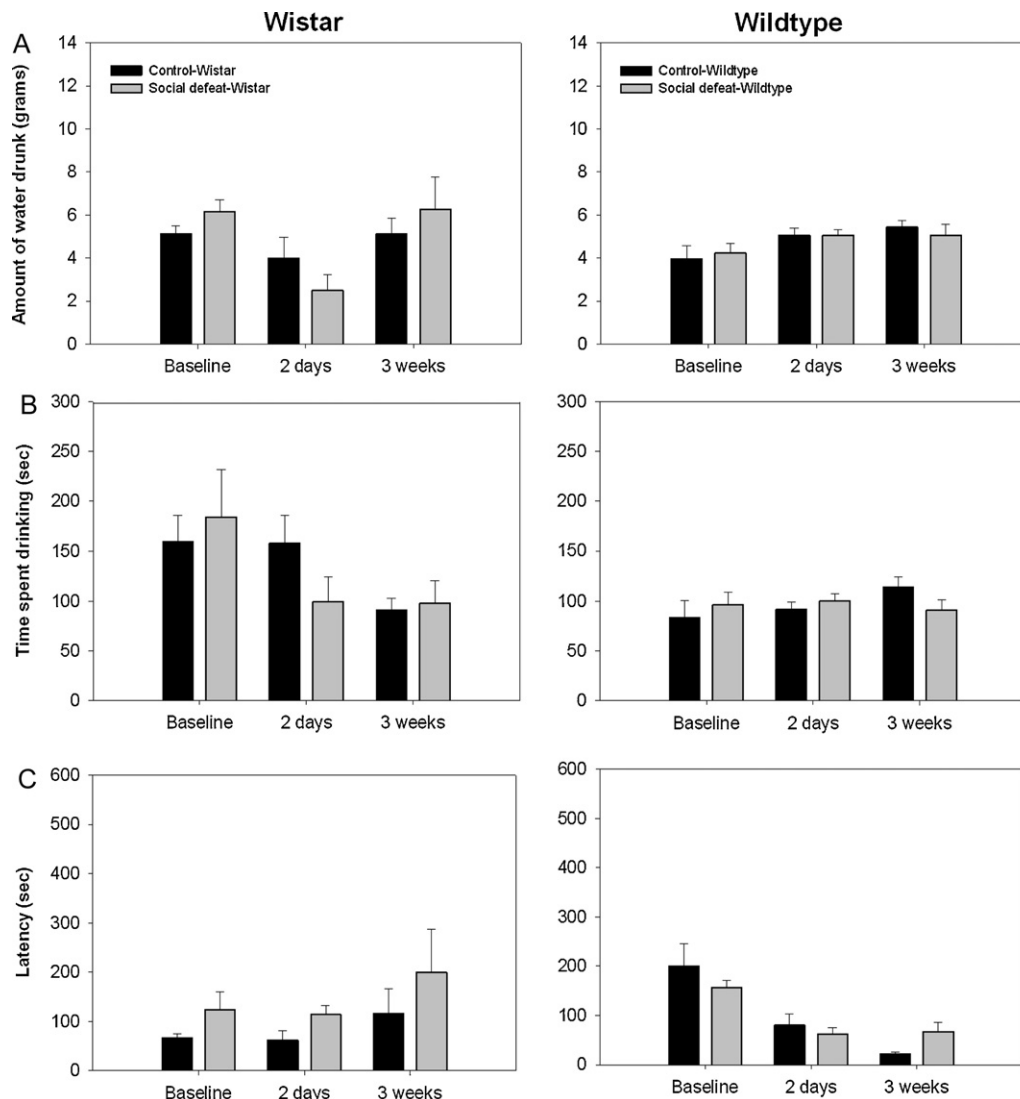
**Amount of water drunk (grams):** Although this unfamiliar cage had a similar size as the one where the defeat took place, neither defeated Wistar nor defeated wild-type rats differed in drink-

ing behavior from control treated rats. ANOVA testing did reveal an interaction between Strain × Session  $F(2,56) = 9.35$ ;  $p < 0.001$ . Wistar rats had a larger water intake in the baseline test than 2 days after the experimental conditions (control and/or defeat) ( $p < 0.001$ ). Interaction effects between the factors Stress, Strain and Session were not significant (see Fig. 3A).

**Time spent drinking (seconds):** Also in time spent drinking there were no significant interactions between stress and session. There was a significant main effect of Strain  $F(1,28) = 5.59$ ;  $p < 0.05$  and a significant interaction between Strain × Session  $F(2,56) = 8.58$ ;  $p < 0.001$ . This was mainly due to the fact that Wistar rats spent more time drinking in the baseline test in comparison with wild-type in the baseline session ( $p < 0.001$ ). Interaction effects between the factors Stress, Strain and Session was not significant (see Fig. 3B).

**Drinking latency (seconds):** No interaction of stress and session was observed in latency to drink. ANOVA revealed a significant main effect of Session ( $F(2,56) = 3.29$ ;  $p < 0.05$ ) and a significant Session × Strain interaction ( $F(2,56) = 9.67$ ;  $p < 0.001$ ). The strain differences were due to a higher drinking latency in the wild-type rats in comparison with Wistars in the baseline session ( $p < 0.01$ ) and lower latencies at 3 weeks ( $p < 0.01$ ). Wild-type rats gradually





**Fig. 3.** Effects of social stress on water conflict test performance in unfamiliar cage: (A) total amount of water intake during the test, (B) time spent drinking, and (C) drinking latency.

reduced their drinking latency in the course of the tests (2 days  $p < 0.001$ , 3 weeks  $p < 0.001$  in comparison with baseline). Interaction effects between the factors Stress, Strain and Session were not significant (see Fig. 3C).

#### 4. Experiment 3: water conflict test in the homecage

##### 4.1. Materials and methods

###### 4.1.1. Animals

16 male Wistar rats (Harlan, NL) and 14 wild-type Groningen (WTG) were used. The housing conditions and the animals' characteristics were the same in experiment 1 and experiment 2 (see experiment 1 for more details).

###### 4.1.2. Experimental procedure

All experimental procedures were conducted during the dark phase. Rats were randomly distributed in social defeat and control groups leading to four experimental groups: control-Wistar ( $n=8$ ), social defeat-Wistar ( $n=8$ ), control-wild-type ( $n=6$ ) and social defeat-wild-type ( $n=8$ ).

**4.1.2.1. Water conflict test.** In experiment 3, the water deprived animals were tested in the home cage and each animal was allowed to drink as much water as needed in 10 min. All the tests were performed in the home cage. After the test the animals had ad libitum access to water. The times that animals were tested were the same in the three experiments. The behaviors analyzed were the same in all the experiments (see experiment 1).

**4.1.2.2. Social stress: resident-intruder paradigm.** The resident-intruder paradigm procedure was the same in all three experiments. For more detailed explanation about the procedure see experiment 1.

##### 4.2. Statistics

The same data analysis techniques were used in experiments 1–3 (for more details see experiment 1).

### 4.3. Results

Similar to experiment 2 where the water conflict test was performed in an unfamiliar cage, no significant interactions were observed between stress and session in amount of water consumed, time spent drinking or latency to start drinking, indicating that no differences were present between defeated and control treated rats of both strains (data not shown).

## 5. Discussion

This study was aimed at individual differences in the short and long term reaction to social stress experienced during adolescence. In a series of experiments, using a water conflict test, the question was addressed to what extent social defeat induced anxiety would be generalized across different contexts. Animals were either exposed to the defeat context, without the presence of the dominant (experiment 1) or a context with similar characteristics as the defeat context but without the smell of a dominant animal (experiment 2). A control experiment on possible effects of social stress on the motivation to quench thirst was tested by measuring drinking behavior in the home cage after water deprivation (experiment 3). The results from these experiments show that Wistar rats that experienced social defeat during adolescence showed a significant decrease in the total amount of water, in the time spent drinking and drinking latency when tested in the context where they received the defeat experience 2 days before but also 3 weeks later (in adulthood) in comparison with non-defeated control Wistar rats. This effect was not observed in the wild-type strain of rats. Moreover, generalization to other contexts did not occur. Also no differences were found between animals which were socially defeated and controls in both strains when the animals were tested in their own cage indicating that stress did not affect motivational aspects of quenching thirst.

These data show that there are considerable differences between strains in the way they react to social stress during adolescence. Only Wistar rats that were socially defeated showed a strong and lasting association with the context. Moreover, there are considerable differences between the two strains in the baseline test at PND42. Wild-type rats have consistently lower values time spent drinking, amount of water intake and drinking latency during the baseline test in comparison to Wistar rats. Based in our data one could think that wild-type rats are more cautious in general when exposed to the different experimental tests. These strain differences in the baseline test and the reaction to social defeat are likely to be attributed to factors such as differences in coping styles and other social competences (der van Vegt et al., 2001; de Boer et al., 2003). Indeed, de Boer et al. (2003) shows that the population of wild-type male rats differs considerably in the frequency distribution of aggressive behavior within the population in comparison to Wistar rats, i.e. the more aggressive phenotype is fully absent in the Wistar strain (de Boer et al., 2003). This data is in line with previous research that has suggested that copying styles may be important to determine our capability to be resistant to stressors. In addition, pre-existing individual differences may contribute to be vulnerable to stressors. The present study supports the view that the understanding of the long term consequences of adolescent social stress requires a thorough understanding of the individual coping styles and social skills.

Generalization of fear to other contexts was not observed in the present study. The absence of “generalization” may be due to the number of exposures to the resident–intruder paradigm. Although the paradigm has been frequently used in adulthood, it is still unknown how many exposures to the resident intruder paradigm will lead to “psychopathology”. For instance, several studies have

shown that one or two exposures to social defeat are sufficient to induce long term, if not permanent changes in behavior and physiology (Meerlo et al., 1996a,b; von Frijtag et al., 2000, 2002). Others have suggested that the resident–intruder paradigm may provide a valid model to understand depressive-like behaviors when the model is used chronically (Rygula et al., 2005, 2006a,b). For instance, Yap et al. (2006) demonstrated that 10 consecutive defeats were necessary to find a decrease in the hippocampal cell proliferation. In the present study we have deliberately chosen for two consecutive defeats. Clearly, this social stress protocol was not enough to cause generalized anxiety.

In our experiments, animals were housed individually from the beginning of the experiment (PND34). Some authors have shown that adolescence is a time for neuronal re-organization (Spear, 2000; Andersen, 2003) and applying social isolation has been reported to have an effect in anxiety-like behaviors in this age (Lukkes et al., 2009). One could think that the effects obtained in these experiments are not due only to social defeat but to the combination between social defeat and social isolation. In our opinion the effects are strictly result of social defeat. The four groups of each experiment were socially isolated but the anxiety-like behaviors were mainly observed in Wistar rats that were socially defeated. If the effect would have been due to the combination between social defeat and social isolation a strong association to the context would have been also observed in wild-type rats who were socially defeated, but that was not the case.

Previous research using a similar protocol found that defeated rats during adolescence spend less time exploring an unfamiliar conspecific when tested in the adulthood (Vidal et al., 2007). From these data one may conclude that the anxiety response may be triggered in particular by social stimuli such as the presence of an other animal or the remaining smell of the animal in the context. Further research should address the nature of the stimuli that may trigger anxiety.

The present study uses a water conflict test to measure effects of social defeat during adolescence on anxiety. Previous studies have addressed the consequences of social stress on anxiety-like behaviors at adulthood using different anxiety tests. For instance, several studies reported that socially defeated rats showed behavioral inhibition when re-exposed to the context where they were defeated even 3 weeks later (Buwalda et al., 2005; Watt et al., 2009). Behavioral inhibition is usually measured using freezing behavior as dependent variable. Although freezing behavior is an indication of contextual fear, this measurement is not standardized for measuring the motivation of the animal to obtain certain necessary resources when needed or to explore novel environments. In addition, most of the paradigms used to measure anxiety such as the open field and the elevated plus maze are based on the idea that rats are afraid of open spaces and/or new environments. Behavioral parameters such as time spent in open arms or time spent in the center of the field are used as a measure of anxiety-like behaviors. Although the open field and the elevated plus-maze give relevant information about anxiety-like behaviors they are hard to standardize for the underlying motivation and they cannot be used repeatedly in the same animal. Therefore, we have used 24 h water deprivation to standardize motivation and measured water intake as the dependent variable. Analyses of baseline intake and of home cage water intake confirm the high degree of standardization. In this type of test the need to get water as an important resource for survival will compete with contextual or generalized fear.

In conclusion, the water conflict test is a useful tool to measure the influence of social defeat on the motivation to obtain resources under conditions with different stimulus properties. In addition, our data suggest the importance of the strain used in adolescent stress experiments. The fact that Wistar rats showed a

strong association with the context at adulthood whereas no effect was observed in the wild-type rats shows that victim characteristics are important determining factors for the long term effects of adolescent social stress.

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