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Abstract

Serotonergic signaling is considered critical for an appropriate adaptation to stress. We have previously observed that in healthy volunteers, prefrontal serotonin transporter (SERT) binding is positively associated with hypothalamic-pituitary-adrenal (HPA)-axis output in terms of the cortisol awakening response (CAR). Here, we tested 1) if such a correlation persists in a human model of chronic serotonin depletion, namely in 3,4-Methylenedioxymethamphetamine (MDMA or "Ecstasy") users, and 2) if CAR differed between MDMA-users (N=18) and non-using healthy volunteers (N=32). **Methods**: Participants underwent imaging with [^{11}C]DASB-PET, and performed home-sampling of CAR, defined as the area under curve with respect to cortisol increase from awakening level. **Results**: When adjusting for age and group, CAR was positively coupled to prefrontal SERT binding (p=0.006) and MDMA users showed significantly higher CAR than the control group (p=0.0003). **Conclusion**: Our data confirm the recently described positive association between prefrontal SERT binding and CAR, this time in a human model of serotonin deficiency. Also, we find that CAR was higher in MDMA-users relative to non-users. We suggest that the inhibitory control on HPA-axis output is less efficient in the off-balance state established by recent MDMA-use most likely through mechanisms other than those that can be compensated by lowering SERT levels.

Introduction

Serotonergic brain signaling is considered critical for an appropriate adaptation to stress. Serotonergic signaling exerts top-down cortical inhibitory control on limbic system functions such as stress and fear responses including hypothalamic-pituitary-adrenal (HPA) axis regulation (1). In general, stress enhances serotonin output, and in turn, serotonin signaling influences the secretion of corticosteroids (2). Rodent studies suggest that serotonergic tone in the medial prefrontal cortex modulates behavioral stress responses by inhibitory actions (3, 4). Such inhibitory control may be critical in the adaption to environmental challenges and potential stressors as supported by the observation that HPA-axis dysregulation is both a state and a trait marker for major depression (5, 6). The majority of antidepressants target serotonergic neurotransmission including selective serotonin transporter inhibitors (SSRIs). Interestingly, the efficacy of SSRIs in maintaining recovery from depression is related to the normalization of HPA-axis reactivity (7), thus, again linking serotonergic signaling to HPA-axis reactivity. Nevertheless, the role of serotonin in regulating HPA-axis activity is far from clear and characterization of ways by which serotonergic signaling and HPA-axis output interact is of particular interest in understanding the risk architecture behind the development of e.g. mood disorders.

The cortisol awakening response represents a measure of HPA-axis output stimulated by awakening. Awakening in the morning provokes a profound 50-75% rise in plasma cortisol that peaks at around 30 minutes after awakening and plasma cortisol returns to baseline levels within approximately 60 minutes (8) and is seen in approximately 75% of healthy individuals under home-sampling conditions (9). CAR, as described by the area under the curve with respect to increase from awakening level

(AUC_i), represents both the rise provoked by awakening and the subsequent restoration to baseline levels. Thus, CAR may also index the regulatory capacity of the HPA-system needed to terminate induced activity. Some studies support that CAR and HPA-axis responses to psychosocial stressors (stress reactivity) are correlated (10, 11), and CAR seemingly indexes HPA-axis reactivity to some extent (9, 12).

In a previous study of healthy volunteers we have shown that prefrontal serotonin transporter (SERT) binding is positively correlated to HPA-axis output in terms of CAR (13). We suggested that the observed association between prefrontal SERT binding and CAR is established in neurodevelopment and/or it could represent an inhibitory capacity of prefrontal serotonin signalling in controlling HPA-axis output (13). In our cross-sectional study of healthy volunteers, we were not able to characterize the relation between prefrontal serotonergic tone and HPA-axis output under more extreme conditions, e.g. an off-balance state of serotonin. Use of 3,4-Methylenedioxymethamphetamine (MDMA or "Ecstasy") offers a human model of chronic serotonin depletion resulting in a transient serotonin deficiency. Animal studies show that long-term exposure to MDMA is associated with a reduction in cerebral serotonin levels and a decreased numbers of SERT binding sites (14). Likewise, human studies in MDMA-users show a dose-dependent decrease in cerebral SERT binding (15), particularly in cortical brain regions (15-17). Interestingly, both acute (18, 19) and recent (20) MDMA-use also show higher basal cortisol excretion. Moreover, recent MDMA-use (3 weeks abstinence) blunts HPA-axis response to psychological induced stress (20), which may be related to pharmacological effects of MDMA still present. However, to our knowledge no studies have directly addressed whether CAR is dysregulated by recent MDMA-use.

Here we investigated if CAR differed between MDMA-users and non-users, and if prefrontal SERT binding is associated with CAR also in a condition with chronically reduced serotonergic neurotransmission, as is the case in MDMA-users. If the coupling is confirmed, this will be supportive of either a sustained prefrontal serotonergic regulatory capacity on CAR and/or a pre-existing coupling of prefrontal SERT and HPA-axis output that is not influenced by serotonin deficiency.

Experimental procedures

Participants

Eighteen MDMA-using volunteers (mean age 24.5±3.8 years (range 20.1 to 33.6), 2 women), and 32 non-using healthy volunteers (mean age 35.3±20.1, range 19.7 to 81.7 years, 7 women) underwent SERT imaging with [^{11}C]DASB-PET and performed home-sampling of CAR during the same investigation period using the exact same techniques. Participants were screened for psychopathology with the symptom check-list (SCL-92) and the major depression inventory (MDI). Levels of perceived stress were scored by Cohen's perceived stress scale (21). To avoid acute drug effects, including SERT occupancy, brain imaging was performed after a minimum drug abstinence period of 11 days. None of the participants had a personal history of present or prior neurological or psychiatric disorders, nor did they receive any psychotropic pharmacological treatment. None of the participants took medicine modulating the immune system, hormone replacement, or anabolic steroids as evaluated by interview. The non-MDMA-user group did not use illicit drugs, but were allowed to have a history of up to 15 exposures to cannabis. Written informed consent was obtained from all participants.

The MDMA-users is a subgroup of a larger group of 24 MDMA- or hallucinogenic-users, published in Erritzoe et al. (2011) (15). Five did not hand in saliva cortisol samples and 1 did not use MDMA, hence a total of 18 individuals were available for the present study. Data on the 32 controls are published in Frokjaer et al. (2012) (13), 15 of these individuals also served as healthy controls in the study by Erritzoe et al. (2011) (15).

Imaging, coregistration, and quantification of SERT

SERT binding was imaged with [^{11}C]DASB PET based on 90 minutes dynamic acquisition starting immediately after bolus injection of 6.4±1.6 and 6.6±1.1 MBq [^{11}C]DASB per kg body weight in the non-user and MDMA-user-group respectively. PET scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, producing 35 image slices with an interslice distance of 4.25mm. The outcome parameter of the [^{11}C]DASB binding is the ratio between specific binding and non-displaceable binding of the tracer, BP_{ND}. We used a modified reference tissue model designed specifically for quantification of [^{11}C]DASB (MRTM/MRTM2) by Ichise et *al*. (22) implemented in PMOD, software version 2.9, build 2 (PMOD Technologies), where cerebellum without vermis, serves as a reference region. Magnetic resonance imaging (MRI) was acquired on a Siemens Magnetom Trio 3T MR scanner (Invivo, FL, USA). As described in Frokjaer et al. (13), two slightly different high-resolution 3D T1-weighted magnetization prepared rapid gradient echo (MPRAGE1 and MPRAGE2) scans were acquired in the non-using healthy volunteers in 8 and 24 individuals respectively. All MDMA-users had the MPRAGE2 acquired. MRI sequence parameters, movement correction, coregistration of [11C]DASB mean image to the T1-weighted image by AIR (23), segmentation of T1-weighted image, and further details on [11C]DASB imaging, MR-imaging, and quantification are described in Frokjaer et al. (24) and (13).

Volumes of interest (VOIs)

Based on the method by Svarer et al. (23) VOIs were automatically delineated on each individual's MRI in a strictly user-independent fashion. Since we aimed at replicating the earlier observation of a coupling between prefrontal SERT and CAR in healthy volunteers with no history of psychoactive drug use (13), the a priori appointed VOI was the prefrontal cortex (computed by pooling orbito-, superior-, and medial- and inferior- (lateral part) frontal cortex). Cerebellum and a high-binding region (pallidostriatum and thalamus) served as a basis for the determination and fixing of k2' (clearance rate constant from cerebellum) which is required in the MRTM/MRTM2 modelling of SERT BP_{ND} (22).

Saliva cortisol

HPA-axis output was characterized by the cortisol awakening response (CAR). CAR was based on 5 serial measurements of the rise in salivary cortisol over the first hour from awakening (ideally, at 0, 15, 30, 45 and 60 minutes after awakening) and computed as the area under curve from 0 to 60 minutes with respect to increase from the baseline awakening cortisol value (AUC_i). Also, 3 saliva samples were collected during the rest of the day at approximately noon, 6 PM, and 11 PM which allowed characterization of an index for the total daily cortisol output as the area under the curve with respect to ground ($AUC_{fullday}$) of the 8 measurements spanning from awakening to approximately 11 PM. $AUC_{fullday}$ measures were standardized across a 960 min time-span.

Participant training, instructions, sampling procedure, storing and cortisol analyses were carried out exactly as described in Frokjaer et al. (2012) (13). Cortisol analyses ran in the same batch and were determined by an Electrochemiluminescence

Immunoassay (ECLIA) method on Modular Analytics E170 equipment (Roche, Mannheim, Germany).

Statistics

We tested if prefrontal SERT BP_{ND} was associated with CAR (AUC_i) in a multiple linear regression analysis adjusted for age and user-group. In addition, we tested for a group by SERT BP_{ND} interaction in predicting CAR in a multiple linear regression analysis adjusted for age. Contingent on observing significant associations between prefrontal SERT BP_{ND} and CAR within the MDMA-user group, we tested if this association depended on measures for proximity to last use of MDMA (days since last use reported at PET-scan date), and accumulated MDMA exposure (number of life-time exposures to MDMA), respectively. Both "days" and "number" were log-transformed as validated in earlier analyses (15). Also, we tested age adjusted group differences in regional SERT binding and CAR, respectively, in separate regression models.

Six supplementary analyses were performed. First, we performed adjustments for additional potentially relevant variables that might confound CAR and SERT (Cohen´s perceived stress score, MDI score, global severity index score (SCL-GSI), absolute cortisol concentration at awakening, $AUC_{fullday}$, gender, BMI, and smoking) to test the robustness of identified significant associations. Second, we tested for effects of prefrontal SERT in predicting $AUC_{fullday}$ in an analysis adjusting for group and age. Third, we tested if the results depended on partial volume correction of SERT BP_{ND}. Fourth, we tested if AUC_i was associated with proxies of non-specific SERT binding (as opposed to our measure of interest; specific binding). Fifth, we tested if the results remained when excluding the observations where more than 100 days separated CAR and

SERT measurements. Sixth, we tested if age differences in our groups drove the results despite including age in the models. Thus, we tested the primary analyses using a restricted control group of the 26 individuals under the age of 40 years (mean age 26.2 ±5.3 years, 6 women).

P-values, as estimated by two-tailed tests, parameter estimates with standard errors (SE) and 95% confidence limits (CI) are reported when appropriate. All models were checked graphically and no gross misspecifications were identified. We compared 95% confidence limits to those obtained using robust standard errors, which yielded very similar results. The analyses and graphical presentations were performed in Instat 3.0b and R3.0.1 (http://www.R-project.org). P-values below 0.05 were considered statistically significant.

Results

Demographic and psychometric profiles are presented in Table 1. MDMA-users were younger, smoked more, and scored higher in symptoms of mental distress relative to non-users. Table 2 shows that age adjusted prefrontal cortex SERT BP_{ND} and CAR (AUC_i) differed between groups as opposed to midbrain BP_{ND} and $AUC_{fullday}$. Adding smoking status to the regression models presented in Table 2 did not change the results or contribute significantly in predicting AUC_i or prefrontal BP_{ND}.

Associations between CAR, prefrontal SERT binding, and user-group

The cortisol awakening response (AUC_i) was positively associated with prefrontal SERT BP_{ND} in a model adjusting for age, and user-group (slope= 1716 per BP_{ND} (unitless), 95% CI [520; 2912] nmol/L cortisol*min., p=0.006, N=50). In the same model, group contributed significantly in predicting CAR, i.e. MDMA-users showed significantly higher CAR than the control group (group mean difference= 340, 95% CI [164; 516] nmol/l*min, p=0.0003, N:18 MDMA-users, 32 non-users). No significant "group by prefrontal SERT BP_{ND}" interaction, i.e., no significant group difference in the slope of the association between prefrontal SERT and CAR, was observed (p=0.71), Figure 1.

A dose-response relation between use of MDMA and CAR could not be demonstrated: Within the MDMA-user group (N=18), neither total number of life time exposures to MDMA, nor time since last MDMA-intake was associated with CAR, p=0.85 and p=0.72 respectively.

Supplementary analyses

In order to address whether the higher level of perceived stress in the MDMA-user group (Table 1) drove the association between CAR and prefrontal SERT binding we added Cohen's perceived stress score to the model. As shown in Table 3, if anything, this strengthened our results. Likewise, when correcting for other measures of subclinical psychological symptoms, namely major depression inventory score (MDI) and the global severity index score (SCL-GSI) the results were sustained (data not shown). As expected, Cohen's perceived stress score contributed significantly in the model (Table 3, B) showing a positive association with CAR (p=0.03), as did the SCL-GSI on a trend-level (p=0.07) in a similar model. On the contrary, MDI did not show such a significant association with CAR (p=0.27) in a similar model. When testing more complex models including additional potentially relevant covariates (cortisol at awakening, gender, BMI, smoking status), the results did not change substantially as compared to the simpler models, Table 3.

We further addressed specificity of CAR as opposed to other HPA-axis measures indexing underlying absolute levels of cortisol. Cortisol at awakening did not contribute significantly (p=0.90) to the association with prefrontal SERT (in a model correcting for age, user-group and Cohen´s score, Table 3, C), indicating that the correlation between prefrontal SERT binding and CAR was largely independent of the underlying absolute level of cortisol. Likewise, adjusting for $AUC_{fullday}$ did not substantially change the associations between SERT BP_{ND} and AUC_i (p=0.004) or the group difference between MDMA-user and non-users (p=0.0007). In addition, $AUC_{fullday}$ was not significantly predicted by prefrontal SERT (p=0.73), or differed between groups (p=0.21) in a simpler regression model adjusting for age.

We confirmed that the results from the primary analysis (Table 3, model A) remained, regardless of partial volume correction of the PET-data (association between partial volume corrected SERT BP_{ND} and AUC_i (p=0.01), and difference between user-groups p=0.001). We excluded that AUC_i coincidentally correlated with a proxy for non-displaceable tracer binding; i.e. the area under the cerebellar time–activity curves normalized to the injected dosage per kg ($AUC_{cerebellumTAC}$) was not correlated with AUC_i (p=0.98). However, we did observe a modest group difference in $AUC_{cerebellumTAC}$ (8.7% (CI: 1.6;15.9%, which was lower in MDMA-users relative to non-users, age adjusted analysis, p=0.02, N=51). Yet, adding this measure to the primary model did not change the results, see Table 3, model E.

The mean time span between CAR and PET measurements was 20 ± 52 days. When excluding the 5 observations where more than 100 days separated those measures the mean time span was 4 ±20 days. We restricted our analyses to the latter group and confirmed the association between prefrontal SERT and CAR and the group difference in CAR between MDMA-users and non-users (N=45), p=0.03 and p=0.0003 respectively. This suggests that observations from individuals with a long gap between CAR and SERT measurements did not drive our results.

Finally, our total group of non-using healthy controls as published in Frokjaer et al. (2012) (13) spanned an age range substantially larger than the MDMA-users. To exclude that age would be a confounder in the group comparison, we tested if restricting the analysis to include only individuals below 40 years of age. With this subset of 26 healthy controls both estimates and significance levels were essentially the same (slope for the association between prefrontal SERT binding and CAR: slope= 1855 per BP_{ND} (unitless), 95% CI [631; 3079] nmol/L cortisol*min., p=0.004, N=44, and, group

difference in CAR = 340, 95% CI [182; 529] nmol/l*min, p=0.0002, N:18 MDMA-users, 26 non-users) as in the original groups (Table 3, A).

Discussion

In a group of mentally healthy MDMA-users, that provide a model of serotonin deficiency, we have demonstrated a positive coupling between prefrontal SERT and HPA-axis output (CAR), similar to the coupling previously seen in healthy volunteers (13). We show that the coupling between CAR and prefrontal SERT was equally strong in MDMA-users and non-using controls, and notably, that CAR was highest in the MDMA-user group. Thus now, in two independent groups differing in their absolute levels of HPA-axis output and prefrontal SERT binding, we have shown a positive coupling between prefrontal SERT availability and CAR.

Coupling of prefrontal SERT and CAR in the context of MDMA-use

In abstinent MDMA-users the extent of MDMA-use is associated with lower cerebral SERT levels and there is reason to believe that the users' habitual serotonergic state prior to MDMA-use does not deviate from that of healthy non-using individuals (15). As such repeated MDMA-use induces a state of compromised serotonergic signalling in the brain. The decrease in SERT density may be due to loss of serotonergic projections (predominantly in cortical regions) or a compensatory phenomenon tending to normalize serotonergic tonus. Our data is consistent with the hypothesis that abstinent MDMA-users sustain some regulatory capacity on CAR, even when serotonergic signaling is compromised. Notably, irrespective of their current prefrontal SERT density, MDMA-users displayed higher CAR than non-users therefore, if implicated at all, the depleted state does not seem to be fully compensated. Therefore, we speculate that the association between prefrontal SERT and HPA-axis output in terms of CAR may well be established

through mechanisms e.g. early in brain development (24, 25), that are not influenced by serotonin deficiency in adult life.

In addition, the coupling of prefrontal SERT and HPA-axis output in both MDMA-users and non-users may also to some extent reflect a serotonin modulated prefrontal inhibitory influence on HPA-axis activity. It is well established that prefrontal cortex is involved in inhibitory feedback control on the HPA-axis (26, 27). Specifically, studies in rats support that serotonin levels in the medial prefrontal cortex are critical for cessation of behavioral stress and fear responses (3, 4, 28). Further, human intervention studies support that serotonergic tonus modulates the HPA-axis activity; lowering central serotonin by acute tryptophan depletion in healthy volunteers leads to a neuroendocrine stress response, in the absence of perceived stress, possibly through a disinhibitory effect on the axis (29). To the extent that serotonergic deficiency induced by recent MDMA-use can be compensated, some serotonergic modulation of prefrontal inhibitory capacity on HPA-axis output may be preserved in MDMA-users. Yet, clearly CAR if off-set in our MDMA-user group as discussed below.

Glucocortocoid exposure in itself may lead to higher SERT protein levels as supported by in vitro studies of human cell lines (30) and lead to higher cortical SERT binding in newborn rats (31). While this may offer an explanation for the positive association between CAR and prefrontal SERT in healthy volunteers, we find it less likely to explain our observations in MDMA-users due to the following; 1) higher CAR and lower prefrontal SERT were seen in MDMA-users relative to non-users, and 2) no significant correlation between total cortisol output ($AUC_{fullday}$) and prefrontal SERT binding was present.

Only one other study in humans in a mixed group of patients with major depression and OCD, has linked HPA-axis dynamics and cerebral SERT availability (32). In contrast to our observations, that study (32) reported a negative association between thalamic SERT binding and HPA-axis output in terms of cortisol response to the dexamethasone (suppression)-corticotrophin test. The study is not directly comparable to our set-up since it evaluated primarily the inhibitory capacity of the HPA-axis system in a highly stimulated condition and focused at a clinical sample that may be influenced by pathophysiological processes or effects of recent medication.

Higher CAR in MDMA-users relative to non-users

Our data indicate that other mechanisms independent on SERT contribute to higher CAR in MDMA-users. No other studies have directly addressed CAR changes in MDMA-users. However, both acute (18, 19) and recent (3 weeks abstinence) MDMA-use (20) is reported to be associated with higher basal cortisol excretion. Some studies implicates dopaminergic and noradrenergic signaling of potential importance for the mechanisms by which MDMA may affect the regulation of HPA-axis activity as indirectly supported by animal work (33). Further, MDMA-induced elevation of total morning cortisol appears to be related to the low activity COMT genotype (34), which is of importance for the degradation of dopamine and noradrenaline and affect prefrontal cortical functions.

Gerra et al. (2003) report that recent MDMA-users show a blunted response to psychologically induced stress (20), whereas we find an elevated HPA-axis output in terms of CAR. However, in Gerra et al., the MDMA-users scored in average above the clinical thresholds for major depression as opposed to our user-group that scored well below clinical cut-off of MDI (mean score 7.3, range 1-19, clinical cut-off 20). Since

blunted HPA-axis responses to psychological stressors are seen in individuals with persistent depressive symptoms (35), this may explain the findings in the depressed MDMA-user group by Gerra et al. (20). Thus, our observation of an elevated CAR in non-depressed MDMA-users suggest that blunted HPA-response does not arise per se via direct pharmacological effects of recent MDMA-use.

Last, we cannot firmly exclude that CAR was elevated in MDMA-users due to preexisting disturbances of HPA-axis or related systems or, that the MDMA-user life style would lead to more physiological or psychological stress that in turn might affect HPA-axis responsiveness to awakening. However, we consider that unlikely given the magnitude of difference in CAR we saw between MDMA-users and non-users, the subacute effects of MDMA on cortisol release demonstrated by others in a longitudinal set-up (20), and given that adjusting for state measures of perceived stress or depressive symptoms if anything strengthened our observations.

Methodological considerations

Although not clearly confirmed (36), withdrawal from MDMA may elicit depressive symptoms (37). We did indeed find higher average scores of depressive symptoms in the MDMA-user group, although clearly, none of the users met criteria for depression. Our analysis showed, however, that the coupling between prefrontal SERT and CAR was not driven by perceived stress or depressive symptoms.

While smoking does not appear to affect SERT binding in a detectable manner (38) it may affect diurnal cortisol profiles although the relationship is complex. Specifically, smoking is associated with both flattened and elevated CAR patterns (39). Since MDMA-users smoked more than our healthy control group, we cannot effectively control for

potential confounds of smoking. However, when performing a linear adjustment for smoking in our regression model smoking did not contribute significantly in predicting CAR or changed the observed associations (Table 3, model F), indicating that smoking did not drive our observations.

The sample size of the MDMA-user group did not allow for a conclusive analysis of the potential dose-response relation between MDMA exposure and CAR. Accordingly, we cannot exclude an association between dose proxies (life time exposure and time since last use of MDMA) and CAR that might become detectable in larger datasets.

Conclusion

Our data support that prefrontal SERT binding is positively associated with cortisol responses to awakening in healthy adults as well as in abstinent MDMA-users and as such may act to balance HPA-axis activity. This coupling is sustained even in the off-balance state of serotonin deficiency induced by recent MDMA-use. The presence of higher CAR in MDMA-users relative to non-users, however, suggests that with MDMA use, CAR is off-set through mechanisms other than prefrontal SERT.

Tables

Table 1: Demographic and psychometric profile.

Parameter	**MDMA-user group** Mean value ± SD (ranges or proportions in brackets)	**Controls** Mean value ± SD (ranges or proportions in brackets)	**Comparison** P-value
N	18	32	NA
Age (years)	24.5±3.8 (20.1;33.6)	35.3 ±20.1 (19.7;81.7)	p=0.006
Sex	11% women (2 of 18)	22% women (7 of 32)	p=0.46
BMI (kg/m^2)	23.6±2.7 (19.5;30.3)	25.0±3.2 (17.9;32.9)	p=0.11
Smoking	44% (8 of 18)	12.5% smokers (4 of 32)	p=0.02
Alcohol (units/week)	9.1±7.3 (0.5;24)	8.6±6.9 (0;25)	p=0.83
SCL_GSI (Global severity index)	0.30±0.21 (0.10;0.68)	0.12±0.11 (0;0.42)	p=0.002
MDI score	7.3±5.5 (1;19)	3.3±2.4 (0;9)	p=0.008
Cohen's perceived stress score	11.0±7.4 (3;30)	4.7±3.6 (0;14)	p=0.002

BMI: Body mass index (weight in kg / (height in $m)^2$). SCL_GSI: Symptom check-list, global severity index score. MDI: Major depression inventory.

Table 2: Age adjusted group comparisons of SERT BP_{ND} and cortisol parameters

Parameter	**MDMA-users** Mean ± SD (ranges or proportions)	**Controls** Mean ± SD (ranges or proportions)	Age adjusted mean difference ±SE	Age adjusted difference p-value
N	18	32	NA	NA
CAR, AUC_i nmol/L*min	222 ±312 (-211;957)	60 ±229 (-468;598)	204 ±79	p=0.01
$AUC_{fullday}$ nmol/L*min	3852 ± 1781 (842;8280)	3052 ±2515 (598;11537)	919 ±717	p=0.20
Prefrontal SERT BP_{ND}	0.19±0.07 (0.07;0.35)	0.25±0.06 (0.15;0.38)	-0.079 ± 0.018	P<0.0001
Midbrain SERT BP_{ND}	1.81±0.21 (1.52;2.19)	1.79 ±0.21 (1.34;2.14)	-0.041 ±0.059	p=0.49
Injected dose of [11C]DASB Mbq per kg body weight	6.6±1.1 (4.3;9.4)	6.4 ±1.6 (3.2;10.1)	NA	p=0.59*
[11C]DASB specific activity (GBq per µmol DASB)**	30.9 ±12.6 (16;53)	30.5 ±13.7 (12;54)	NA	p=0.91*

Mean difference is calculated as MDMA-users minus healthy non-using controls. CAR: Cortisol awakening response. AUC: Area under curve. AUC_i: AUC with respect to increase from awakening. $AUC_{fullday}$: AUC with respect to ground estimated from 8 diurnal measures. BP_{ND}: Binding potential of specific tracer binding (unitless). *Not age adjusted, **Specific activity at injection time.

Table 3: Effect of prefrontal cortex serotonin transporter (SERT) binding on cortisol awakening response (CAR) and user-group differences in CAR when analyzed in alternative models of various complexity

Model (covariates)	**Effect of SERT BP_{ND} on CAR (AUC_i)** Slope estimate (nmol cortisol/L*min per BPnd), [95% CL], p-value	**Group difference in CAR (AUC_i)** Mean difference (MDMA-users minus healthy controls), [95% CL], p-value
A. (BP_{ND}, age, user group)	1716 [520; 2912], p=0.006	340 [164; 516], p=0.0003
B. (BP_{ND}, age, user group, Cohen score)	1809 [603; 3017], p=0.004	284 [88; 480], p=0.005
C. (BP_{ND}, age, user group, Cohen score, cortisol at awakening)	1794 [541; 3046], p=0.006	285 [86; 483], p=0.006
D. (BP_{ND}, age, user group, Cohen score, BMI, gender)	1846 [604; 3087], p=0.005	260 [55; 465], p=0.01
E. (BP_{ND}, age, user group, $AUC_{cerebellumTAC}$)	1710 [498; 2923], p=0.007	344 [159; 523], p=0.0005
F. (BP_{ND}, age, user group, smoking)	1721 [490; 2953], p=0.007	342 [149;534], p=0.0008

95% CL: 95% confidence limits. AUC_i: The cortisol awakening response calculated as area under curve with respect to increase from awakening, unit nmol cortisol/L*min. SERT BP_{ND}: Serotonin transporter binding potential (unitless). Cohen score: Cohen´s perceived stress score at the cortisol home-sampling day. BMI: Body mass index (weight in kg / (height in m) ^{2}). $AUC_{cerebellumTAC}$: Area under curve under the cerebellar time activity curve which provides a proxy for non-specific binding. Smoking: Smoking status (current smoker or not).

Figure legends

Figure 1. Scatter plot of the association between Cortisol awakening response (CAR) and prefrontal SERT-binding in healthy volunteers (black circles) and MDMA-users (open circles) modeled as a "prefrontal SERT by group" interaction (adjusted for age). No significant interaction is present. Shaded areas represent point-wise 95% confidence bands.

Figure 2. Cortisol awakening response (CAR) in 18 MDMA-users (A) versus 32 non-using controls (B). Average curve estimated from an additive mixed model of salivary cortisol concentration over time. Dashed lines represent point-wise 95% confidence bands.

COMPETING INTEREST STATEMENT

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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