Chapter 3: Developing predictors of alcohol use disorder in a chronic alcohol taking rat model with social defeat experience

Yong-Mei Sun, Danai Riga, August B. Smit, Sabine Spijker
**Abstract**

Alcohol is a highly addictive substance, however, it does not necessarily cause alcohol use disorder (AUD). For instance, individual vulnerability to addiction exists and social stress may affect the severity of AUD. Specific personality traits (related to impulsivity, compulsivity and inability to cope with stress) are important predictors for the severity of AUD in later life. Here, we searched for potential behavioral predictors by first examining the baseline value of four different tests assessing spatial and social interaction before rats were exposed to social stress and alcohol. Long-term social stress was induced by exposing rats to the social defeat-induced persistent stress (SDPS) paradigm after which they had long-term access to alcohol using home cage two-bottle free-choice and operant self-administration paradigms. Using a 5-criteria classification of according to DSM-V, AUD severity was assessed in the control and SDPS-exposed groups. Using these 5-criteria a higher incidence of the AUD phenotype was detected in the SDPS group. Baseline affective behavior, measured as interaction with an adult conspecific (social approach avoidance, SAA), prior to stress exposure was significantly correlated with most AUD criteria and opposite in control and some in SDPS-exposed groups. Furthermore, a low SAA interaction rate correlated with protection from alcohol addiction, whereas exposure to stressful life events increased addictive behavior later on in this group. We conclude that most AUD sub-phenotypes are based on prior genetic and environmental factors that may have shaped the individual’s affective behavior. Subsequent exposure to social stress is able to transform the AUD predictive ability of affective behavior. Disrupted affective behavior might therefore be an interesting parameter in the prediction of developing AUD in humans.

**Keywords**

Alcohol abuse disorder, addiction vulnerability, behavioral predictors, addiction prediction, depression model
1. Introduction

Many people come into contact with potentially addictive drugs, whereas a few of these become addicted. Drug use does not necessarily lead to addiction. It is clear that individual vulnerability to addiction exists [1, 2]. The interplay between genetic, epigenetic, environmental and neural-systems-level factors generates susceptibility to addiction [2-5]. One of the key questions in addiction research is why some individuals undergo the transition from casual drug use to compulsive patterns of drug use [6]. Identifying the addiction-prone or -resilient individuals at an early stage of drug taking, or even before drug taking starts, could help to prevent this behavioral transition.

Alcohol use disorder (AUD) is characterized by chronic alcohol abuse, extreme preoccupation with alcohol-related activities, and frequent episodes of relapse into alcohol use (American Psychiatric Association, 2013). Twin studies show that drug addiction is heritable [4] and environmental factors, such as social culture, peer influence and parenting contribute to AUD [3]. Evidence from humans [7, 8] suggests that a set of psychiatric disorders, including major depressive disorder with onset in early childhood, significantly increase the propensity of affected children to develop substance use disorders earlier or faster once they have initiated substance use.

Addictive behaviors are also found in animals after access to addictive drugs [9-11]. In a rat addiction model [9], four groups of rats with different vulnerability or resilience to cocaine addiction were categorized based on the fourth version of the Diagnostic and Statistical Manual of mental disorders (DSM-IV). Subsequently, a follow-up study using this animal model [12] showed that addiction severity in rats could be predicted by drug-taking behaviors displayed during the first 17 days of self-administration. Other studies showed that animals with certain traits, such as increased impulsivity, were more likely to take drugs or to show addictive-like behavior [13-15]. Together, this implies that either the addiction-vulnerable subjects had an addiction-predictive signature prior to exposure of addictive substances, or that the exposure to addictive substances induced these addictive behaviors specifically in these subjects.

Previously, a rat model with long-term access to alcohol after repeated social defeat was established to study the impact of chronic social stress on AUD [16]. Here, we used a scaling
method to quantify the severity of rat alcohol addiction based on different operant alcohol SA parameters, approximating the criteria currently considered hallmarks of AUD in DSM-V. As Glickman and Schiff (1967) suggested that approach behaviors and operant reinforcement are directed by a common neural mechanism, we measured with our test battery peer influence, social recognition, and object location memory, all of which reflected approach behaviors. We then tested the hypothesis that addiction severity can be predicted before subjects are exposed to alcohol, based on a series of behavioral tests using approach behavior.

2. Animals, Methods and Materials

2.1. Animals & social defeat-induced persistent stress (SDPS)

Pair-housed male Wistar rats (Harlan CPB, Horst, Netherlands) 6–7 weeks old, weighing < 200 g upon arrival were habituated in the vivarium (2 weeks) and subsequently exposed to social defeat-induced persistent stress (SDPS, ≥ 10 weeks old) [16, 17]. Social defeat is used to model the effects of physical and psychological stress on drug abuse-related behaviors in animals. Male Long-Evans rats (Charles River, UK, weighing > 500 g), which were paired-housed with age-matched tube-ligated females (Wistar, Harlan), were used as residents. SDPS rats (n=48) were exposed to five 15-minute daily defeat sessions, which included a 5-minute fight phase. A different resident was matched to each Wistar rat per day. Social defeat episodes were monitored by two researchers and the latency to submission during the fight phase was recorded for each rat in each of the five sessions provided. At defeat days, control animals (n=32) were exposed to an empty defeat cage for 15 minutes. From the first defeat session or cage exposure onwards, all animals were single-housed and remained in social isolation for the rest of the experimental conditions, in absence of further sensory interaction with the stressor (residents).

Due to the large number of animals included in the initial experiment, social defeat and subsequent behavioral assessment were performed in 3 consecutive weeks, as animals arrived in the vivarium in 3 independent batches, separated by 1 week each. All experimental manipulations were conducted during the dark phase of a reversed 12-h light-dark cycle (lights on at 19.00 h). 32 SDPS-experienced rats and 16 randomly selected control rats were
kept in one room; the behavioral data of these animals are used in this study, which is published recently [18]. For the whole experimental period, animals received food and water *ad libitum*. All experiments were approved by the VU University Amsterdam Animal Users Care Committee.

2.2. Affective and emotional tests before exposure to alcohol

Before participating in the behavioral assays, all animals were transferred to the video-recording room and habituated to the test arena (plastic, opaque, 79 x 57 x 42 cm) for at least 10 min during 3 consecutive days. Animals were subjected to the social approach-avoidance (SAA), the social interaction (SI), the social memory (SM) and the object place recognition (OPR) tasks in the week prior to social defeat. All video recordings were analyzed with Viewer² software (BiObserve GmbH, Bonn, Germany).

Social approach-avoidance test (SAA) (total 5 min) – Approach-avoidance behavior was estimated using an unfamiliar Long-Evans adult male rat (resident) as previously described [16, 17]. In brief, rats were habituated and allowed to explore two empty target boxes (TBs, perforated, metal, 16 x 7 x 8 cm) located in the opposite sides of the testing arena (sampling phase). Subsequently, an unfamiliar resident was introduced to one of the TBs and rats were allowed to explore and interact with the target, in absence of direct physical contact (testing phase). Interaction index was calculated as time spent in active zone (resident zone)/total exploration time (resident + neutral zone), in a 5-min test. Active and inactive zones were randomly assigned, in all tests provided and between groups, to avoid development of preference.

Social interaction (SI) – The equipment was the same as used in SAA described above. This test is similar to SAA, with the difference that the social target is not an aggressor but an unfamiliar juvenile rat of the same strain, namely a Wistar pup (4 – 6 weeks old). The interaction was calculated based on how much time a rat spent around the box with the pup vs. the empty box in a 5-min test.

Social memory (SM) – Long-term social discrimination memory [19] was tested using a social recognition (SR) test following first exposure to a juvenile Wistar rat. This test took place 24 h after the SI test. A novel juvenile Wistar pup was introduced into the empty box in
the SI tests. Animals were habituated to the testing arena as described above (SAA). For each animal and test, a different (novel) juvenile rat was introduced as the unfamiliar target. Discrimination between familiar (inactive zone) and novel (active zone) social targets was used as measurement of SM (interaction rate = time spent in active zone/total interaction time) in a 5-min test.

Object place recognition (OPR) – Hippocampal-dependent short-term memory [20] was assessed by the object place recognition task using a 15-min retention interval as previously described [16, 17]. In brief, following habituation, rats were allowed to explore two identical objects (cylinders or cubes, metal, 8 x 8 x 35 cm), located in two opposite corners of the arena (sampling phase). After a 15-min time interval, both objects were replaced with 2 identical ones, and one was displaced to a different position. Discrimination between the spatial locations of the two objects was used to assess spatial memory (exploration index = time spent in novel location / total exploration time (novel + familiar location)) in a 4-min test. The position of novel and familiar locations and the choice of object shapes were random in all tests provided and between groups to avoid development of preference.

In this study, the baseline values of the four behavioral tests before social defeat were measured and used in this study to predict the later addiction severity.

2.3. Procedure for alcohol exposure

After finishing a battery of behavioral tests, rats were exposed to alcohol in their home-cage and subsequently in the operant alcohol self-administration box for a total of 60 sessions.

Home-cage consumption – All animals were habituated to alcohol consumption using the two-bottle free/limited access paradigm (Wouda et al, 2011). The position of the alcohol/water-bottles was alternated to avoid preference, and both solutions’ consumption was monitored during the whole 5-week period. Rats were exposed to gradually elevating alcohol concentrations (2–12% v/v) in the home cage. During the last 2 weeks alcohol availability was limited to 1 h/day.

Operant alcohol self-administration (SA) – Fixed Ratio – Rats were trained to nose-poke for a 0.20 mL 12% alcohol reward in 1-h sessions every other day. Alcohol delivery (US) was accompanied by discrete audiovisual stimuli (CS, 4-s active hole illumination and tone
Different reinforcement schedules (fixed ratio, FR) were used (FR1–3). Initially, a continuous reinforcement (fixed ratio 1, FR1) schedule was implemented, in which each reward delivery was followed by a 15-s time-out period, during which nose-poking had no programmed consequences. Responding on the inactive hole was monitored, but had no consequences. When FR1 performance reached stable levels (Criteria: 1) Average consumption 0.35 g/kg; 2) > 10 active responses per session; 3) No significant difference between the last 3 sessions when tested with repeated measures ANOVA), animals were introduced to FR2 and subsequently FR3 training schedules. Consolidation of alcohol SA was estimated by peak performance at FR3 (45 ± 7 active responses/session for control and 64 ± 7 for SDPS). All animals were trained under FR training schedule for 8 weeks.

Progressive ratio (PR) – Animals were subjected to six 2-h progressive ratio (PR) sessions, during which the effort (number of nose-pokes) to obtain a reward was progressively increased according to: response ratio = \(5^{e(0.2 \cdot \text{reward number})} - 5\), rounded to the nearest integer.

Extinction – After PR sessions, all animals were retrained (FR1) to minimize between-group differences that could affect subsequent analysis of extinction performance. Extinction training consisted of 1-h exposure to the training context in absence of alcohol and alcohol-associated cues. Following 15 daily sessions, operant responding was successfully extinguished (< 5 active responses/session) and all animals participated.

Relapse – At the start of this session, the house and cue lights were turned on and nose-poking resulted in presentation of the discrete compound audiovisual cues (but no alcohol reward) on an FR1 schedule.

2.4. Assessment of addiction severity

The addiction-like criteria in rats were based on DSM-V for alcohol use disorders (AUD) and the criteria were based on the following five parameters: 1) motivation to acquire alcohol, 2) drug seeking behavior during the non-drug period, 3) alcohol intake, 4) difficulty to stop drug seeking, and 5) difficulty to stop drug use (relapse). In detail, these parameters were related to the following tasks during operant SA:
1. The average active nose-pokes of session 27–29 in the PR tests (PR) were used to measure the motivation to acquire the addictive substance.

2. The average active nose-pokes during a time out period of SA sessions 41–43 (non-drug; ND) were used to reflect drug seeking, comparable with the non-drug period active nose-pokes in the cocaine addiction-like model [9].

3. The average injections of SA sessions 41–43 (Average injections) were used to reflect the load of alcohol consumption and the intention to take more alcohol.

4. The average active nose-pokes of the first 3 extinction sessions (Extinction) were used to measure the difficulty to stop drug seeking.

5. The active nose-pokes in the relapse test (Relapse) were used to model relapse in human addicts, or the difficulty to quit drug taking.

Rats were classified as meeting zero to five ‘addiction criteria’. A rat was classified as positive for an addiction criterion if its performance on a given parameter mentioned-above was in the top 35% of the total sample [9].

2.5. Predicting parameters for alcohol addiction-severity

The four baseline values of each behavioral test (SAA, SI, SM and OPR) were analyzed for predicting addiction severity using correlation analysis. Since the SDPS groups received chronic social stress this correlation analysis was carried out in the control and the SDPS groups separately.

2.6. Statistical analyses

All behavioral data collected from SAA, SI, SM and OPR were analyzed using Student's t-test to compare the baseline differences between social defeat and control groups with all of the assumptions (outliers, normality and homogeneity of variances) checked. If the assumption of homogeneity of variances was violated, then the post-hoc test was a Games-Howell test. Principal component analysis was carried out to analyze whether these four behavioral tests measured the same affective/cognitive component. To quantify the differences in addiction severity, one-way ANOVAs or Kruskal-Wallis H-tests were performed to compare the
differences among the three groups. To check whether the four behavioral tests could predict the later addiction criteria, Pearson's correlations were carried out in the control and SDPS groups. Only with significant correlation pairs, linear regressions or standard multiple regressions were carried out. All statistics were performed using IBM SPSS Statistics 22. All of the data are expressed as mean ± S.E.M. For all tests, the significance level (α) was set to 0.05. During assessment of the four behavioral tests, the tracking software was erroneously terminated, leaving datasets for the following tests at the baseline level incomplete: SI, n=1; OPR, n=2.

3. Results

In this study, we investigated whether behavioral tests performed prior to social defeat and alcohol exposure (Fig. 1A) predict the severity of subsequent alcohol abuse disorder, as defined according to the DSM-V criteria. These four behavioral tests (SAA, SI, SM and OPR) measured peer influence, social recognition, and object location memory, all of which require approach behavior [21]. First, we assessed how the total population of rats scored based on the 5 AUD criteria. Then, we analyzed whether the AUD criteria were able to discriminate different degrees of AUD severity based on the total group, as well as for the SDPS and control groups separately. Finally, we used the values obtained for each of these 5 addiction criteria to correlate to the baseline values of the four behavioral tests, after first assessing whether they measured any overlapping affective and cognitive aspects.

3.1. Addiction criteria are valid to assess AUD severity

Based on the DSM-V and a previous cocaine-addiction model [9], we characterized all alcohol taking rats using the set of five addiction criteria, which yielded six subgroups (Table 1). In the DSM-V, AUD is defined as being mild, moderate or severe based on 11 criteria. Therefore, we formulated here 5 criteria for rats to define addiction severity accordingly (absent, 0 criteria; mild–moderate, 1–3 criteria; severe, 4–5 criteria) (Table 2). The majority of the animals (33.3%) did not comply with any criterion (Fig. 1B). A small proportion of animals fulfilled all 5 criteria (6.3%), and the group fulfilling 1–4 criteria ranged from 10–19%. Based on this, the AUD
group fell apart in the severe phenotype that was applicable to 20.8% of all animals, and the AUD mild–moderate phenotype for 45.8% (Fig. 1C).

To further quantify the behavior within the two AUD subgroups and the AUD absent group of the entire population, we performed a one-way ANOVA or Kruskal-Wallis H test for each addiction criterion. After checking the assumptions for one-way ANOVA, only the data of average injection and relapse met all of the assumptions. For the extinction test and the progressive ratio test, the assumption of homogeneity of variances was violated, and the non-drug period lever presses violated the assumption of normality.

For the progressive ratio test (Fig. 2A), there were significant differences among the three groups (F(2,45)=31.037, P<0.001). The AUD absent group pressed significantly less levers than both the AUD severe (P=0.001) and the AUD mild-moderate group (P<0.001). In addition, the AUD severe group pressed more levers than the AUD mild-moderate group (P=0.043). The median number of lever presses during the non-drug (ND) period (Fig. 2B) was significantly different between groups (Kruskal-Wallis H test, $H(2)=29.437$, P<0.001). Pair-wise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons. This post-hoc analysis revealed statistically significant differences in median non-drug period lever presses between the AUD severe (30.7) and the AUD absent (6.3; P<0.001), as well as between the AUD mild–moderate (23.3) and the AUD absent (P<0.001) groups. However, a significant difference between the AUD severe and the AUD mild–moderate group was absent (P=0.077).
Figure 1. Distribution of addiction criteria and AUD phenotype across the total set of rats used. A) Experimental timeline. Wistar rats were tested in four behavioral tests, and were then exposed to 5 daily social defeat sessions (n=32) or used as controls (n=16) (in week 0–1). They were subsequently single-housed for a period of ~6 months, during which alcohol habituation and alcohol SA took place. B) The total set of 48 rats was screened for compliance to the 5 addiction criteria (see Table 1). C) The resulting division for the two AUD phenotypes (mild–moderate, severe) and the absence of AUD is shown (see Table 2).

<table>
<thead>
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<th>2 crit</th>
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<td>5</td>
<td>8</td>
<td>9</td>
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<td>10.4</td>
<td>16.7</td>
<td>18.8</td>
<td>33.3</td>
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</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>SDPS</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 1. The distribution of the 5 addiction criteria in the control (n=16) and SDPS (n=32) group, resulting in 6 different bins.
Regarding average injections in the late stage of SA (Fig. 2C), significant differences were found among the three AUD groups (F(2,45)=46.348, P<0.001), with all groups being significantly different from each other upon post-hoc testing (t-tests, P<0.001).

Regarding the measures of extinction (Fig. 2D), significant differences were found among three AUD groups (F(2,45)=36.616, P<0.001). In the post-hoc tests (Games-Howell test), the AUD severe group pressed significantly more at the levers than the AUD mild–moderate (P=0.050) and the AUD absent group (P<0.001). Moreover, the AUD mild–moderate group showed higher lever presses than the AUD absent group (P<0.001).

Concerning relapse, significant differences were found among the three AUD groups (F(2,45)=14.945, P<0.001) (Fig. 2E). Lever presses of the AUD absent rats were significantly less than those of both AUD subgroups (P<0.001). However, there was no significant difference between the AUD mild–moderate and the AUD severe group (P=0.205).

Because the AUD phenotype classification was able to distinguish all contained parameters, we subsequently assessed the distribution of the five addiction-criteria and the AUD phenotypes across the SDPS and control groups. We expected that in the SDPS group more individuals would fall into the AUD severity phenotype, as previously it was shown that SDPS increases motivation and relapse to alcohol seeking [16]. In the SDPS group, 78% of the animals (25/32) scored positively on at least one addiction criterion compared with 44% (7/16) in the control group (Table 1). This indicates that 5 single sessions of social defeat that

<table>
<thead>
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<th>Criteria</th>
<th>AUD severe</th>
<th>AUD mild–moderate</th>
<th>AUD absent</th>
<th>AUD severe (%)</th>
<th>AUD mild–moderate (%)</th>
<th>AUD absent (%)</th>
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<tr>
<td>Total</td>
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<td>22</td>
<td>16</td>
<td>20.8</td>
<td>45.8</td>
<td>33.3</td>
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<tr>
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<td>2</td>
<td>5</td>
<td>9</td>
<td>12.5</td>
<td>31.3</td>
<td>56.3</td>
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<td>8</td>
<td>17</td>
<td>7</td>
<td>25.0</td>
<td>53.1</td>
<td>21.9</td>
</tr>
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</table>

Table 2. Addiction severity in terms of the DSM-V AUD score, and distribution in the control (n=16) and SDPS (n=32) groups, as well as in the subgroups of SDPS (prone (n=10); moderate (n=12); resilient (n=10); see supplemental table 2). The number of animals (left), and the % of the total group (right) are shown.
were employed 4 months prior to alcohol SA worsen the later abuse of alcohol in male rats. Social defeat stress doubled the proportion of those with a severe AUD-like profile (12.5% in control and 25.0% in SDPS), and almost doubled the proportion of mild-moderate AUD (31.3% in control and 53.1% in SDPS) at the expense of the non-AUD individuals (56.3% in control and 21.2% in SDPS). These results demonstrate that prior stress (SDPS paradigm) doubles the chance of developing alcohol addiction-like behaviors. The fact that pre-existing stress increased the likelihood of developing AUD is similar to the situation in humans [22-24].

Previously, we have shown that within a large population of rats individual differences exist in terms of susceptibility to depression [18]. To check whether this susceptibility had an effect on the AUD-like score, we selected 10 SDPS-prone and 10 SDPS-resilient rats based on a deficit in both affective and cognitive behavior [18]. The remaining 12 SDPS rats only showed a deficit in either the affective or the cognitive domain (SDPS-moderate). Analysis of the proportion of the severe, and mild–moderate AUD-like phenotype or the absence of an AUD-like phenotype confirmed that SDPS susceptibility contributed to the AUD-score (Supplemental Tables 1, 2), with SDPS-prone and SDPS-normal groups having a higher proportion of the AUD-like phenotype at the expense of the AUD absent group.

In SDPS-resilient rats, which do not show any deficit in the long-term after social stress, there was only a small shift from the AUD absent phenotype to the AUD mild–moderate and severe phenotype. Thus, prior social stress and the way it is perceived by the individual affects the liability and severity to develop AUD, albeit that it is not known to what extent this is driven by the same underlying mechanism.

3.2. Behavioral parameters measured at baseline are predictive of conforming to several addiction criteria

Having shown that the 5 addiction criteria yield individuals with a valid and expected distribution of control and SDPS rats, we continued to correlate the behavioral parameters measured at baseline to the values specified by these criteria. Before performing the correlation, we had to first assess whether there were significant differences of these parameters across the control and SDPS groups at baseline. There were no significant differences in the baseline mean scores between control and the SDPS groups [18] for the
SAA (control 0.660±0.072, SDPS 0.728±0.034, t(46)=0.979, P=0.333; Fig. 3A), and the OPR test (control 0.579±0.046, SDPS 0.582±0.025, t(46)=0.065, P=0.949; Fig. 3B). In addition, both the baseline of SI (control 0.789±0.036, SDPS 0.728±0.034, t(45)= -0.176, P=0.861; Fig. 3C) and that of SM (control 0.579±0.046 and SDPS 0.582 ±0.025, t(46)=0.065, P=0.949; Fig. 3D) were similar. However, as individual differences exist, we hypothesized that this individual variation might be at the basis for the later observed different stress and drug reactivity.

Social approach avoidance (SAA), social interaction (SI), social memory (SM) and object place recognition (OPR) all measures of behavior that is dependent on horizontal locomotion of animals. Before analyzing the predictive value of each parameter of addiction-severity, it is necessary to check whether these four tests measure different behavioral aspects of affective or cognitive domains.

Alternatively, the underlying summary variables from a principle component analysis (PCA) could be used. The suitability of PCA was assessed by measuring the proportion of variance among the variables that might be common. Inspection of the correlation matrix showed that all variables showed correlation coefficients smaller than 0.3. The overall Kaiser-Meyer-Olkin (KMO) test was 0.475 with some of the individual KMO tests less than 0.50, and hence are "unacceptable" [25]. Bartlett's test of sphericity was not significant (P=0.843). This analysis revealed that the four components explained 30.5%, 27.5%, 23.2% and 18.8% of the total variance, respectively. More importantly, as shown in Fig. 4, there was no inflection point. Thus, the baseline values of the four behavioral tests actually measure independent affective and cognitive behavioral aspects.
Next, we continued to correlate each of the 4 behavioral baseline parameters to the absolute value of the individual’s score on the 5 addiction criteria. We separated the 48 rats into the SDPS (n=32) and control (n=16) group. Then, we performed linear regression analyses with the indexes of the four behavioral tests as the predictive parameters. To prepare for the regression analysis, we first examined the Pearson correlation relationship between each of the four predictive parameters and the scores of the individual animals on the five addictive criteria in the two groups (Table 3, Table 4). In the control group (Table 3, Supplemental Fig. 1), significant positive correlations existed between SAA and values obtained using four of the addiction criteria. Whereas the correlations of average injections ($r(16)=0.632$, P=0.009) and extinction ($r(16)=0.543$, P=0.030) were very strong, moderate positive correlations existed between SAA and ND ($r(16)=0.495$, P=0.050) and between SAA and relapse ($r(16)=0.497$, P=0.050). PR showed a trend in the same direction as the other parameters ($r(16)=0.460$, P=0.073). No other behavioral parameter showed a correlation with values obtained using the addiction criteria.
Figure 3. Comparisons of the baseline data of four behavioral tests between control and SDPS groups. 
A–D) Social approach avoidance (SAA; A), object place recognition (OPR; B), social interaction (SI; C), social memory (SM; D). Tests were performed during the week before social defeat. No differences were found in the social defeat and control groups using Student's t-tests.

In the SDPS group (Table 4, Supplemental Fig. 1), there were statistically significant moderate correlations between SAA and PR ($r(32)=-0.472$, $P=0.006$) and ND ($r(32)=-0.490$, $P=0.004$), albeit that they were negative, instead of positive as for the control group (Table 4). Only with a trend, baseline SAA behavior correlated negatively with average injections ($r(32)=-0.304$, $P=0.091$), and with extinction ($r(32)=-0.334$, $P=0.062$). Also, unlike the control group, there was no correlation between SAA and relapse in the SDPS group. Specifically in this group, there were significant positive correlations between SM and ND ($r(32)=0.481$, $P=0.005$) and between SM and average injections ($r(32)=0.465$, $P=0.007$), as well as a significant moderate negative correlation between SI and extinction ($r(32)=-0.363$, $P=0.045$).
Thus, albeit in different directions, only affective behavior and affective memory were correlated with values obtained using the AUD criteria. Short-term object location memory was not predictive.

After this correlation analysis, we performed linear regression or multiple regression analyses to establish the prediction relationships between the behavioral tests and the addiction criteria for those having a significant Pearson correlation in the two groups. In the control group, linear regression analysis established that the baseline value of SAA could significantly predict individual values obtained using several addiction criteria. The baseline

Figure 4. The scree plot of the baseline values of SAA, SI, SM and OPR tests. There is no inflection point, which implies that the four variables could not be reduced to a common principle component and hence the variables reflect different affective or cognitive behavioral dimensions.
<table>
<thead>
<tr>
<th>r (P-value)</th>
<th>PR</th>
<th>ND</th>
<th>Average injections</th>
<th>Extinction</th>
<th>Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SAA</strong></td>
<td>0.460 (0.073)</td>
<td>0.495 (0.050) *</td>
<td>0.632 (0.008) **</td>
<td>0.543 (0.029) *</td>
<td>0.497 (0.050) *</td>
</tr>
<tr>
<td><strong>SI</strong></td>
<td>-0.417 (0.108)</td>
<td>-0.117 (0.666)</td>
<td>-0.042 (0.877)</td>
<td>-0.304 (0.252)</td>
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<tr>
<td><strong>SM</strong></td>
<td>0.053 (0.846)</td>
<td>-0.008 (0.976)</td>
<td>0.025 (0.928)</td>
<td>-0.050 (0.855)</td>
<td>0.071 (0.793)</td>
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<tr>
<td><strong>OPR</strong></td>
<td>0.102 (0.706)</td>
<td>0.232 (0.388)</td>
<td>0.235 (0.381)</td>
<td>0.131 (0.623)</td>
<td>0.196 (0.467)</td>
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</table>

Table 3. The correlation matrix of the control group. The Pearson correlation coefficient (r) between baseline behavior and AUD parameters and p-values are indicated for the control group. ** P<0.01; * P<0.05.

<table>
<thead>
<tr>
<th>r (P-value)</th>
<th>PR</th>
<th>ND</th>
<th>Average injections</th>
<th>Extinction</th>
<th>Relapse</th>
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</thead>
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<tr>
<td><strong>SAA</strong></td>
<td>-0.472 (0.006) **</td>
<td>-0.490 (0.004) **</td>
<td>-0.304 (0.091)</td>
<td>-0.334 (0.062)</td>
<td>0.003 (0.987)</td>
</tr>
<tr>
<td><strong>SI</strong></td>
<td>0.082 (0.661)</td>
<td>-0.134 (0.471)</td>
<td>-0.1324 (0.478)</td>
<td>-0.363 (0.045) *</td>
<td>-0.092 (0.630)</td>
</tr>
<tr>
<td><strong>SM</strong></td>
<td>0.311 (0.083)</td>
<td>0.481 (0.005) **</td>
<td>0.465 (0.007) **</td>
<td>0.285 (0.114)</td>
<td>0.083 (0.650)</td>
</tr>
<tr>
<td><strong>OPR</strong></td>
<td>0.008 (0.968)</td>
<td>0.079 (0.679)</td>
<td>0.176 (0.351)</td>
<td>-0.038 (0.841)</td>
<td>0.272 (0.154)</td>
</tr>
</tbody>
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Table 4. The correlation matrix of the SDPS group. The Pearson correlation coefficient (r) between baseline behavior and AUD parameters and p-values are indicated for the SDPS group. ** P<0.01; * P<0.05.
Figure 5. The baseline value of SAA predicts values based on the addiction criteria in the control group. A–D) The baseline value of SAA predicted the ND criterion at a marginal significance level (F(1,14)=4.589, P=0.050) (A), whereas the average injections criterion (F(1,14)=9.372, P=0.008) (B) and the extinction criterion (F(1,14)=5.897, P=0.029) (C) were significant. In addition, the relapse criterion was predicted at a marginal significance level (F(1,14)=4.494, P=0.050) (D).

The baseline value of SAA could predict the ND, average injection, extinction, and relapse criteria (Figure 5) explaining 24.7%, 40.1%, 29.6% and for 24.3% of the variability, respectively. In the SDPS group (Figure 6), linear regression analysis revealed significant prediction relationships between the baseline values of SAA and the PR criterion, between SI and the extinction criterion, and of SM and the average injection criterion, accounting for 22.9%, 13.1%, and 21.6% of the explained variability, respectively.

Since the baseline values of SAA and SM both had significant correlations with the ND criterion, a multiple regression analysis was carried out. The multiple regression model (Table 5) statistically significantly predicted ND (F(2,29)=11.59, P<0.001, R²=0.444). Both the SAA and SM values added significantly to the prediction (P<0.05).
Comparing the correlation analyses from the control and SDPS group (Supplemental Figure 1), showed that with an opposite correlation between the two groups, the difference stemmed mostly from the low-scoring individuals. To substantiate this, we assigned within each group the proportion of animals with low SAA (interaction value < 0.6), and those with high SAA (interaction value > 0.6) for the SAA parameter. For PR, ND and average injections, the 4 different SAA subgroups all showed an overall group effect (Kruskal-Wallis one way ANOVA; \( P=0.011, P=0.003, P=0.003 \), respectively). Subsequent testing revealed that the ND and average injections parameters were significantly different for low SAA controls vs. low SAA SDPS animals (MWU test, \( p=0.015, p=0.021 \), respectively) and PR showed a trend (MWU test, \( p=0.065 \)), but not for the high SAA subgroups (\( P=0.639, P=0.751, P=0.903 \)). As we did not see any obvious ‘low SAA’-effect for the relapse parameter, we took this one along as a ‘negative’ control. Although both the extinction and relapse criteria showed a significant overall group effect (one way ANOVA, extinction: \( F(3,44)=4.289, P=0.010 \); relapse: \( F(3,44)=3.234, P=0.031 \), post-hoc testing only revealed the ‘low SAA’-effect for relapse and not for extinction (Bonferroni, \( P=0.028, P=1.000 \), respectively). There was no significant difference in the high SAA subgroups (Bonferroni, \( P=1.000, P=1.000 \), respectively).

Figure 6. The baseline values of SAA, SI and SM predict the addiction criteria in the SDPS group. A) The baseline value of SAA significantly predicted the PR criterion (\( F(1,30)=8.917, P=0.006 \)). B) The baseline value of SI significantly predicted the extinction criterion (\( F(1,29)=4.375, P=0.045 \)). C) The baseline values of SM predicted the average injection criterion (\( F(1,30)=8.264, P=0.007 \)).
<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>SE_B</th>
<th>ß</th>
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<tr>
<td>Intercept</td>
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<td>9.85</td>
<td></td>
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<tr>
<td>SAA</td>
<td>-29.44</td>
<td>8.82</td>
<td>-0.463**</td>
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<tr>
<td>SM</td>
<td>38.68</td>
<td>11.85</td>
<td>0.453**</td>
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</tbody>
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Table 6. Summary of multiple regression analysis in the SDPS group. B, not standardized regression coefficient; SE_B, standard error of the coefficient; ß, standardized coefficient. ** P<0.01.

Similarly, when comparing the correlation analyses from the control and SDPS group (Supplemental Fig. 1) for social memory, the high skilled individuals in combination with stress were more likely to score high on the ND criterion, whereas low skilled individuals were more prone to an increase in average injections upon stress. Only for ND responses the 4 different SM subgroups showed an overall significant group effect, whereas this was a trend for average injections (Kruskal-Wallis one way ANOVA; P=0.022, P=0.080 respectively). Subsequent testing for the ND responses criterion revealed that it was indeed significantly different for high SM controls vs. high SM SDPS animals and not for their low SM counterparts (MWU test, P=0.006; P=0.116, respectively). For average injections, there was a significant difference for high SM controls vs. high SM SDPS animals and not for their low SM counterparts (MWU test, P=0.016; p=0.127, respectively).

4. Discussion

In the present study, a large set of rats (n=48) was tested for their baseline data in four behavioral tests and a part of this group (n=32) was subjected to the SDPS paradigm. All of the animals were exposed to alcohol for a long period. The severity of alcohol abuse disorder was assessed based on the human DSM-V AUD criteria and we used five criteria with overlap in the rodent self-administration model and human addiction, similar to previous studies [9, 10]. We then verified its potential by assessing the SDPS vs. control groups in relation to the severity of alcohol abuse. Finally, to test the predictive potential of four
Figure 7. Different sociability skills in combination with lasting social stress leads to addiction vulnerability. A) After splitting the control and SDPS group on low (SAA<0.6) and high (SAA>0.6) SAA score (control LowSAA n=6; control highSAA n=10; SDPS LowSAA n=6; SDPS highSAA n=26), all five addiction criteria were tested across the 4 groups. As expected from the inverse correlation of SAA with the values obtained using these addiction criteria (PR, ND, Average injections, Extinction) in the control and SDPS group (Supplemental Fig. 1), specifically the low SAA group in control and SDPS were significantly different (post-hoc testing; P<0.050), or showed a trend (P<0.100). B) After splitting the control and SDPS group on low (SM<0.6) and high (SM>0.6) SM score (control LowSM n=6; control highSM n=10; SDPS LowSM n=6; SDPS highSM n=26), the ND and average injection criteria were tested across the 4 groups. After a significant one-way ANOVA (Kruskal-Wallis), a post-hoc test showed a significant difference in the high SM group between control and SDPS for the ND criterion. The average injections showed a trend (one-way ANOVA), however, was significant with post-hoc testing in the high SM group between control and SDPS. Post-hoc testing; * P<0.10, * P<0.05).

affective and cognitive tests measured at baseline we carried out regression analyses between these baseline behavioral values and the individual values obtained using these addiction criteria.

4.1. A rodent AUD classification instrument

In humans, there are different classification instruments to score the severity of AUD. Here we used five criteria based on the DSM-V to reflect the individual AUD severity of each rat and then binned these animals into three different groups representing severe AUD,
mild-moderate AUD, AUD absent. Notably, for each of the five criteria, the quantitative measurements for the AUD severe and AUD mild-moderate groups were significantly higher than that for the AUD absent group. Interestingly, in two out of five criteria (i.e., ND and relapse) there were no statistical differences between the AUD severe and AUD mild-moderate groups. As AUD is a chronic disease with different dimensions, it might well be that after 60 days alcohol exposure only some of the AUD criteria in this mild-moderate group might have reached the maximal severity level. As persistent alcohol intake might be the most important aspect affecting AUD severity, the mild-moderate group might transform to the AUD severe group after longer alcohol exposure. If so, than more early interventions with the AUD mild-moderate group could potentially disrupt the development of severe alcohol addiction.

Population-based studies support a positive association of social stress and severity of AUD [26, 27] and the level of stress is related to alcohol use problems [28, 29]. In rodent studies, the SDPS paradigm of social defeat, in which animals undergo only 5-10 defeat sessions, mimics a state of enduring social stress in humans that might elicit alcohol use and abuse. This form of social defeat increased the alcohol preference in mice several weeks after the last social defeat [30, 31]. Our results showed that the SDPS paradigm almost doubled the occurrence of the AUD phenotype in rats. It is of note that in the current paradigm intermittent alcohol was given for 18 weeks after the last defeat [16]. Thus, the results provide evidence that chronic social stress has long-term adverse effects on AUD.

Psychiatric disorders, such as depression, commonly coexist with addictive disorders. Both AUD and major depression pose a significant risk for the development of the reciprocal disorder [32]. In humans, when the same personal, psychosocial, lifestyle and physical health measures were used to explain the comorbidity of depression and alcohol dependence, perceived stress is the only factor that was consistently associated with comorbidity [22]. Here we specifically tested the relation of prior social stress to addiction in terms of a 5-criteria model according to the DSM-V. In the SDPS group, about 78.1% rats classified as AUD, which is higher than the 42.8% in the control group. Knowing that social stress before alcohol exposure increases motivation and relapse [16], we here give proof of concept for the validity of using rodent AUD criteria.
4.2. Baseline behavioral parameters

As novelty seeking in humans predicts drug use [33], the behavioral tests used for subsequent prediction of AUD vulnerability in our experiment measured the preference to novel objects (OPR) or conspecifics (SAA, SI), and memory (SM). Although the four behavioral tests all measured horizontal locomotion of animals, the principal component analysis revealed that they reflected four different aspects of affective and cognitive behavior. This despite the fact that common aspects of learning and memory are involved in both OPR and SM tests. The spontaneous preference for an object in a new place is measured in the OPR test [20], and reflects a form of declarative memory [34]. This form of memory requires a functional hippocampus and connectivity to relevant cortical regions [35]. In the social memory test (SM) the ability to recognize a novel juvenile conspecific vs. a familiar one after a 24 h interval is measured in terms of approach behavior, which requires the ability to identify and remember conspecifics [36]. Specifically the CA2 region in the hippocampus is critical for this socially essential type of memory [37]. Most likely the specific requirement of hippocampal subregions and cellular connectivity with other brain regions make these two types of memory tests (OPR vs. SM) independent from each other. Social interaction and recognition is critical for the structure and stability of the social networks and relationships that define societies. The social approach avoidance test (SAA) in rodents measures approach behavior towards a potential aggressor, and hence reflects the intricate balance between curiosity-driven exploration and stress-driven inhibition of exploration. On the other hand, the social interaction test (SI), which measures the exploration of a juvenile conspecific, is devoid of this potential danger, and therefore the interaction rate with the juvenile is likely higher than with the adult. Although the control group and the SDPS group showed no group differences (cf. Fig. 3), there was enough individual variability to potentially predict the addiction criteria.

4.3. Predicting addiction from sociability parameters

Self-recognition of an alcohol use disorder is often late in the natural course of alcohol use. Efforts of early detection and brief intervention for AUD [38] have been made to reduce alcohol consumption and thereby avoid progression to dependence and the development of physical, mental and social problems. These screening and detection methods were based on
measuring the consumption of alcohol in the early stage of AUD. However, not every individual might be at risk for progression to addiction. Because disruptions in social behavior and social recognition characterize a variety of neuropsychiatric disorders, including depression, addiction, and obsessive-compulsive disorders [39, 40], we here used behavioral performances in different affective and cognitive domains that preceded alcohol exposure by several months to predict the alcohol use severity in rats. If future severe AUD and absent subjects could be identified before drug exposure, it would benefit many AUD vulnerable people by designing and implementing effective prevention strategies. We found different relationships of social behavior and the addiction parameters. In controls, social interaction with an adult conspecific (SAA test) regressed significantly with values obtained on 4 out of 5 addiction criteria, in which a higher interaction rate predicted higher addict-like behavior later on. It is of note that in the control group only a marginal portion showed a full addict-like profile according to the DSM-V criteria, with their average high score for any criterion being in the mid-low range of the values obtained for the SDPS group. In this respect, the relation of the same interaction trait with addict-like behavior in the SDPS group was opposite, namely a low interaction rate predicted high addict-like behavior for at least 2 addiction criteria (PR and ND). Our assumption that only low sociable individuals were at risk for addiction upon stress was true for all SAA–addiction correlations that showed an opposite pattern in controls vs. SDPS animals. Also for social interaction with a juvenile conspecific, low sociability predicted addict-like behavior in terms of extinction responding, namely the search for alcohol. Although here a similar correlation was obtained for control and SDPS animals, only the SDPS animals showed a significant correlation. Together, this indicates that animals with a low social interaction score are specifically at risk for addict-like behavior, in part due to prior stress.

With respect to social recognition memory of a juvenile conspecific, no significant correlation was obtained for any of the addiction criteria in controls. In SDPS animals, positive correlations were obtained for values related to 4 out of 5 addiction criteria, with 2 criteria (ND responding and average injections) being significant. For the ND criterion, specifically the high recognition animals shifted their scoring after stress, with low memory to protect from more impulsive-like drug taking and higher memory seeming to enhance this.
Given the social aspect of alcohol drinking in the western society, many future addicts start this way. Yet, alcohol drinking has reciprocal connections with social behavior depending on the dose used, i.e. more social and relaxed at a low dose but possibly more anti-social (e.g. aggressive behavior) at a higher dose. When classifying features of alcoholism in humans, a three-dimensional structure appeared in which one was dominated by males, taking alcohol in pubs, and scoring high on ‘fighting when drinking’ as well [41]. As we found opposing addiction criteria relations between social interaction and social recognition memory, it is clear that more in depth studies are required to tease out the precise interrelationships of specific behaviors that determine increased addiction vulnerability, as well as to demonstrate this in rodents and humans alike.

To conclude, we provided proof of concept for a DSM-V-based classification instrument in rats, and evidence that affective parameters can predict later AUD severity in rats not exposed to social stress. Moreover, specific affective parameters gained predictive value upon prior social stress exposure, and hence were able to predict risk for developing addiction according specific DSM-V addiction-criteria.
References


**Supplementary materials**

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<th>4 crit</th>
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<td>1</td>
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**Supplementary table 1.** The distribution of the individual rats with the scores on the 5 addiction criteria, resulting in 6 different bins for the SDPS subgroups of SDPS prone (n=10), SDPS moderate (n=12), and SDPS prone (n=10). The control group (n=16) is shown for comparison (see Table 1).

<table>
<thead>
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<th>Criteria</th>
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<th>AUD mild-moderate</th>
<th>AUD absent (%)</th>
<th>AUD severe</th>
<th>AUD mild-moderate</th>
<th>AUD absent (%)</th>
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<td>70.0</td>
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<td>6</td>
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<td>25.0</td>
<td>50.0</td>
<td>25.0</td>
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<tr>
<td>SDPS resilient</td>
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<td>4</td>
<td>4</td>
<td>20.0</td>
<td>40.0</td>
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**Supplementary table 2.** Addiction severity in terms of the DSM-V AUD score, and distribution in the SDPS subgroups of SDPS prone (n=10), SDPS moderate (n=12), and SDPS prone (n=10), resulting in 6 different bins. The control group (n=16) is shown for comparison (see Table 2). The number of animals (left), and the % of the total group (right) is shown.
### Supplementary table 3 The differences among the three SDPS groups in the five addiction criteria.

Only group differences were found in the ND response. Data for five criteria were presented as mean ± SEM. *P<0.05 compared with SDPS-resilient group (post-hoc LSD).

<table>
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<tr>
<th>Criteria</th>
<th>SDPS-prone (n=10)</th>
<th>SDPS-moderate (n=12)</th>
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<th>F-value F(2,29)</th>
<th>P-value</th>
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<td>PR response</td>
<td>64.4 ± 5.1</td>
<td>71.5 ± 16.4</td>
<td>59.1 ± 12.3</td>
<td>F(2,29) = 0.24</td>
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<tr>
<td>ND response</td>
<td>27.6 ± 2.5*</td>
<td>27.8 ± 4.4*</td>
<td>15.9 ± 2.8</td>
<td>F(2,29) = 3.70</td>
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<tr>
<td>Average injections</td>
<td>17.7 ± 1.5</td>
<td>16.0 ± 1.7</td>
<td>13.4 ± 1.8</td>
<td>F(2,29) = 1.56</td>
<td>0.228</td>
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<tr>
<td>Extinction</td>
<td>18.7 ± 1.6</td>
<td>17.6 ± 3.0</td>
<td>14.8 ± 2.6</td>
<td>F(2,29) = 0.60</td>
<td>0.555</td>
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<tr>
<td>Relapse</td>
<td>18.6 ± 2.9</td>
<td>14.9 ± 1.8</td>
<td>12.4 ± 2.1</td>
<td>F(2,28) = 1.78</td>
<td>0.187</td>
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Supplementary Figure 1. Correlations between the significant affective measures at baseline and the individual’s values obtained at four addiction criteria for the control (green) and SDPS (red) group. A–C) Scatter plots are shown for SAA (A), SI (B), SM (C) and the addiction criteria for control (left) and SDPS (right) animals. A trend line is indicated together with its $r^2$ value. For the exact correlation and P-value, see main Tables 3 and 4.