Alterations in brain microstructure in rats that develop abnormal aggression following peripubertal stress

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Keywords – aggression, brain structure, corticosterone, early life stress, individual differences, MRI

Abstract: 233 words

Article: 5269 words

Figures: 5

Supplemental information: 650 words

Running title: Variable brain & behavior response to early stress

Abstract

Exposure to early adversity is implicated in the development of aggressive behavior later in life in some but not all individuals. The reasons for the variability in response to such experiences are not clear but may relate to pre-existing individual differences that influence its downstream effects. Applying structural magnetic resonance imaging (MRI) to a rat model of abnormal aggression induced by peripubertal stress, we examined whether individual differences in the development of an aggressive phenotype following stress exposure were underpinned by variation in the structure of aggression-associated, cortico-limbic brain regions. We also assessed whether responsiveness of the hypothalamic-pituitary-adrenal axis to stress was associated with neurobehavioral outcome following adversity. A subset of the rats exposed to peripubertal stress developed an aggressive phenotype, while the remaining rats were affected in other behavioral domains, such as increased anxiety-like behaviors and reduced sociability. Peripubertal stress led to changes in tissue microstructure within prefrontal cortex, amygdala and hippocampal formation *only* in those individuals displaying an aggressive phenotype. Attenuated glucocorticoid response to stress during juvenility predicted the subsequent development of an aggressive phenotype in peripubertal stress-exposed rats. Our study establishes a link between peripubertal stress exposure in rats and structural deviations in brain regions linked to abnormal aggression, and points toward low glucocorticoid responsiveness to stress as a potential underlying mechanism. We additionally highlight the importance of considering individual differences in behavioral response to stress when determining neurobiological correlates.

Introduction

There is growing interest in identifying risk factors and neurobiological mechanisms associated with pathological aggression (Haller, 2013; Dorfman *et al.*, 2014; Glenn & Raine, 2014; Waltes *et al.*, 2016). Compelling evidence implicates early adversity in the subsequent development of aggressive and anti-social behaviors (Widom & Maxfield, 1996; Caspi *et al.*, 2002; Weder *et al.*, 2009; Beach *et al.*, 2011; Viding & McCrory, 2012; Fanning *et al.*, 2014; Haller *et al.*, 2014; Lee *et al.*, 2014; Provencal *et al.*, 2015; Tzanoulinou & Sandi, 2017). However, there are clear individual differences in vulnerability to develop aggression following early life stress exposure (Caspi *et al.*, 2002; Odgers *et al.*, 2008; Green *et al.*, 2010). Achieving a better understanding of the neurobiology underlying this variability may allow progress in the prevention and treatment of pathological aggression.

Magnetic resonance imaging (MRI) of individuals diagnosed with aggression-related psychopathologies has highlighted variation in brain structure in areas engaged in socio-emotional functions, including prefrontal cortex (PFC), hippocampus, and amygdala (Raine *et al.*, 2000; Dolan *et al.*, 2002; Barkataki *et al.*, 2006; Zetzsche *et al.*, 2007; Coccaro *et al.*, 2015; Coccaro *et al.*, 2016), in association with pathological aggression. The PFC, hippocampus and amygdala form part of a corticolimbic circuit that is functionally implicated in aggression (Haller, 2014; van der Kooij *et al.*, 2014; Kohl *et al.*, 2015; White *et al.*, 2016). All three regions undergo continuous development early in life, rendering them susceptible to the impact of stress (Spear, 2000; Casey *et al.*, 2008). Strikingly, structural variation in these brain regions has been reported to correlate with relative severity of early life adversity in humans (Cohen *et al.*, 2006; Pechtel *et al.*, 2014), suggesting that early life stress might contribute to structural variation observed in certain types of pathological aggression. This possibility is supported by studies that have documented greater structural differences in aggressive individuals with early life stress exposure versus those without (Sala *et al.*, 2011; Morandotti *et al.*, 2013).

The timing of exposure to adversity during early life is associated with the nature of social dysfunctions subsequently developed (Haller *et al.*, 2014; Sandi & Haller, 2015; Walker *et al.*, 2016; Tzanoulinou & Sandi, 2017). Here, we decided to focus on the peripubertal period as evidence from both animal and human studies highlight it as a sensitive period for the development of stress-induced anti-sociality (Sandi & Haller, 2015). In humans, exposure to adversity prior to and during puberty increases risk for psychopathological alterations – such as borderline personality disorder (Newnham & Janca, 2014) or intermittent explosive disorder (Fanning *et al.*, 2014) – that present a high prevalence of 'reactive' aggression (McCloskey *et al.*, 2009; Coccaro *et al.*, 2015). Such individuals show functional alterations in the amygdala and PFC in association with increased likelihood of aggressive behaviors (Coccaro *et al.*, 2007; Rosell & Siever, 2015). Similar amygdala and PFC dysfunctions were observed in adult rats submitted to fearful experiences during the peripubertal period (Marquez *et al.*, 2013). Behaviorally, peripubertally stressed rats display reactive aggression towards conspecifics that goes far beyond species-specific norms (Haller, 2017). Importantly, although development of such behavior following peripubertal stress has been observed a number of times (Cordero *et al.*, 2012; Cordero *et al.*, 2013; Marquez *et al.*, 2013; Tzanoulinou *et al.*, 2014; Cordero *et al.*, 2016), as in humans, there is substantial variability in the data implying that it is but a fraction of stressed individuals that develop subsequent aggressiveness (Tzanoulinou *et al.*, 2014; Cordero *et al.*, 2016).

Here, we used structural MRI to investigate whether individual differences in the development of an aggressive phenotype in rats following peripubertal stress are associated with individual differences in brain structure. Rather than relying on a single measure of aggression, we adopted a profiling approach to achieve a more holistic assessment of the aggressiveness of individual rats. Previous application of a profiling approach has enabled the determination of neurobiologically meaningful subtypes of response to trauma (Cohen *et al.*, 2004; Anacker *et al.*, 2016; Ritov *et al.*, 2016). We focused on the medial prefrontal cortex (mPFC), amygdala, and hippocampal formation, as these brain regions are: (i) involved in the

regulation of aggressive behavior (Haller, 2014; van der Kooij *et al.*, 2014; Kohl *et al.*, 2015); (ii) subject to ongoing development during the peripubertal period (Spear, 2000; Casey *et al.*, 2008); and iii) susceptible to stress influences during this period (Isgor *et al.*, 2004; Eiland *et al.*, 2012; Marquez *et al.*, 2013). Furthermore, we analyzed the link between the emerging phenotype and glucocorticoid responsiveness to early stress exposure.

Methods & Materials

Subjects

Experimental subjects (N=24 [n=12/group]) were male offspring of Wistar Han rats (Charles River, France) bred in our animal facility. Stimulus animals (i.e. juveniles [n=6], intruders [n=24] and females [n=24]) were bought from the same supplier. All were maintained on a 12-h light-dark cycle (lights on: 0700 h). At weaning on postnatal day (p)21, pairs of rats from different litters were matched according to weight and housed together. Rats remained undisturbed, except for the peripubertal stress protocol and standard husbandry, until p90. Experiments were performed between 0800 and 1200 h, except where otherwise stated. All procedures were conducted in accordance with the Swiss National Institutional Guidelines on Animal Experimentation and approved by a license from the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Peripubertal Stress protocol

The stress protocol was performed as previously described (Marquez *et al.*, 2013). Briefly, following exposure to an open field for five minutes on p28, two different stressors were presented intermittently between p28-42, each one lasting 25 minutes (see Fig.1 for the schema). Stressors were either exposure to the synthetic fox odor, trimethylthiazoline (Phero Tech Inc., Canada), or to an elevated platform. To assess the effect of stress exposure on hypothalamic-pituitary-adrenal axis activity we took tail-blood samples following stress on p28, p30 and p42. Blood samples were collected by tail nick: rats were

wrapped in a cloth and, within 1 minute, up to 100µl of blood was collected from a small incision made in one of the tail arteries. Control rats underwent brief handling on stress days, no blood samples were taken.

Behavioral procedures

This study focused on the identification of neurodevelopmental trajectories that lead to differential aggression following exposure to peripubertal stress. To gain a better understanding of the behavioral phenotype associated with differential aggression, animals were characterized with a battery of behavioral tests. The sequence of behavioral tests progressed from low to increasing stressfulness, with a one-week break imposed between tests (see Fig.1 for details).

Novelty stress

Following 20 minutes of exposure to a dimly-lit (30 lx) novel environment (circular plastic container; 35 cm x 25 cm), blood samples were obtained via tail-nick. A second tail-blood sample was obtained from the same tail-nick following 30 minutes in a neutral holding cage.

Elevated plus maze (EPM)

Anxiety-like behavior was evaluated using the EPM test (Pellow & File, 1986). The EPM used consisted of two opposing open arms (50 x 10 x 50 cm) perpendicular to two enclosed arms (50 x 10 x 50 cm) that extend from a central platform (10 x 10 cm) elevated 65 cm above the floor. Light levels were maintained at 14-16 lx on the open arms and 5-7 lx on the closed arms. At the start of the test, the rat was placed on the central platform facing a closed arm and allowed to explore the maze for five minutes. The maze was cleaned with 5% ethanol solution, and thoroughly dried, between subjects. Behavior was monitored using a ceiling-mounted video camera and analyzed with a computerized tracking system (Ethovision 9; Noldus IT, Netherlands). The time spent and entries in the open and closed arms, and distance moved, were automatically recorded.

Social preference test

The social preference test was performed in a rectangular, polycarbonate, three-chambered box that included a central compartment (20 x 35 x 35 cm) and two side compartments (30 x 35 x 35 cm). After five minutes of habituation to the central chamber, retractable doors were removed and the rat was allowed to explore the whole apparatus for 10 minutes. Side compartments were each equipped with a central, floor-fixed, transparent, perforated cylinder that contained either an unfamiliar male juvenile rat or an unfamiliar object. The apparatus was cleaned with 5% ethanol solution, and thoroughly dried, between subjects. Each trial was video-recorded (MediaCruise, Canopus Co. Ltd, Japan) and manually scored offline by an experimenter blind to experimental group. The percentage of time spent exploring (snout <2 cm from the cylinder) either the juvenile or the novel object was recorded, and a social preference ratio calculated according to the formula: time spent exploring the juvenile/time spent exploring the juvenile + object.

Resident-intruder test

Prior to the resident-intruder test, experimental rats cohabited with a female partner for 10 days to encourage territoriality. The female was removed 30 minutes prior to the test, and then replaced afterwards. Tests were performed between 1900 and 2200 h. The resident was exposed in its home cage to a lighter (5-10%), unfamiliar male for 30 minutes. Intruders were used only once.

Encounters were video-recorded and scored offline by an experimenter blind to the experimental group, assisted by Observer software (Noldus IT, Netherlands). The following parameters were quantified in terms of frequency and duration: attack (a rapid and intense contact with the intruder, often involving a clinch), offensive upright (pushing the intruder away whilst both are standing on hindpaws), lateral threat (approaching the intruder laterally, with arched back), keeping down (actively pinning the intruder on its back), biting, social investigation (sniffing and grooming the intruder), non-social investigation (exploring

the cage) and auto-grooming. The cumulative frequency and duration of the first four behaviors were summed to provide measures of total offensive behavior. Latency to the first offensive event initiated by the resident was also recorded.

Additionally, detailed video analysis of biting attacks was performed to assess their signaling, targeting and intensity (Toth *et al.*, 2012; Haller, 2017). Specifically, a bite was considered to be signaled when it occurred in the context of an ongoing bout of offensive behavior. Bites were scored as targeted toward vulnerable (head, throat and belly) or non-vulnerable (back or flanks) parts of the opponent. Bites were also scored as hard or soft, depending on the response elicited by the bite. A hard bite was scored when the bite evoked a strong startle response from the opponent. Soft bites elicited little or no response from the opponent. The ratio of each of the following was calculated for all bites performed by one rat: i) unsignalled versus signaled bites; ii) bites targeted to vulnerable versus non-vulnerable areas; iii) hard versus soft bites. For bite-related measurements the number of rats in the control group reduced to eight, since three control rats did not perform any bites and to include them in the analysis with scores of zero would have biased results to make the peripubertal stress group appear more aggressive by comparison.

Profiling for aggression

Many behaviors exhibited during a social encounter are deemed aggressive. Aggressive behaviors can be both 'normal' (i.e. within species-typical norms) and 'abnormal' in nature (Haller, 2017). Here, in line with the literature, we considered abnormal forms of aggression to include attacks that were excessively violent (i.e. causing a strong reaction in the bitten rat), unsignaled (i.e. not occurring in the context of an ongoing bout of offensive behavior) or targeted towards vulnerable body parts (i.e. head, belly or genitals) (Haller, 2017).

To measure holistically the development of an aggressive phenotype, an individual profiling approach was applied (Cohen *et al.*, 2004; Ritov *et al.*, 2016). Classification criteria were defined according to the

extremes (20th or 80th percentile, depending on index) of the control group's distribution for each measure, including: offensive behavior duration; offensive behavior frequency; latency to offend; frequency of bites with any abnormal component; proportion of all bites that were unsignaled, targeted toward vulnerable body parts or excessively 'hard'. Rats scoring above the cutoff for a particular measure received an 'aggressive' score. Any rat accruing five such scores from seven was considered an 'aggressive' rat overall.

Aggression *z* scores were calculated from the raw scores for the **seven** variables described above using the formula: ((score – mean of all scores) / standard deviation of all scores). The *z* scores were averaged to derive a single aggression score (Guilloux *et al.*, 2011), subsequently used as a continuous variable against which corticosterone responses to stress were correlated.

Forced swimming test (FST)

Whilst still cohabitating with females, rats were submitted to the FST to evaluate coping-style (Porsolt *et al.*, 1978). Animals were placed in a plastic beaker (25 cm diameter x 46 cm) containing 30 cm of water (25°C) for 15 minutes. The following day, rats were re-exposed under the same conditions for a further five-minute session. The apparatus was cleaned with 5% ethanol solution, and dried, between subjects. Both sessions were recorded using a ceiling mounted video camera, and the times spent immobile (making only those movements necessary to keep the snout above the water), swimming or climbing were quantified by an experimenter blind to the condition using in-house software (Clicker; EPFL, Switzerland).

Perfusion

Two weeks after the FST, rats were anesthetized with a lethal dose of pentobarbital (Esconarkon, Streuli Pharma, Switzerland, 150 mg/kg) and transcardially perfused using 0.9% saline solution followed by a fixative solution of paraformaldehyde 4% in phosphate-buffered saline (pH=7.5). Heads were stored in 4% paraformaldehyde overnight and rehydrated in phosphate-buffered saline containing 0.05% sodium azide for at least one week prior to scanning.

Ex vivo MRI

Before scanning, the lower jaw was removed from each head to reduce the required field-of-view. The skull and brain were then immersed in fluorinated fluid (Galden, Solvay, Belgium) to reduce susceptibility artefacts, and imaged with a 7-Telsa pre-clinical scanner (Agilent Technologies, UK) and 39 mm diameter birdcage radiofrequency coil (Rapid GmbH, Germany). A 3D Fast Spin-Echo (FSE) image was acquired with TE/TR=60/2000 ms, echo-train-length 8, echo-spacing 15 ms, matrix 192x128x192, isotropic 150 µm voxel size, and acquisition time 104 minutes. A diffusion-weighted segmented echo-planar image was acquired with TE/TR=35/5000 ms, 4 segments, 10 averages, matrix 128x96, 40 slices, voxel size 200x200x500 µm, 30 diffusion directions with b=2000 s/mm2, 4 b=0 images, δ/Δ=4/16 ms and acquisition time 234 minutes.

Image processing

Diffusion tensor indices were calculated from diffusion imaging using previously published methods (Wood *et al.*, 2016). The FSE images were used to construct a study-specific template image (Avants *et al.*, 2010) which was then registered to an atlas image (Valdes-Hernandez *et al.*, 2011).

Regions of interest (ROIs) covering the mPFC (prelimbic and infralimbic cortex), hippocampal formation (hippocampus, subiculum), amygdala and globus pallidus (equivalent to external globus pallidus in primates) were drawn on the atlas with Jim (Xinapse Systems, UK). The globus pallidus, a region still developing during adolescence but not implicated in aggressive behavior, was selected as a control region.

The inverse transforms from subject to atlas space were applied to the ROIs to move them to individual subject space, where their volumes were calculated. In addition, for each ROI the mean value of mean diffusivity (MD) and fractional anisotropy (FA) were extracted from their respective quantitative image.

Corticosterone measurement

Total corticosterone was measured from blood plasma samples via enzymatic immunoassay performed according to manufacturer's instructions (Enzo Life Sciences, Switzerland). Levels were calculated using a standard curve method.

Statistics

Data were analyzed using either SPSS 17.0 (Chicago, USA; behavioral) or Python (Anaconda Software Distribution Version 4.3.29; MRI). Both behavioral and MRI variables were analyzed using two-tailed Mann-Whitney tests (median and interquartile range are shown), with correction for multiple comparisons applied using the Holm-Bonferroni method (corrected p-values are shown). Two-way repeated measures analysis of variance (ANOVA) was used to analyze corticosterone measurements, with group as the between-subjects factor and postnatal day as the within-subjects factor (mean ± SEM). Correlations were performed using Pearson's method. Statistical significance was set at $p<0.05$. Given the risk of Type II error owing to low sample size, findings at $p<0.1$ are reported as marginally significant. One rat was excluded from the control group as it was an outlier (defined as being >3 standard deviations from the mean) in the key measure of several behavioral tests.

Results

Exposure to peripubertal stress induced an aggressive phenotype

We first confirmed that rats exposed to peripubertal stress showed increased aggression relative to the control group, and independent of an individual differences approach. In accordance with previously published data, peripubertally stressed rats displayed an aggressive phenotype (Aggression *z score*: U=13, p=0.007).

Individual differences in development of an aggressive phenotype indicated two subtypes of behavioral response to peripubertal stress

As predicted, and as previously observed, variability in aggressiveness of individuals exposed to peripubertal stress was evident. To discern aggressive individuals, we applied a profiling approach according to the distribution of scores from the control group. Classification was made according to the extremes of the control distribution (see Supplemental Information [SI] for cutoff values) of several variables (Fig.2A-C, 'normal' aggression; Fig.2D-G, 'abnormal' aggression). Every rat achieving an 'aggressive' score in five of the seven variables was classified as an aggressive rat overall. This delineated two subpopulations within the peripubertal stress group, depicted in Fig.2H, one defined as aggressive (n=5; 'aggressive PPS') and the other as non-aggressive (n=7; 'non-aggressive PPS').

Underlining the validity of the profiling approach, a normalized aggression score considering all seven aggression variables was higher in aggressive PPS rats than in control or non-aggressive PPS rats (U=39, p=0.027 and U=32, p=0.072, respectively; Fig.2I). The increased 'violence' of aggressive PPS rats relative to controls appeared to be driven by qualitatively 'abnormal' aggression (U=39, p=0.009) rather than by 'normal' aggression (U=27, p=0.843; Fig.2K-L; see SI Table 1 for all group comparisons).

Non-aggressive peripubertally stressed rats were affected by stress in other behavioral domains

Responses of the two peripubertal stress subgroups were compared to control animals in other behavioral tests. Differences between groups were observed in the social preference test. Non-aggressive PPS rats showed reduced social preference compared to the control rats (Fig.3C: U=5, p=0.003), whereas the aggressive group did not (U=17, p=0.534). Decreased social preference ratio in non-aggressive rats appeared primarily driven by increased exploration of the object (SI Table 1: U=69, p=0.012) rather than decreased exploration of the juvenile (U=16, p=0.132).

Differences in anxiety-like behaviour on the EPM were also evident. Specifically, non-aggressive PPS rats tended to spend less time on the open arms of the EPM than control rats (Fig.3B: U=13, p=0.060) and aggressive PPS rats (U=30, p=0.096). Control and aggressive PPS rats did not differ (U=27, p=1.000). Differences in locomotion did not account for this disparity, control and non-aggressive PPS rats travelled comparable distances during EPM testing (SI Table 1: U=53, p=0.633).

Peripubertal stress experience did not appear to alter corticosterone responsiveness to acute novelty stress (Fig.3A, SI Table 1), nor the time spent immobile during a forced swimming test (Fig.3D, SI Table 1).

Individual differences in aggression following peripubertal stress were associated with differences in tissue microstructure in stress-sensitive brain regions

Ex vivo MRI revealed a lack of significant differences in regional brain volume between the control group and either of the peripubertal stress subgroups (see SI Table 2 for full details).

Reduced FA was observed in the amygdalae of aggressive PPS rats relative to non-aggressive PPS rats (U=33, p=0.045). No additional differences in FA were observed (Fig.4; see SI Table 3 for full details).

Reduced MD was observed in the subiculum of aggressive PPS rats relative to both control rats (U=54, p=0.001) and non-aggressive PPS rats (U=34, p=0.019). Similar reductions in MD were observed in the infralimbic cortex and hippocampus of aggressive PPS rats versus control rats (infralimbic: U=46, p=0.083;

hippocampus: U=48, p=0.069) and non-aggressive PPS rats (infralimbic: U=32, p=0.069; hippocampus: U=32, p=0.069), although these findings were not statistically significant after correction for multiple comparisons. Additionally, reduced MD was found in prelimbic cortex of aggressive PPS rats as compared to non-aggressive PPS rats (U=35, p=0.017). Mean diffusivity did not differ between control rats and non-aggressive PPS rats in any region studied (see SI Table 4).

Glucocorticoid responsiveness to peripubertal stress exposure was associated with adult aggressiveness.

Corticosterone response to peripubertal stress declined from first to last stress exposure (Fig.5A: effect of day: $F_{(1,20)}$=20.86, p<0.001). Whilst the pattern of glucocorticoid responsiveness did not differ between peripubertal stress subgroups across the stress protocol (day*group: $F_{(1,20)}$=1.03, p=0.322), aggressive rats had a blunted corticosterone response to stress relative to non-aggressive rats (effect of group: $F_{(1,20)}$=36.06, p<0.0001).

Rats' corticosterone response to the first stress exposure (i.e. on p28) was significantly negatively correlated with aggressiveness at adulthood (Fig.5B: p28 CORT*aggression *z* score: r=-0.61, p=0.034), whereas rats' corticosterone response to the last stress exposure (i.e. on p42) was not (Fig.5C: p42 CORT*aggression *z* score: r=-0.43, p=0.159).

Discussion

Using a well characterized rat model of peripubertal stress-induced abnormal aggression (Cordero *et al.*, 2012; Cordero *et al.*, 2013; Marquez *et al.*, 2013; Cordero *et al.*, 2016), we show here that peripubertal stress leads to structural alterations in selected brain regions *only* in those individuals in which adversity triggers an abnormal aggression phenotype. Structural alterations were found in brain regions implicated in the regulation of aggression, such as the mPFC, amygdala, and hippocampus, but not in an aggression unrelated region such as the globus pallidus. In addition to this aggression-related neurodevelopmental trajectory, we identify an alternative one also triggered by peripubertal stress. This second one comprises animals devoid of abnormal aggression but showing increased anxiety-like behavior, reduced sociability, and absence of structural changes in the brain regions examined.

In line with previous findings from our laboratory, we report here that, when examined at the group level, peripubertal stress exposure in rats leads to abnormal aggression, increased anxiety and reduced sociability (Cordero *et al.*, 2013; Marquez *et al.*, 2013; Tzanoulinou *et al.*, 2014). Crucially, by applying a profiling approach, we were able to identify different profiles in the long-term response to peripubertal stress that were related to variability in concomitant brain structural changes. This approach adds to earlier contributions to the literature that have emphasized the importance of profiling for individual differences when examining neurobiology associated to a behavioral outcome (Cohen *et al.*, 2004; Anacker *et al.*, 2016; Ritov *et al.*, 2016).

We focused our structural analyses in several candidate brain regions, including different subdivisions of the mPFC, amygdala, and hippocampus, all brain regions subject to ongoing development during adolescence; functionally affected by peripubertal stress; and involved in the regulation of aggression in both humans and animals (Spear, 2000; Gregg & Siegel, 2001; Andersen & Teicher, 2008; Casey *et al.*, 2008; Marquez *et al.*, 2013; Haller, 2014; van der Kooij *et al.*, 2014; Kohl *et al.*, 2015; White *et al.*, 2016).

No significant volumetric differences were found between peripubertally stressed and control animals, in contrast to volumetric reductions reported in PFC (Raine *et al.*, 2000; Sala *et al.*, 2011), hippocampus (Dolan *et al.*, 2002; Barkataki *et al.*, 2006; Zetzsche *et al.*, 2007; Sala *et al.*, 2011; Morandotti *et al.*, 2013; Coccaro *et al.*, 2015), and amygdala (Coccaro *et al.*, 2015) in patients with aggression-related psychopathologies. Furthermore, volume decrements in aggressive, borderline personality disordered individuals in the PFC appeared to be exacerbated by a history of early adversity (Sala *et al.*, 2011; Morandotti *et al.*, 2013). In our study, we cannot exclude that the lack of detection of volumetric differences, particularly in the subiculum where the data depicts a picture for smaller volume in the aggressive PPS group, is due to the small sample size. Previous studies in rats in which brain structure was analyzed using MRI following chronic stress exposure at adulthood have depicted mixed results. Following 10 days of immobilization stress, (Henckens *et al.*, 2015) identified increased volume and diffusivity of the lateral ventricles, whereas no other volumetric changes in specific brain regions were observed. On the contrary, following 3-weeks of exposure to chronic unpredictable stress, (Magalhaes *et al.*, 2017a) reported small structural reductions in a large number of brain regions. The experimental approach of these two studies differs in a number of ways. In addition to the differences in length and nature of the stressors applied, the former study applied a deformation-based morphometry analyses to MRI data, whereas the latter one used a voxel-based morphometry analysis. Rodent brain morphometric analysis remains a new field. In the future, it will be important to standardize experimental procedures in animal MRI studies and to apply stringent statistical analyses that guarantee validity of the reported conclusions.

The amygdala was the only brain region in which group differences of FA were observed, with reduced values in aggressive PPS rats relative to non-aggressive rats. The exact biological basis of FA is complex, but it is likely related to axonal density and weakly to myelination (Jones *et al.*, 2013; De Santis *et al.*, 2014). Early life stress has been shown to increase FA in hippocampal CA1 in correspondence with a reduction in total apical dendritic length (Molet *et al.*, 2016). Although no data regarding amygdala have

yet been reported, our findings indicating reduced FA in the amygdala in aggressive PPS rats add to several examples whereby stress leads to opposite effects in hippocampus and amygdala at the structural level (Chattarji *et al.*, 2015; McEwen *et al.*, 2016). Of note, the amygdala projects to the hypothalamic attack area (Toth *et al.*, 2010); structural alterations in the amygdala in aggressive PPS rats might thus contribute to the abnormal aggressive behaviors observed in this subset of rats.

Regarding MD, we found aggression-related reductions in hippocampus and subiculum, as well as in the infralimbic and prelimbic regions of the mPFC, but not in the globus pallidus. Though some of these findings did not survive correction for multiple comparison, in light of the relatively low number of animals and the conservative statistical approach used in the study, the potential for Type II error is high and we do not therefore disregard them. Studies in which tissue properties were assessed jointly with diffusion tensor imaging and histology indicated that diffusivity measures, as well as deriving from myelination and neuronal density, may also derive from cellularity, and neurite density (Khan *et al.*, 2016; Tu *et al.*, 2016). Diffusivity reductions observed here might therefore reflect decreased alignment of neurites, increased complexity of neuronal processes or increased glial cells (Beaulieu, 2002; Delgado y Palacios *et al.*, 2011; Evans, 2013; Hemanth Kumar *et al.*, 2014; Khan *et al.*, 2016). Identification of sources of diffusivity fluctuations may be complicated by concurrent changes in several such parameters (Tu *et al.*, 2016). In accordance with this, species-atypical aggressive behavior displayed by rats exposed to post-weaning social isolation was associated with several structural alterations in the mPFC, including a reduction in thickness, a decrease in dendritic and glial density, and reduced vascularization (Biro *et al.*, 2017).

We additionally asked whether individual differences in glucocorticoid responsivity to stress during peripuberty might be associated with the development of an aggressive phenotype and found that corticosterone response to stress indeed differed between aggressive and non-aggressive PPS subgroups, in a manner that was associated with subsequent aggressiveness. Our data is in line with previous work highlighting a link between abnormal glucocorticoid levels and aggressive behavior (Haller *et al.*, 2000;

Kruk *et al.*, 2013; Haller, 2014) and our own work using the peripubertal stress model revealing a role for glucocorticoids during peripubertal stress on the long-term programming of aggressive behaviors (Veenit *et al.*, 2013; Papilloud *et al.*, 2018; Walker & Sandi, 2018).

In line with our findings, repetitive stress has been found to have differential impact on brain structure in more versus less stress responsive rat strains (Bourgin *et al.*, 2015; Magalhaes *et al.*, 2017a; Magalhaes *et al.*, 2017b). Non-aggressive PPS rats, that had greater corticosterone responses to peripubertal stress, displayed more anxiety-like and less social behavior, in accordance with the phenotype of recently developed high-corticosterone rat lines (Walker *et al.*, 2017; Walker & Sandi, 2018). The brain regions studied here are particularly responsive to the programming effects of stress and are still maturing during the peripubertal period (Spear, 2000; Andersen & Teicher, 2008; Romeo *et al.*, 2013). Glucocorticoids are potent modulators of biological processes, including neuroanatomical plasticity (de Kloet *et al.*, 2005; Eiland & Romeo, 2013; McEwen, 2016), and could conceivably induce brain structure changes associated with aggressive phenotypes. Interestingly, experiments determining the impact of stress exposure timing on brain microstructure implicated pre-puberty as a moment of heightened vulnerability to stress-induced alterations (Zalsman *et al.*, 2015). Many neurodevelopmental processes take place during this narrow window including synaptic overproduction, synaptic pruning, and myelination (Andersen & Teicher, 2008; Liston & Gan, 2011) and all are sensitive to disruption by stress (Liston & Gan, 2011; Pattwell *et al.*, 2016).

Several clinical studies have indicated a relationship between glucocorticoid reactivity and brain tissue microstructure. For example, in older men, a relationship was found between higher cortisol responses to mild stressors and higher MD in white matter (Cox *et al.*, 2015). Moreover, patients with Cushing's disease (with a history of endogenous hypercortisolism but presently in remission) showed widespread reductions in FA throughout the brain, indicative of persistent structural effects of hypercortisolism (van der Werff *et al.*, 2015). Our finding that only a subset of individuals showed structural and behavioral susceptibility to early life stress, and that those individuals already presented lower glucocorticoid responsiveness early in

life, suggests that gene x environment interactions could account for the findings. Indeed, similar interactions have been reported in human studies. For example, possession of a single nucleotide polymorphism of the FKBP5 gene in conjunction with experience of childhood maltreatment was reported to predict structural changes in brain regions involved in emotional processing in depression (Tozzi *et al.*, 2016).

A limitation of this study is that we cannot determine the causal relationships between aggressive behavioral phenotype, stress responsiveness, and brain structure. Indeed, a longitudinal chronic social defeat stress study in mice indicated that pre-existing differences in hippocampal structure, as well as magnitude of stress-induced volume change, predicted behavioral susceptibility to stress (Tse *et al.*, 2014).

Emerging clinical evidence highlights alterations in brain structure in individuals diagnosed with aggression-related psychopathologies typically associated with exposure to early life trauma (Widom & Maxfield, 1996; Raine *et al.*, 2000; Barkataki *et al.*, 2006; Zetzsche *et al.*, 2007; Sala *et al.*, 2011; Viding & McCrory, 2012; Morandotti *et al.*, 2013; Fanning *et al.*, 2014; Lee *et al.*, 2014; Provencal *et al.*, 2015). However, establishing a direct association between stress exposure and the interrelated emergence of both behavioral and brain structural phenotypes is difficult due to limitations associated with human studies. Our preclinical study in rats takes a step toward closing this gap.

In summary, we present evidence of two distinct neurodevelopmental trajectories arising from peripubertal stress in rats, one of them leading to abnormal aggression and structural alterations including reduced FA in the amygdala and reduced MD in the PFC and hippocampal formation. The second one, low in aggression and devoid of structural changes in the brain regions examined, exhibited increased levels of anxiety-like behavior and reduced sociability. Interestingly, all brain regions showing structural changes in aggressive PPS individuals have been highlighted in structural and functional human studies as altered in individuals showing emotion dysregulation and psychopathology following early life stress exposure (Tottenham & Sheridan, 2009; VanTieghem & Tottenham, 2017) and abnormal levels of reactive

aggression (Coccaro *et al.*, 2007; Coccaro *et al.*, 2015; Rosell & Siever, 2015). Our study establishes a link between peripubertal stress exposure and structural deviations in these brain regions in association with abnormal aggression, and points toward differential glucocorticoid responsiveness across stressful challenges encountered throughout life as a potential contributing mechanism. Our data, obtained under controlled laboratory conditions in a rodent model of reactive aggression, support the view that alterations in brain structure described in aggressive humans subjected to early life adversity may indeed reflect their prior stress exposure and underlie their behavioral dysfunctions.

Acknowledgements

We thank Dr. Aurélie Papilloud, Dr. Damien Huzard, Jocelyn Grosse and Olivia Zanoletti for their valuable technical assistance**.** This project has been supported by grants from the Swiss National Science Foundation (31003A-152614; and NCCR Synapsy, grant no. 51NF40-158776), European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no. 603016 (MATRICS), and intramural funding from the EPFL to CS. The funding sources had no additional role in study design, in the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the paper for publication. This paper reflects only the authors' views and the European Union is not liable for any use that may be made of the information contained therein.

Abbreviations

ANOVA	Analysis of variance	**mPFC**	Medial prefrontal cortex
EPM	Elevated plus maze	**MRI**	Magnetic resonance imaging
FA	Fractional anisotropy	**p**	Post-natal day
Fig.	Figure	**PFC**	Prefrontal cortex
FST	Forced swimming test	**ROI**	Region of interest
MD	Mean diffusivity	**SI**	Supplemental information

Financial disclosures

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1 Experimental design. Animals were weaned at postnatal day (p)21 and assigned to Control or Peripubertal Stress (PPS) groups (n=12/group). The stress protocol consisted of exposure to an open field (OF) for 5 minutes on p28, followed by an elevated platform (EP, 25 minutes), with predator odor (trimethylthiazoline; TMT, 25 minutes) also used as a stressor. Stressors were presented as depicted in the schema. Blood sampling days are indicated with a red drop. Control animals were handled briefly on the days on which their experimental counterparts were exposed to stress but no blood samples were taken. Behavioral testing started at p90, with a delay of one week imposed between each test in the series of tests.

Figure 2 There were individual differences in the development of an aggressive phenotype following exposure to peripubertal stress (PPS). When exposed to an unfamiliar intruder, adult PPS rats did not differ at the group level from the control group in terms of the total amount of time spent engaged in offensive behavior (A), nor in the frequency of offensive behaviors (B). However, PPS rats did offend more readily (C). Compared to control rats, the attacks of PPS rats tended to be more frequently abnormal in nature (D), with a non-significant trend to target vulnerable body parts more readily (E). A higher proportion of biting attacks performed by PPS rats were 'hard', eliciting a strong startle response from the opponent (F). Control and PPS rats showed similar signaling of their intent to attack (G). Statistical differences between groups are indicated by red symbols (A-C: n: Control = 11, PPS = 12; D-G, I-K: n: Control = 8 [non-biting rats not included], PPS = 12; Mann-Whitney tests: * = significantly different, # = marginally significant). Large inter-individual variability was evident in all aspects of aggressive behavior. Profiling was conducted using the values of the control group as a reference. Dashed lines indicate the 80^{th} (A, B, D, E, F, G) or 20^{th} (C) percentile for each variable considered within the profile. A rat was considered to be aggressive overall when it exceeded the cutoff in a minimum of five of these indices. This yielded two subgroups amongst PPS-exposed rats, the non-aggressive (n=7) and aggressive (n=5)

individuals (H). Aggressive PPS rats had a higher aggression score than control and non-aggressive PPS rats when all variables were considered (I). This difference was driven more by abnormal forms of aggression (L: frequency of bites having an abnormal aspect, bite targeting to vulnerable parts, hard bites and unsignaled bites) than by normal aggression (K: duration and frequency of offensive behavior and latency to offend).

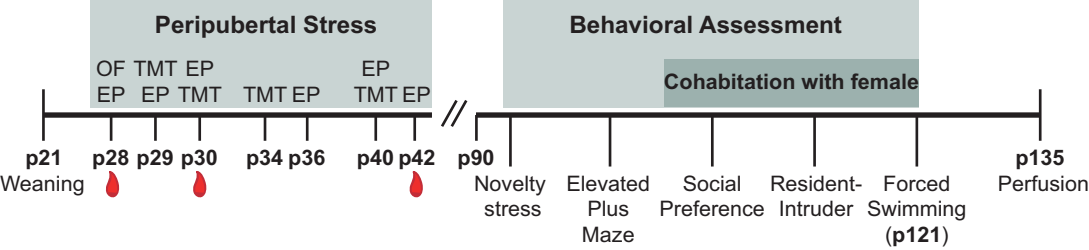
Figure 3 Individual differences in behavior were found following exposure to peripubertal stress (PPS) in other measures of emotionality. Non-aggressive PPS rats tended to spend less time on the open arm of an elevated plus maze (B) and showed reduced preference for a social target in a test of sociability (C) relative to the control group. These differences were not evident in PPS rats classified as aggressive. No differences were found between either of the peripubertal stress groups and the control rats in corticosterone response to novelty stress (A), or in immobility during the second exposure to forced swimming (D). Statistical differences between groups are indicated by red symbols (n: Control = 11, PPS non-aggressive = 7, PPS aggressive = 5; Mann-Whitney tests: * = significantly different, # = marginally significant).

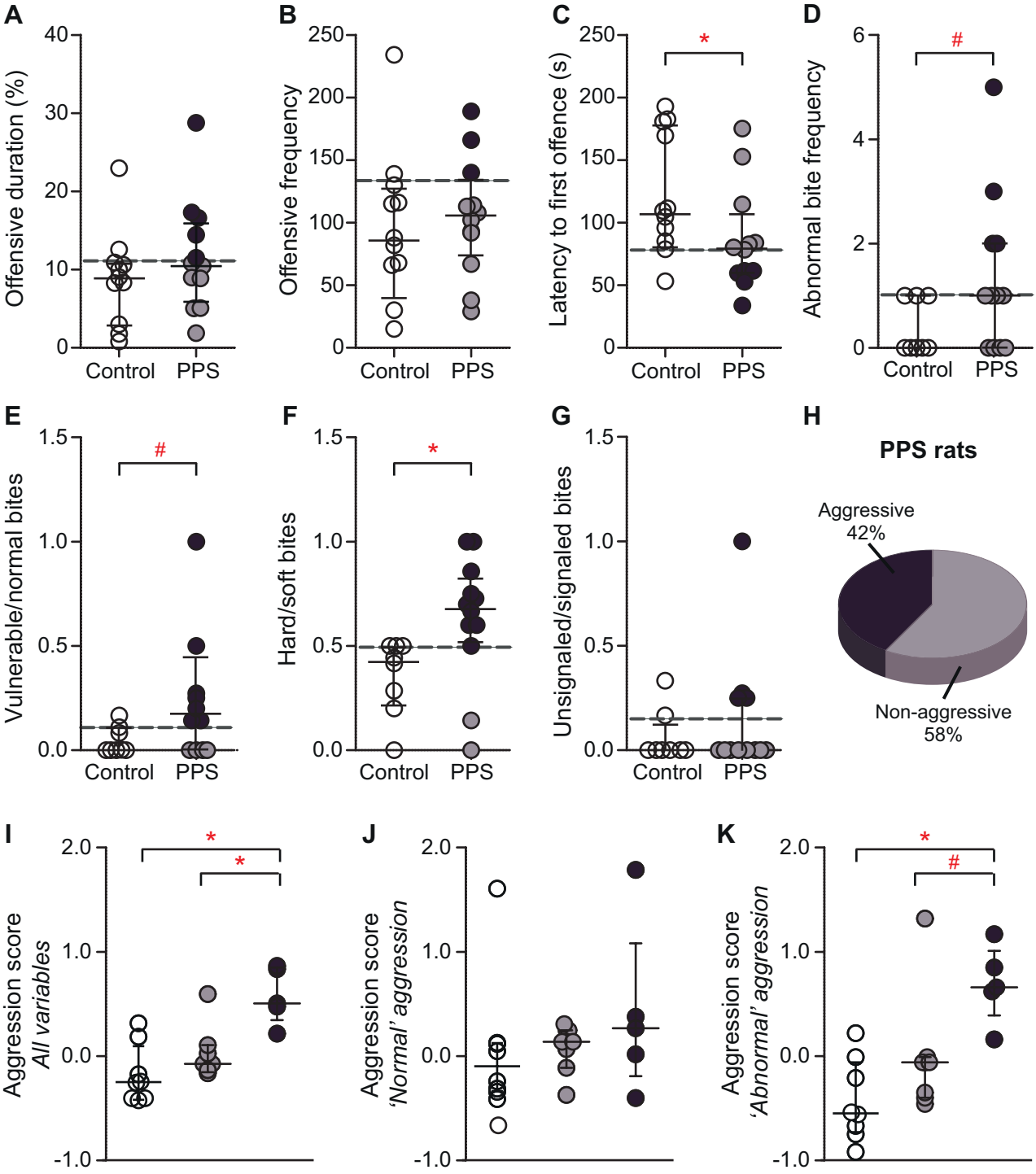
Figure 4 Development of an aggressive phenotype following peripubertal stress (PPS) exposure was associated with reductions in mean diffusivity (MD) in subcortical brain regions often associated with aggression but not in a control region not associated with aggression. Statistical differences between groups are indicated by red symbols (n: Control = 11, PPS non-aggressive = 7, PPS aggressive = 5; Mann-Whitney tests: * = significantly different, # = marginally significant; see text for further details). Abbreviations: FA = Fractional anisotropy; MD = Mean diffusivity.

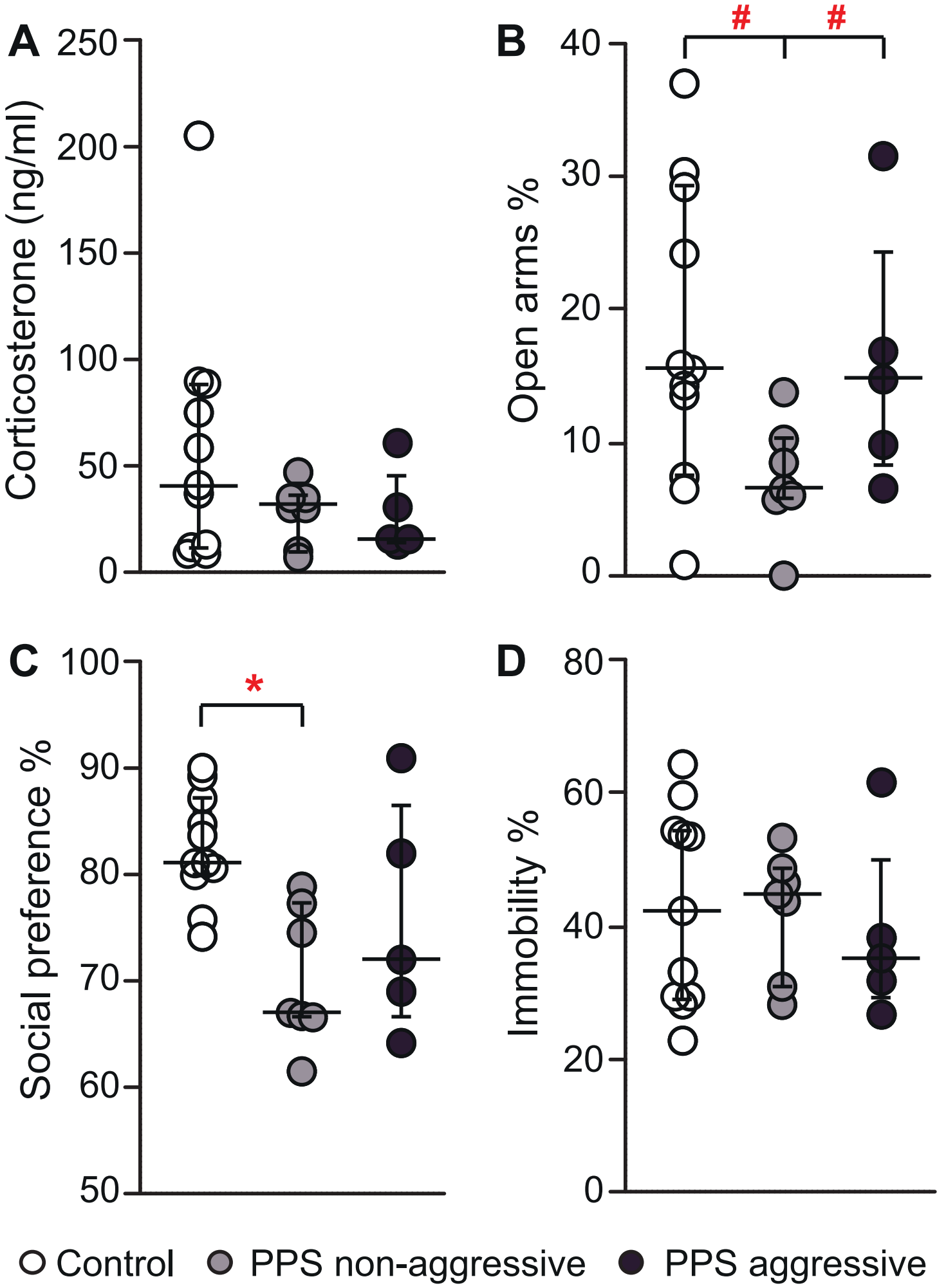
Figure 5 Development of an aggressive phenotype following peripubertal stress (PPS) exposure was associated with differential corticosterone (CORT) responsiveness to that stress exposure. Rats from the PPS aggressive subgroup had lower CORT at the offset of stressors on postnatal day (p) 28 and p42 than those from the PPS non-aggressive subgroup (A). CORT response on p28 was significantly correlated

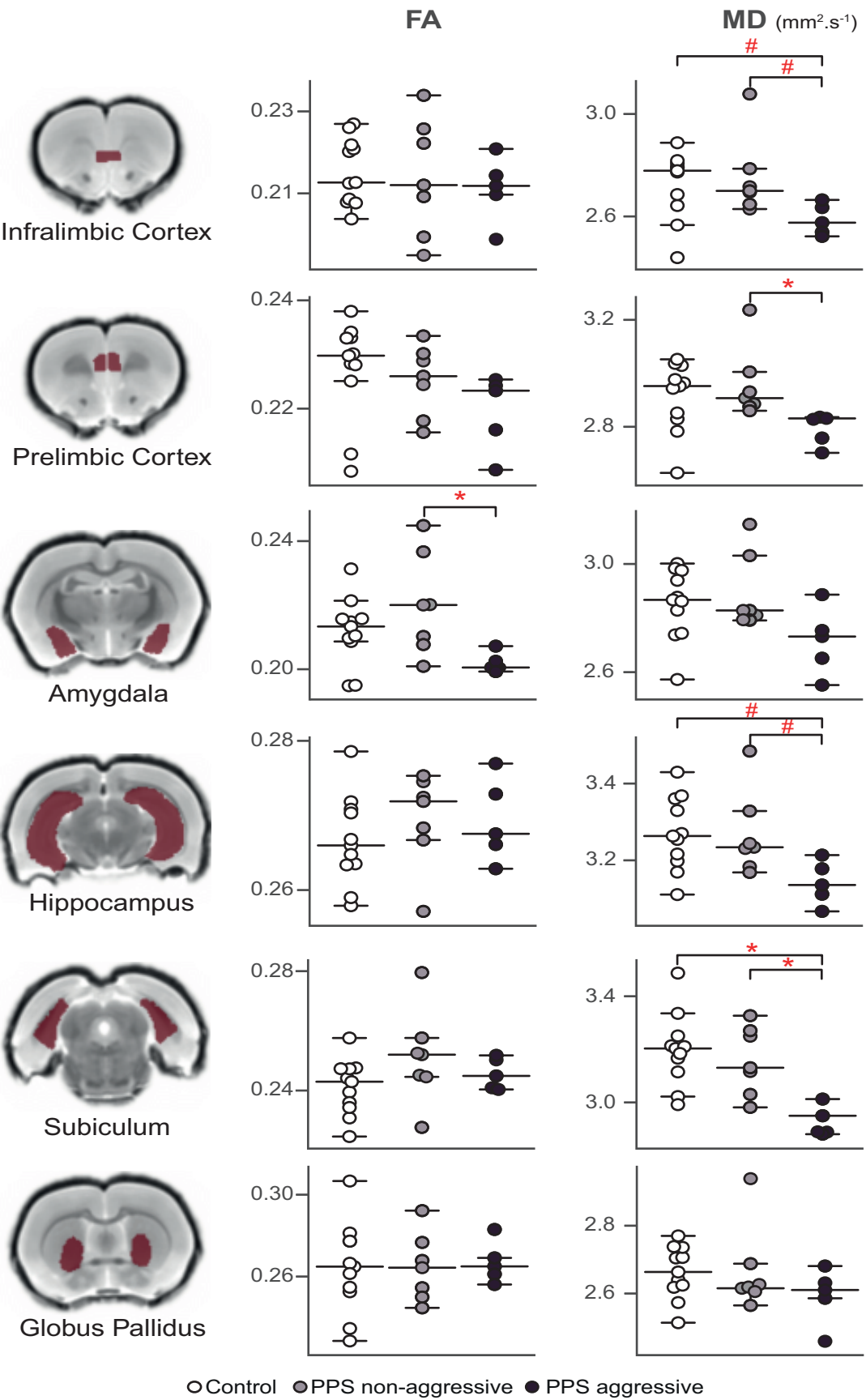
with overall aggressiveness in the resident-intruder test (B), whereas CORT response on p42 was not (C). Pearson's correlations (r) and significant p-values are shown on graphs (n: PPS non-aggressive = 7, PPS aggressive = 5).

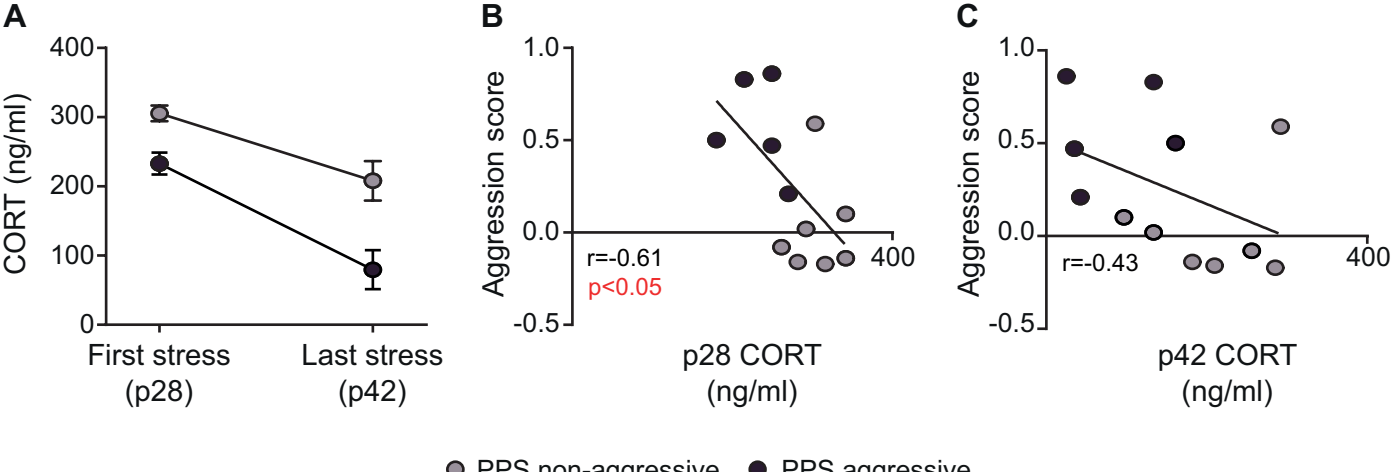
.


Peripubertal Stress
OF EP
TMT EP
EP TMT
TMT EP
EP TMT EP
Behavioral Assessment
Cohabitation with female
p21 Weaning
p28
p29
p30
p34
p36
p40
p42
p90
Novelty stress
Elevated Plus Maze
Social Preference
Resident-Intruder
Forced Swimming (p121)
p135 Perfusion


A
Offensive duration (%)
40
30
20
10
0
Control
PPS
B
Offensive frequency
250
200
150
100
50
0
Control
PPS
C
Latency to first offence (s)
250
200
150
100
50
0
*
Control
PPS
D
Abnormal bite frequency
6
4
2
0
#
Control
PPS
E
Vulnerable/normal bites
1.5
1.0
0.5
0.0
#
Control
PPS
F
Hard/soft bites
1.5
1.0
0.5
0.0
*
Control
PPS
G
Unsignaled/signaled bites
1.5
1.0
0.5
0.0
Control
PPS
H
PPS rats
Aggressive
42%
Non-aggressive
58%
I
Aggression score
All variables
2.0
1.0
0.0
-1.0
*
*
J
Aggression score
'Normal' aggression
2.0
1.0
0.0
-1.0
K
Aggression score
'Abnormal' aggression
2.0
1.0
0.0
-1.0
*
#


A
Corticosterone (ng/ml)
250
200
150
100
50
0
B
Open arms %
40
30
20
10
0
#
#
C
Social preference %
100
90
80
70
60
50
*
D
Immobility %
80
60
40
20
0
Control
PPS non-aggressive
PPS aggressive


FA
MD (mm².s⁻¹)
Infralimbic Cortex
Prelimbic Cortex
Amygdala
Hippocampus
Subiculum
Globus Pallidus
0.23
0.21
0.24
0.22
0.24
0.20
0.28
0.26
0.28
0.24
0.30
0.26
3.0
2.6
3.2
2.8
3.0
2.6
3.4
3.2
3.4
3.0
2.8
2.6
#
#
*
*
#
#
*
*
Control
PPS non-aggressive
PPS aggressive


A
CORT (ng/ml)
400
300
200
100
0
First stress
(p28)
Last stress
(p42)
B
Aggression score
1.0
0.5
0.0
-0.5
r=-0.61
p<0.05
400
p28 CORT
(ng/ml)
C
Aggression score
1.0
0.5
0.0
-0.5
r=-0.43
400
p42 CORT
(ng/ml)
PPS non-aggressive
PPS aggressive

Supplemental information

Results

Breakdown of the aggressive phenotype of peripubertal stress exposed rats

Breaking down the cumulative *z* score into individual variables, group differences in total percentage of time engaged in offensive behavior (Fig. 2A: U=49, p=0.295) and frequency of offensive behaviors (Fig. 2B: U=59.5, p=0.689) were not evident. However, peripubertally stressed rats engaged in offensive behavior more readily (Fig 2C: U=32, p=0.036), and the biting attacks they performed were of greater intensity (Fig 2G: U=16, p=0.013). Peripubertally stressed rats also displayed non-significant trends toward higher proportion of biting attacks targeted to vulnerable body parts of the opponent (Fig 2F: U=24, p=0.052), as well as higher frequency of attacks characterized as abnormal in terms of targeting or signaling (Fig 2E: U=28, p=0.097). Biting attacks were equally well signaled to the opponent (Fig 2H: U=43, p=0.634).

Cutoffs used to determine 'aggressiveness'

Cutoffs related to the 20^{th} or 80^{th} percentile (depending on index) of the control group's scores were used to determine the aggressiveness of peripubertally stressed rats in several variables. These cutoffs were, specifically: total duration of offensive behavior (>10.92%), frequency of offensive behaviors (>130), latency to first offence (<78.8s), frequency of abnormal bites (>1), proportion of all biting attacks that were unsignalled (>0.17), proportion of all biting attacks targeted toward vulnerable parts (>0.11), and proportion of all biting attacks that were 'hard' (>0.5).

Behavioral results

Table 1 - Behavior

	Comparison					
	Control vs. Non-aggressive		**Control vs. Aggressive**		**Non-aggressive vs. Aggressive**	
Parameter	U	p-value	U	p-value	U	p-value
Cort response to novelty	24	0.633	22	1.000	16	1.000
EPM OA%	13	0.060	27	1.000	30	0.096
EPM distance	53	0.633	30	0.827	11	0.686
SP ratio	5	0.003	17	0.534	12	0.534
SP juvenile %	16	0.132	27	1.000	29	0.146
SP object %	69	0.012	41	0.290	19	0.876
FST floating %	35	1.000	25	1.000	13	1.000
RI z-score all variables	44	0.072	39	0.027	32	0.072
RI z-score 'normal' aggression	38	0.843	27	0.843	22	0.843
RI z-score 'abnormal' aggression	42	0.121	39	0.009	30	0.096

Cort = Corticosterone; EPM = Elevated plus maze; FST = Forced swimming test; OA = Open arm; RI = Resident intruder; SP = Social preference; U = Mann-Whitney statistic.
Note: p-values shown are Holm-Bonferroni corrected.

MRI results

Table 2 - Volume

	Comparison					
	Control vs. Non-aggressive		**Control vs. Aggressive**		**Non-aggressive vs. Aggressive**	
ROI	U	p-value	U	p-value	U	p-value
ILCx	18	0.210	23.5	0.691	27	0.288
PLCx	35	1.000	25.5	1.000	21	1.000
Hippocampus	24	0.614	28	1.000	25	0.614
Amygdala	31	1.000	34	1.000	23	1.000
Subiculum	32	0.587	44	0.140	31	0.104
GP	35	1.000	30	1.000	22	1.000

GP = Globus pallidus; ILCx = Infralimbic cortex; PLCx = Prelimbic cortex; ROI = Region of interest; U = Mann-Whitney statistic.
Note: p-values shown are Holm-Bonferroni corrected.

Table 3 - Fractional anisotropy

	Comparison					
	Control vs. Non-aggressive		**Control vs. Aggressive**		**Non-aggressive vs. Aggressive**	
ROI	U	p-value	U	p-value	U	p-value
ILCx	40	1.000	33	1.000	20	1.000
PLCx	47	0.469	45	0.162	27	0.288
Hippocampus	24	0.614	20	0.856	19	0.871
Amygdala	29	0.415	45	0.108	33	0.045
Subiculum	20	0.309	18	0.511	25	0.511
GP	41	1.000	24	1.000	14	1.000

GP = Globus pallidus; ILCx = Infralimbic cortex; PLCx = Prelimbic cortex; ROI = Region of interest; U = Mann-Whitney statistic.
Note: p-values shown are Holm-Bonferroni corrected.

Table 4 - Mean diffusivity

	Comparison					
	Control vs. Non-aggressive		**Control vs. Aggressive**		**Non-aggressive vs. Aggressive**	
ROI	U	p-value	U	p-value	U	p-value
ILCx	43	0.717	46	0.083	32	0.069
PLCx	38	1.000	44	0.140	35	0.017
Hippocampus	44	0.651	48	0.069	32	0.069
Amygdala	40	0.928	43	0.179	30	0.154
Subiculum	43	0.717	54	0.001	34	0.019
GP	49	0.730	41	0.422	22	0.730

GP = Globus pallidus; ILCx = Infralimbic cortex; PLCx = Prelimbic cortex; ROI = Region of interest; U = Mann-Whitney statistic.
Note: p-values shown are Holm-Bonferroni corrected.