

Genetic vulnerability, timing of short-term stress and mood regulation: A rodent diffusion tensor imaging study



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Abstract

Early stressful life events predict depression and anxiety in carriers of specific polymorphisms and alter brain responses but brain structural phenotypes are largely unknown. We studied the interaction between short-term stress during specific time-windows and emotion-regulation using a genetic animal model of depression, the Wistar-Kyoto (WKY) rat. Brain structural alterations were analyzed using Diffusion Tensor Imaging (DTI). WKY ($n=49$) and Wistar ($n=55$) rats were divided into experimental groups: Early stress (ES): From postnatal day (PND) 27 rats were exposed to three consecutive days of stressors; Late stress (LS): From PND 44 rats were exposed to the same protocol; Control: No stressors. From PND 50, all animals were behaviorally tested for levels of anxiety and despair-like behaviors and then scanned. Gene \times Environment \times Timing ($G \times E \times T$) interactions ($p=0.00022$ after Hochberg correction) were found in ventral orbital cortex, cingulate cortex, external capsule, amygdala and dentate gyrus and in the emotion regulation measures. WKY showed longer immobility in forced swim test, but no effect of ES was detected. ES increased open-field anxiety-like behaviors in Wistar rats but not in WKY, possibly indicating a ceiling effect in WKY. Stress in pre-pubertal or adolescent phases in development may influence structural integrity of specific brain regions and emotion regulation behaviors depending on genetic vulnerability, consistent with a $G \times E \times T$ interaction in mood dysregulation.

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1. Introduction

Vulnerability to depression and anxiety is partly genetic and partly non-genetic due to familial and environmental risk factors (Kendler and Prescott, 1999; Kessler, 1997; Lupien et al., 2009; Nestler et al., 2002; Pine et al., 2002; Zalsman et al., 2006b). Stress-related neuroplastic changes in the brain of depressed patients have been reported (Nestler et al., 2002). Mood disorders result from genes that increase one's predisposition, gene-gene interactions, environmental risk factors (including early and current stressors) and gene-environment interactions (Costello et al., 2002; Mill and Petronis, 2007). Early stress (ES) produces alterations in brain structure and function that mediate the environment's effect on mood regulation and response to stress in adulthood. Brain vulnerability to ES is thought to decline with age of exposure, but little attention has been devoted to the relationship between age of ES and its impact on mood regulation and brain integrity (Baker et al., 2013). Mood dysregulation is a trait considered to be an early manifestation of mood disorders (Deveney et al., 2013; Posner et al., 2013; Ryan, 2013) and DSM 5 (APA, 2013) recently proposed a specific diagnosis, Disruptive Mood Dysregulation Disorder (DMDD), that may lead to a mood disorder later in life (Leibenluft, 2014).

The most studied candidate gene polymorphism in depression as an example of gene \times environment interaction is the *5HTTLPR* in the promotor of the serotonin transporter gene. Children, adolescents and young adult carriers of the short (S)-allele are reported to be vulnerable to major depression when exposed to adverse events during childhood (Caspi et al., 2003; Eley et al., 2004; Kaufman et al., 2004; Kendler et al., 2005; Zalsman et al., 2006a). We replicated these findings (Zalsman et al., 2006a) in terms of severity of depression. Karg et al. (2011) in a meta-analysis of 54 studies confirmed that 5-HTTLPR polymorphism moderates the relationship between childhood stress and adult depression ($p=0.00002$). When stratifying the analysis by the type of stressor studied, they found strong evidence for an association between the S allele and increased stress sensitivity to childhood maltreatment ($p=0.00007$), but it was early but not late stress that was pathogenic.

During development there are sensitive periods in which the brain is more vulnerable to environmental influences (Knudsen, 2004). Giedd et al. (1999) used magnetic resonance imaging (MRI) in humans and showed a linear change in white matter across ages of 4 to 22, and nonlinear changes in cortical gray matter, with a preadolescence increase followed by a post adolescence decrease. Those developmental brain changes are evident not only in humans, but across species ranging from rodents to nonhuman primates (Spear, 2009) (for review see Buwalda et al., 2011). Stress during sensitive early childhood and adolescent periods affects normal brain development (Jankord et al., 2011; Lupien et al., 2009; Spear, 2009; Tottenham and Sheridan, 2009). Early-life stress with increased reactivity to stress may create cognitive and mood regulation deficits, with altered stress responses, in adulthood (Lupien et al., 2009).

Timing of childhood stress may be a crucial factor in modulating the gene \times environment interaction. The present study hypothesizes (following Zalsman, 2010), that a

study of gene \times environment interaction must consider the timing of childhood stressors. We hypothesized that only when an individual with a specific genotype is exposed to a specific environmental risk, during a critical period of brain development, will depressive and anxiety symptoms emerge in adulthood.

In order to test this hypothesis we used Diffusion Tensor Imaging (DTI) analysis, which provides specific information on the architecture/microstructure of the tissue, based on measuring water molecules' motion or diffusion along multiple directions. We extracted two parameters: apparent diffusion coefficient (ADC), which measures the mean diffusivity, and fractional anisotropy (FA), which measures the directional preference of diffusion. Higher ADC levels refer to greater free diffusion, suggesting sparse cells or axons; higher FA levels suggest that the tissue is more organized (Assaf, 2008). DTI studies of mood disorders report lower anisotropy in the frontal and temporal lobes (Sexton et al., 2009).

Homologous brain structures are implicated in mood regulation for both humans and animals (Berridge and Waterhouse, 2003), allowing animal models to be used in depression research (Nestler and Hyman, 2010; Willner and Mitchell, 2002). The Wistar Kyoto (WKY) rat, a breed from the Wistar line, is stress-hyper-reactive, and is considered a "genetic animal model of depression", with anxiety-like behaviors (Malkesman and Weller, 2009; Pare, 1989a, 1989b, 1994a, 1994b). WKY rats demonstrate "behavioral despair" and "anhedonia" on several behavioral tests (Pare, 1989a, 1989b, 1994a, 1994b), two central criteria for depression diagnosis (Malkesman et al., 2006; Malkesman and Weller, 2009), as well as depression-like physiological and behavioral symptoms such as reduced body weight, disturbed REM sleep, lower levels of social behavior and social play than controls (Malkesman et al., 2006; Pare, 2000), and abnormalities in some central (monoaminergic) and peripheral (HPA axis) neurochemical systems (Jiao et al., 2003; O'Mahony et al., 2011; Scholl et al., 2010). We studied the interaction between genotype and short-term stress during specific time-windows on emotion-regulation and activity measures, using WKY and Wistar (control strain) rats. We then analyzed the changes in brain structure following early and late stress using DTI.

2. Experimental procedures

Rats from the "normal" control strain Wistar, and from the derived "depressive/anxious-like" line, WKY, were purchased from Harlan Labs Inc. and bred at the university's specific pathogen free animal housing facility. The facility meets NIH regulations; animal care is in accordance with the guidelines of the Society for Neuroscience and the American Psychological Association. Experiments meet National and European Animal Care regulations and were approved by the University's Animal Care and Use Committee. Rats were housed in a temperature controlled vivarium at 20-23 °C, under 12-h light-dark cycle (lights on at 0700). Food and water were available ad libitum. Housing was in Techniplast 42.5 \times 26.6 \times 18.5 cm cages with a shelter made from PVC piping. Litter size was culled on postnatal day (PND) 1-2 to 10+2, with approximately balanced sex-distribution. Pups were weaned on PND 22. For this study only males were selected. After weaning, animals were marked on the base of the tail with a color code using non-toxic markers in order to identify each individual. The cages were changed weekly;

animals were weighed, marked and handled in order to reduce handling stress on the testing days.

2.1. Experimental groups

1. Early stress (ES): On PND 27 ± 1 to 29 ± 1 rats were exposed to 3 consecutive days of stressors. Day one included elevated platform, day two restraint stress and day three wet cage stress.
2. Late stress (LS): On PND 44 ± 1 to 46 ± 1 rats were exposed to the same stress protocol as the ES group.
3. Control (C): Were left undisturbed in their home cages except for once a week cage change and handling. See Table 1.

2.2. Procedure

After weaning, male rats were housed in groups of 2 or 3 animals from the same strain and experimental condition. Care was taken to assure 1-2 siblings per sex per test in each group, to minimize "litter effects". The subjects from both strains were assigned to the above-mentioned conditions: C, ES & LS ($n=12-18$ rats per group per strain). When reaching late adolescence (PND 50-55), all animals were behaviorally tested in order to evaluate levels of anxiety- and "despair"-like behaviors (see Figure 1 and details below). On testing days, animals were removed from their home cages, weighed and administered a test, and then returned to their home cage. On day one animals were tested in the open field test (OF). On the second testing day all animals underwent a 24 h overnight saccharin preference test. Animals completed the test in the morning of the third test day, were returned to their home

Table 1 Number of subjects that participated in behavioral tests (number of subjects scanned) from each stress exposure group for both WKY and Wistar strains.

	Control	Early stress	Late stress	Total
Wistar	18 (9)	18 (6)	19 (7)	55 (22)
WKY	16 (6)	18 (6)	15 (7)	49 (19)
Total scans	15	12	14	41
Grand total				104 (41)

Footnotes: WKY=Wistar Kyoto depressed-like rat. In parenthesis: number of rats that underwent MRI scan.

cages and were left undisturbed for the remainder of the day. On the fourth and fifth test days animals were tested on the forced swim test (FST). On day six (PND 56 ± 1) rats were left undisturbed to rest in their home cages and on the following day (57 ± 1) half the animals from each group ($n=6-9$ rats per group) were transported to another facility for the DTI procedure. After a day for accommodation, the rats were anesthetized with 1-2% isoflurane and oxygen, and were maintained at 37°C . Breathing was monitored with a breathing sensor and their brains were scanned by MRI.

For timeline of experimental procedure see Figure 1.

2.3. Stress exposure protocol

The stress protocol used in the current research included three different unpredictable stressors applied over 3 consecutive days, a schedule reported to generate long-lasting alterations in rats' behavior, extending into adulthood (Tsory and Richter-Levin, 2006).

2.3.1. First stressor—Elevated platform

The elevated platform is a known stressor, shown to generate significant physiological stress responses (Degroot et al., 2004). The procedure was adopted from a protocol introduced by Richter-Levin (Maroun and Richter-Levin, 2003; Tsory and Richter-Levin, 2006) and included three 30 min trials, separated by 60 min inter-trial interval (ITI) in the animals' home cage. During trials, the rat was placed on an elevated platform (70 cm height of 12×12 cm) located in the middle of a small room with bright illumination. The animal was left alone in the room and its behavior was monitored by the experimenter from the outside from a window. In case the animal fell or jumped from the platform, the experimenter picked it up and put it back on the platform immediately. The platform was wiped clean between subjects using 70% ethanol.

2.3.2. Second stressor—Restraint

Restraint sessions are very commonly used as stressors and were shown to generate significant physiological stress responses as well as alterations in behavior (for review see Buynitsky and Mostofsky, 2009). In the current research, animals were restrained once, for duration of 120 min using a restrainer made from PVC piping fitting rats' size (Bhat et al., 2007; Schroeder et al., 2013).

2.3.3. Third stressor—Wet cage

The wet cage stressor was adopted from chronic mild stress (CMS) protocols and involved soaking the home cage bedding with water

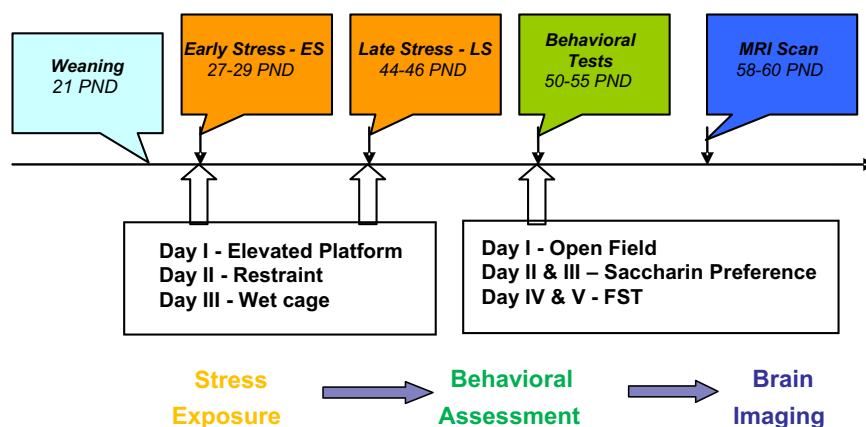


Figure 1 Timeline of the experimental procedure.

and leaving it wet over-night (Azpiroz et al., 1999; Duncko et al., 2001). Though considered mild, this type of stressor, when combined with other stressogenic experiences, can enhance the behavioral and physiological consequences of stressful experiences.

The use of three different stressors prevents habituation to the stressor and therefore generates stronger effects compared to one type of stressor applied repeatedly (Prieto et al., 2003; Simpkins and Devine, 2003). Moreover, it may model a series of adverse life events occurring unpredictably and beyond the individual's control. The relatively short duration of the procedure allowed us to examine and compare the influence of adverse environment at specific points throughout development (such as juvenile vs. adolescence periods Tsoorj and Richter-Levin, 2006).

2.4. Behavioral tests

The behavioral tests included the open field (OF), and the saccharin and forced swim (FST) tests.

2.4.1. Open field test (OF)

The arena was built from black plastic polymer (62 × 62 cm) enclosed by walls (30 cm high) (Schroeder and Weller, 2010). The rat was placed in the center of the arena and allowed to explore the surrounding area freely for 5 min. Behavior was recorded with a web camera (Microsoft Life-cam VX 1000). Digital recordings of the animals' behaviors were analyzed using Noldus Information Technology's "Ethovision" system. Measures included: (Degroot et al., 2004) total distance traversed (Maroun and Richter-Levin, 2003) mean velocity (Tsoorj and Richter-Levin, 2006) time spent in the center square; and other measures coded manually by the experimenter while the Noldus was running: (Buynitsky and Mostofsky, 2009) number of rearing (Schroeder et al., 2013) amount of grooming (Bhat et al., 2007) number of visits to the center square.

2.4.2. Saccharin preference test

A day after the open field test, rats' preference for saccharin over water was tested (as previously performed in our lab on WKY and Wistar rats Malkesman et al., 2005). Animals were tested individually after 1 h of habituation to a cage similar to their home cage. The animals were not previously deprived of food or water but had no access to water during the habituation period. Each animal was presented with a bottle (200 ml) of saccharin 0.04% (for both strains, based on pre-tests performed prior to the experiment) and a bottle of drinking water (200 ml). The animals had the opportunity to drink as much of the solutions as they wanted for 24 h. The bottles were weighed before the experiment and then again after 24 h. Total consumption of water and saccharin in grams were measured and used to calculate the preference ratio as follows: (consumed saccharin × 100)/(consumed water + consumed saccharin). Saccharin (2,3-dihydro-3-oxonenzisulfonazole from Sigma) was dissolved in tap water. Food was available throughout the test.

2.4.3. Forced swim test (FST)

A day after the saccharin test, rats' behavior and floating were tested. Each rat was immersed alone for 5 min in a Plexiglas cylinder, 45.5 cm tall, 19 cm diameter, filled to 30 cm with 24 ± 0.5 °C water. The time the rat spent immobile during the 5-min test period was measured. The criterion for immobility was making only the minimal movements necessary to keep the head above water (Malkesman et al., 2006). The water was changed between test animals. After the test, rats were dried off with a towel and put back in their home cages. All tests took place during the light phase of the light-dark cycle, but were conducted in a dark

room. A day later this test was repeated. Figure 1 summarizes all the manipulation of exposure and tests.

2.5. Imaging

MRI was performed with a 7 T MRI scanner (Bruker, Karlsruhe, Germany) with a 30-cm bore and a gradient strength of up to 400 mT/m. The MRI protocol included diffusion tensor imaging (DTI) acquisition with a diffusion-weighted (DW) spin-echo echo-planar-imaging (EPI) pulse sequence having the following parameters: TR/TE = 8000/23 ms, 4 EPI segments, $\Delta/\delta = 10/4.5$ ms, 15 non-collinear gradient directions with a single b value shell at 1000 s/mm² and one image with b value of 0 s/mm² (referred to as b_0). Geometrical parameters were: 17 slices of 0.8 mm thickness (brain volume) and in-plane resolution of 0.156 × 0.156 mm² (matrix size of 128 × 128 and FOV of 20 mm²). The imaging protocol was repeated three times for signal averaging. Each DTI acquisition took 4.5 min and the entire MRI protocol lasted about 20 min.

2.6. MRI image analysis

Image analysis included DTI analysis of the DW-EPI images to produce the fractional anisotropy (FA) and apparent diffusion coefficient (ADC). The DTI analysis was implemented in Matlab (©Mathworks, USA) using in-house software. In order to compensate for motion artifacts, each image (for each slice and each gradient direction) was automatically screened, prior to DTI analysis, for motion artifacts. The signal profile along the phase-encoding direction was measured, and if the signal found outside the borders of the brain was significant the slice was omitted from the analysis. To compensate for this, DTI acquisition was repeated three times. The remaining images were corrected for linear (motion) and non-linear (eddy currents/susceptibility) artifacts using SPM2 (Wellcome Trust Centre for Neuroimaging, London, UK). Image analysis included DTI analysis of the DW-EPI images to produce the Fractional Anisotropy (FA) and Apparent Diffusion Coefficient (ADC) indexed maps. The DTI analysis was implemented in Matlab, using in-house software.

2.7. Statistical analysis

For statistical comparisons between rats, each rat brain volume was normalized with a template rat atlas allowing voxel-based statistics. All image transformations and statistical analyses described below were calculated using SPM (version 2, UCL, London, UK). The rat brain template was created from a dataset of one representative rat that was registered with a digitized version of the stereotactic atlas (Paxinos and Watson, 2005) and included a registered template b_0 and FA images. Each rat data set was normalized to the template images. The normalization procedure included the following steps: (a) All b_0 zero images underwent bias correction before subsequent steps. (b) For each rat, the b_0 image was co-registered with the b_0 template. The co-registration parameters were applied on the different DTI indexed maps (FA and ADC). (c) The co-registered FA maps were normalized to the FA template. The normalization parameters were then applied on all DTI indexed maps. (d) Smoothing of the normalized indexed maps with 0.3 mm Gaussian kernel.

Results were analyzed using ANOVAs and post hoc comparisons when relevant with a significance level of 0.05 or less. All tests were two-tailed. The focus was on differences between the two stress groups and the control group. ANOVA voxel-based analysis (VBA) was performed using in-house software written in MATLAB between the groups as described previously (Blumenfeld-Katzir et al., 2011). To reduce the number of comparisons in the statistical

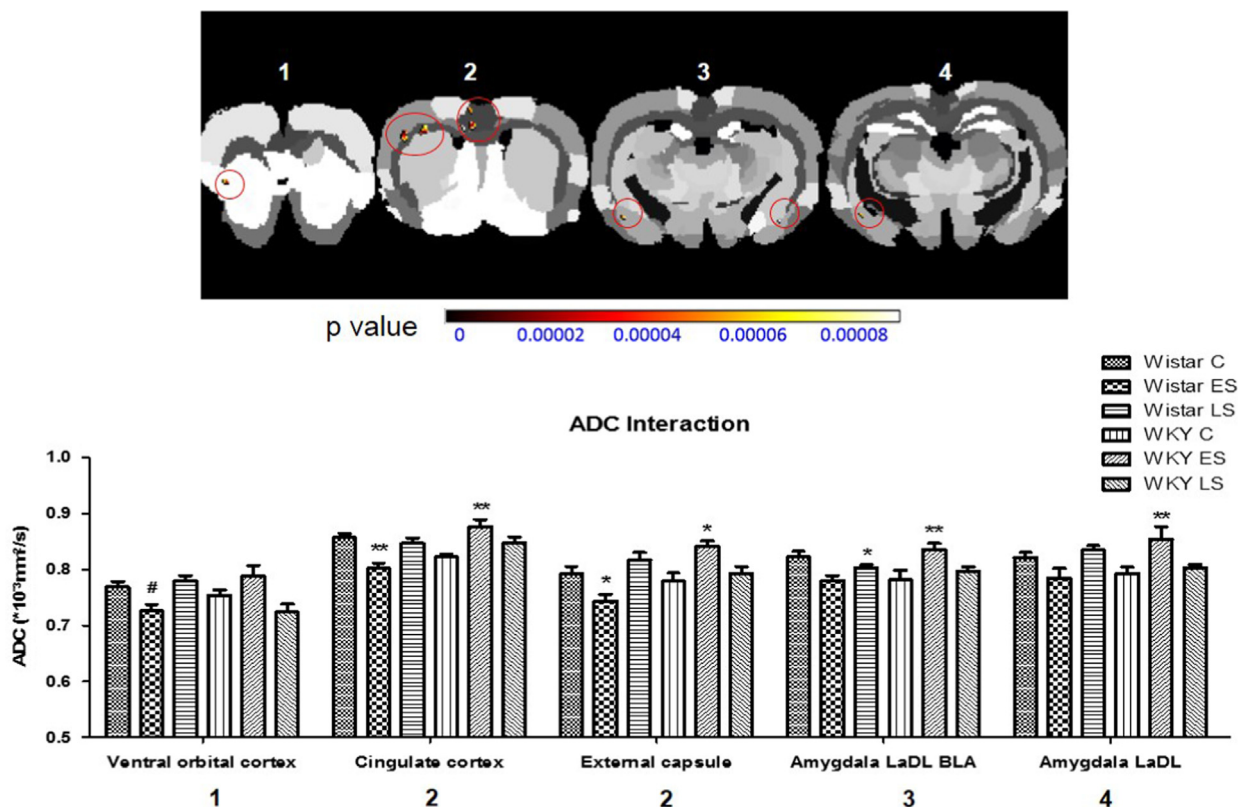


Figure 2 (A and B) Mean ADC level for Wistar (control) and WKY (depressed-like) rats exposed to early, late and no stress, in 4 brain regions. Footnote to (A and B): for each graph the corresponding region is shown on the statistical parametric map above. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to same-strain control group. Results after Hochberg correction for multiple testing. WKY=Wistar Kyoto strain, ES=Early exposure to stress, LS=Late exposure to stress.

analysis, the analysis was restricted to brain areas related to emotional processing: cingulate cortex, orbitofrontal cortex, insular cortex, hippocampus & pathways from them (CA1, CA3, entorhinal cortex, dentate gyrus (DG), dorsal hippocampal commissure (DHC), fimbria, cingulum), amygdala, external capsule (EC), anterior commissure, and corpus callosum (CC). A cluster size threshold of 5 voxels was used.

Hochberg correction for multiple comparisons was performed on the interaction and main effects maps (Hochberg, 1988; Hochberg and Benjamini, 1990). Significant clusters are presented superimposed on a rat atlas. The ADC and FA values from each brain were extracted in the clusters passing the statistical threshold and their averages are represented in graphs.

The imaging data analysis was performed by BiImage—Professional Imaging Services (Haifa, Israel).

3. Results

3.1. MRI results

3.1.1. Apparent diffusion coefficient (ADC) Interactions
A voxel based ANOVA (VBA) revealed a significant strain \times condition interaction in the following regions: ventral orbital cortex (VO), cingulate cortex (Cg1 and Cg2), external capsule and the amygdala (lateral amygdaloid nucleus, dorsolateral part—LaDL; baso-lateral-amygdaloid nucleus, anterior part—BLA). The ADC values are presented

in Figure 2A; the significant clusters with interaction of the ADC parameter are presented on a brain atlas (Figure 2B). In total, 52 voxels were significant after correction for multiple comparisons, the threshold p -value is: 0.00095.

The significant interaction came from different patterns of influence of ES between strains: While in WKY rats, ES produced significantly higher ADC values compared to control in amygdala, cingulate cortex and external capsule (tests for simple main effects with Bonferroni adjustment; $p < 0.01$ for the amygdala and cingulate cortex, $p < 0.05$ for external capsule), Wistar rats showed the opposite pattern ($p < 0.01$ for the cingulate cortex, $p < 0.05$ the amygdala (only BLA) and external capsule). The significant interaction for VO came from a different pattern: a difference between ES and LS groups (not compared to the control group) among strains ($p < 0.05$). No significant differences were found in the LS group compared to the control group in both strains.

3.1.2. ADC main effect—Stress

VBA revealed a significant ADC parameter main effect for stress only in the fimbria (data not shown). Five voxels survived correction for multiple comparisons, the threshold p -value is: 0.0083. Duncan's post-hoc test further clarified that only the LS group had higher ADC values compared to the control group ($p < 0.05$).

3.1.3. FA interaction

Voxel based ANOVA revealed a significant $G \times E \times T$ interaction in the external capsule and the dentate gyrus. 31 voxels survived correction for multiple comparisons, the threshold p -value is: 0.0015.

The FA values are presented in Figure 3A; the significant clusters with interaction of the FA parameter are presented on a brain atlas (Figure 3B).

A test of simple main effects with Bonferroni adjustment further clarifies that the significant effect in the external capsule was in the Wistar strain where the ES and LS groups had lower FA values compared to the control group ($p < 0.001$ for ES; $p < 0.01$ for LS). In the dentate gyrus the significant effect was in the WKY strain: the ES group had higher FA values compared to control group ($p < 0.01$).

3.1.4. FA main effect—Stress

VBA revealed a significant effect for stress in the anterior commissure, external capsule and corpus callosum (data not shown). Twenty voxels survived correction for multiple comparisons, the threshold p -value is: 0.0024. The FA values in the anterior commissure (anterior part) and in the external capsule were significantly lower in ES and LS groups compared to control groups (Duncan's post-hoc test, $p < 0.05$ for both regions).

In the corpus callosum, ES groups had significantly higher FA values than control groups (Duncan post-hoc, $p < 0.05$).

3.2. Behavioral results

In the open field, a multivariate ANOVA on distance traversed, number of rearing events and number of visits to the center square revealed a $G \times E \times T$ interaction ($F(6, 130) = 3.20$, $p < 0.01$), as well as gene ($p < 0.001$) and manipulation ($E \times T$, $p < 0.05$) effects. Breakdown by individual measures (Figure 4a-c) showed that in the Wistar strain, the ES (but not the LS) group entered the center fewer times, traveled a shorter distance over the test and performed less rearing behavior (tests for simple effects with Bonferroni adjustment, $p < 0.05$). In contrast, no $E \times T$ effects were found in the WKY rats. The pattern of effects on the velocity measure was similar to that of the distance measure, and no interaction effect was found in grooming and time in the center (not shown).

On test day 1 of the FST, WKY rats floated longer than Wistar rats ($F(1, 93) = 12.97$, $p < 0.001$), as shown in Figure 4d. $E \times T$ and gene $\times E \times T$ interactions showed only trends ($F(1, 93) = 2.883$, $p < 0.07$ and $F(2, 93) = 2.425$, $p < 0.1$, respectively). LS Wistar rats floated for less time than their matched controls (test for simple main effects with Bonferroni adjustment, $p < 0.05$). The pattern of results on test day 2 was similar (not shown).

As in previous studies, WKY rats showed less saccharin preference than Wistar controls. No $G \times E \times T$ interaction was found (not shown).

4. Discussion

We found that stressful exposures in pre-pubertal or adolescent phases in development may influence emotional regulation and the structural integrity of relevant brain

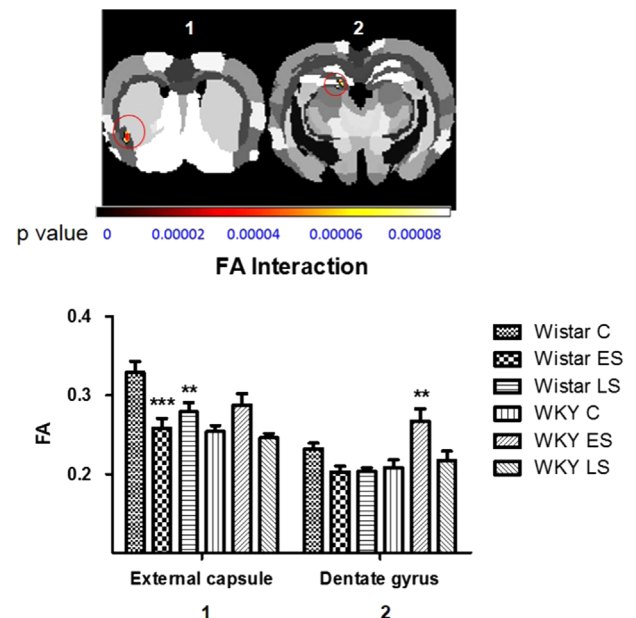


Figure 3 FA interactions. Footnote to this figure: for each graph the corresponding region is shown on the statistical parametric map above. ** $p < 0.01$; *** $p < 0.001$ compared to same-strain control group. Results after Hochberg correction for multiple testing. WKY=Wistar Kyoto strain, ES=Early exposure to stress, LS=Late exposure to stress.

regions: ventral orbital cortex, cingulate cortex, external capsule, amygdala and dentate gyrus. The effect of stress was different in the WKY compared to Wistar rats suggesting that genetic vulnerability to depression and anxiety results in a different effect on brain structural integrity and behavior. These findings indicate a possible $G \times E \times T$ interaction in mood dysregulation during development. The behavioral measures of emotion regulation validated the WKY as a model of depression and anxiety and lend validity to the structural findings.

We detected a $G \times E \times T$ interaction in the above mentioned brain areas regarding the impact of stress on brain structure, as can be seen in the ADC results. In the genetically depressive-like WKY rats, ES produced higher ADC indicating distorted tissue (possibly reflecting fewer cells or axons) while LS did not. In the control Wistar rats the pattern was the opposite: ES presented lower ADC (probably reflecting greater cell density or cell swelling). Lower ADC reflects higher cellular density, and higher ADC is associated with lower tissue density as shown previously in in-vitro and in-vivo studies in various pathological conditions (Knight et al., 1994; Latour et al., 1994; Le Bihan, 1995; Le Bihan et al., 1992; Lyng et al., 2000). We now discuss the significance of the findings in each brain area:

4.1. Amygdala

In WKY rats, ES produced significantly higher ADC values than controls in amygdala, while in Wistar rats we found the opposite pattern.

The amygdala is well-known for its role in emotional processing (LeDoux, 2000). Depressed patients are reported to

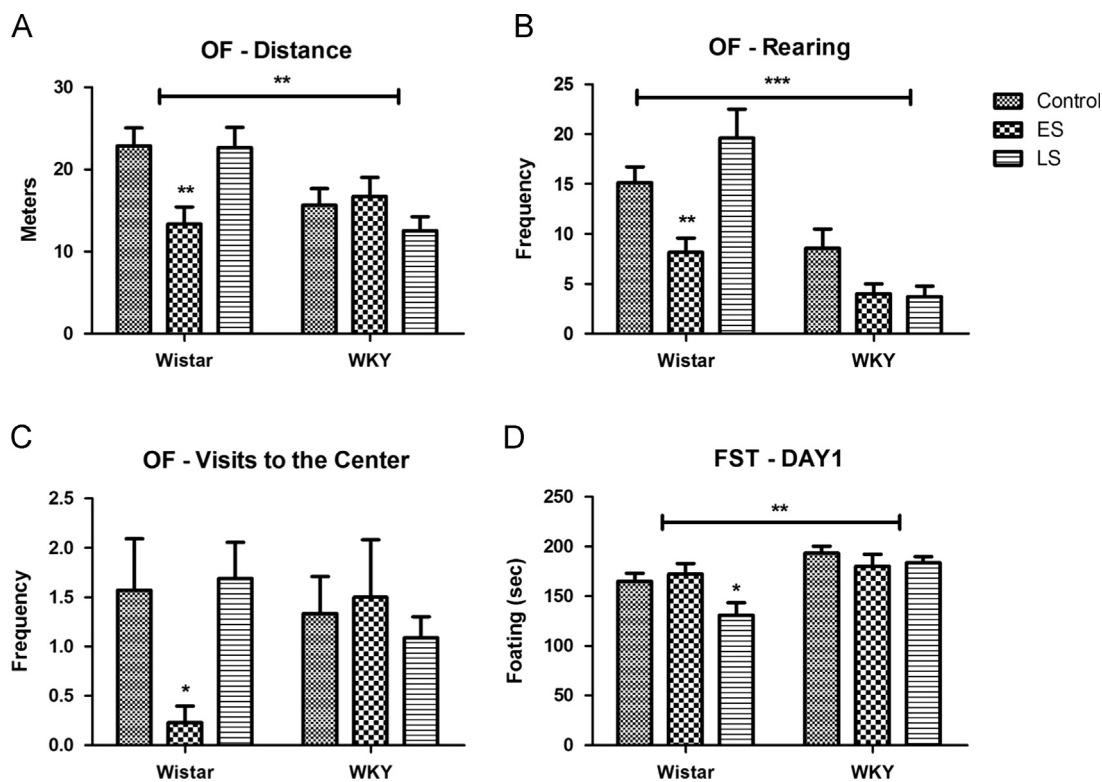


Figure 4 Behavioral tests Footnote to this figure: means+S.E.M of distance traversed, number of rearings, and number of entrances into the center of the Open Field (a-c); and of duration of floating in the Forced Swim Test (d), for rats of both strains in the control, ES and LS conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

have over-responsiveness to fearful stimuli, that may result in excessive and persistent negative mood, and amygdala volume has been reported to be larger, smaller or not different from controls (Andersen and Teicher, 2008; Frodl et al., 2002; Hulvershorn et al., 2011; Price and Drevets, 2010). The amygdala can be viewed as part of a larger circuit (the extended amygdala), which also includes the nucleus accumbens (NAc), striatum, bed nucleus of stria terminalis and other brain regions, and plays a critical role in emotion memory, thus mediating the anhedonia, anxiety and reduced motivation seen in depression (Nestler et al., 2002).

The two specific amygdala areas manifesting a $G \times E \times T$ interaction were within the lateral amygdala, and area known to accommodate synaptic plasticity associated with fear-learning (Johansen et al., 2011; Kim et al., 2014).

4.2. Cingulate cortex

In cingulate cortex, ES increased ADC in WKY and decreased this measure in Wistar rats. This was detected in a relatively anterior part of the rat's cingulate cortex, an area that has been implicated in major depression on the basis of neuroimaging studies in humans and mood regulation in lesion studies of animals (Drevets et al., 2008b; Franklin et al., 2015).

4.3. Hippocampus

In dentate gyrus WKY exposed to ES had higher FA (indicating greater directionality of the tissue). The hippocampus as

part of the limbic system, participates in emotion regulation (LeDoux, 2000; Drevets et al., 2008a) and some studies have reported a smaller hippocampal size in major depression (Bremner et al., 2000; Tottenham and Sheridan, 2009). The current pattern of findings suggests that structural changes in the dentate gyrus emerged uniquely in the "stress-sensitive" WKY rats that experienced the ES.

4.4. External capsule

The same ADC interaction seen in the amygdala and cingulate cortex was evident; exposure to childhood stress increased ADC levels within WKY rats, while decreased it within Wistar rats. An interaction effect was also found in the FA parameter; both early and late stress procedures decreased FA levels among Wistar rats, while FA was left unchanged among WKY. The cortico-cortical association fibers of the external capsule connect one cortex of the brain to another and this is a route for cholinergic fibers from the basal forebrain to the cerebral cortex (Bessette et al., 2014; Duffy et al., 2014; McDonald et al., 2012). Several studies have found decreased FA in the external capsule among depressed patients (e.g. Bessette et al., 2014; Guo et al., 2012; Xiao et al., 2015), while others reported an increase (Sacchet et al., 2014; Zhou et al., 2013). FA differences indicate decrease in the directionality of the tissue among Wistar rats, suggesting that stress during both childhood and adolescence alters external capsule connectivity when genetic background for depression is absent.

4.5. Anterior commissure and corpus callosum

In the anterior commissure, stress was found to affect diffusion directionality across strains; both childhood and adolescence stress decreased FA levels, regardless of genetic background to depression. The anterior commissure is a bundle of white matter fibers connecting right and left temporal lobes between hemispheres. Therefore, it plays a role in the interhemispheric transfer of visual, auditory and olfactory information (Wilde et al., 2006). However, connections have also been reported between the anterior commissure and the medial frontal cortex and amygdala (Condes-Lara et al., 2003; Martinez-Lorenzana et al., 2004), thus it is part of the emotional circuitry of the brain. Prenatal stress in rats, considered a model of depression, has been shown to change the size of the rostral anterior commissure (Jones et al., 1997).

In corpus callosum, ES produced significantly higher FA values than in non-stressed controls (Bessette et al., 2014; Duffy et al., 2014; McDonald et al., 2012). The corpus callosum is a major white matter tract that connects right and left homologous cerebral areas between hemispheres. Our results suggest that the early stress experience increases inter-hemispheric connections.

4.6. Human MRI findings in early depression

Depressed children and adolescents show functional and anatomical abnormalities in PFC sub regions, such as orbitofrontal cortex (OFC), dorsolateral PFC (DLPFC) and ACC (Hulvershorn et al., 2011; Price and Drevets, 2010). One of the primary roles of the PFC is to modulate activity of the limbic system, thus it serves as an emotion regulator (Drevets, 2001; Miller and Cohen, 2001; Price and Drevets, 2010). The development of emotion regulation is also a cognitive process, which includes regulation of attention, inhibitory control and executive functions (Fox et al., 2001; Kopp, 2009; Posner et al., 2013). Childhood stress shrinks the hippocampus, whereas stress during adolescence resulted in reduced PFC volume. Childhood stress predisposes to depression over a long incubation period potentially by altering hippocampal development, while exposure to stress during adolescence might precipitate depression over a shorter incubation period by directly affecting the PFC (Andersen and Teicher, 2008; Buwalda et al., 2011; Tottenham and Sheridan, 2009).

4.7. The behavioral tests

The behavioral tests validated the strain differences as expected. WKY rats characteristically floated longer in the FST, had lower saccharin preference, and showed less activity in the OF. This is in accordance with previous studies (Malkesman et al., 2005; Malkesman and Weller, 2009). The $G \times E \times T$ interaction was evident in the OF, meaning that Wistar ES rats entered the center of the arena less and were less active in the test compared to controls, while in the WKY strain there were no $T \times E$ effects. Thus, the early, but not the late stress window increased anxiety-like behavior in the control strain. This is in general accordance with previous reports (e.g., Tsory and

Richter-Levin, 2006). The lack of effect in the WKY strain may imply a ceiling effect in this strain.

The FST indicated less despair-like behavior in the Wistar LS group compared to controls. This appears to be a resilience adjustment to LS. The same pattern was found in brain areas that showed a $G \times E \times T$ in the ADC analysis: LS is associated with a better outcome relative to controls in the Wistar rats. The finding that stress at different developmental times may be associated with a resilience or better outcome is in line with a recent model proposed by Nederhof and Schmidt (Nederhof, 2012). According to their theory, individuals are more likely to suffer from disease if a mismatch occurs between the early programming environment and the later adult environment—the mismatch hypothesis. This contrasts with the cumulative stress hypothesis, where individuals are more likely to suffer from disease as adversity accumulates. These seemingly contradicting hypotheses are integrated into a new model proposing that the cumulative stress hypothesis applies to individuals who were not or only to a small extent programmed by their early environment, while the mismatch hypothesis applies to individuals who experienced strong childhood programming effects (Nederhof and Schmidt, 2012). This theory may also explain why the WKY ES group showed higher FA in dentate gyrus compared to controls. Experiencing ES in the WKY strain may result in greater structural integrity reflected by increased myelinated tissue.

4.8. Timing of ES

Previous studies of ES in humans suggest that it is associated with alterations in brain structure. However, less attention has been devoted to the timing of the ES onset (Nestler et al., 2002). Baker et al. (2013) examined whether ES onset in older ages of youth rather than younger ages is associated with smaller limbic and basal ganglia volumes as measured by MRI. A total of 173 individuals were divided into three groups based on the age of self-reported ES. The three groups included individuals only experiencing early childhood ES (1 month–7 years), those only experiencing later childhood ELS (8–17 years), and those who have not experienced ELS. ACC, hippocampus, amygdala, insula and caudate volumes were measured using MRI. Later childhood ELS was associated with smaller ACC and insula volumes, while ELS (between the ages of 1 month and 7 years) was not associated with smaller ACC and insula. The results may reflect the influence of more fully developed emotional processing of ES on the developing brain (Baker et al., 2013).

4.9. Limitations

Generalization from an animal model to human subjects is limited. Yet the WKY model was validated in our study. While the complete phenotype of a complex disorder as depression cannot be encompassed by a single animal model, it is possible to study specific behavioral domains in relation to psychiatric disorders (Gottesman and Gould, 2003). There have been a number of reports of $G \times E$ interactions in animal models of depression (El Khoury

et al., 2006; Martin and Ceuterick, 2002; Musazzi et al., 2010). Another limitation is that the time that passed between the stress and the DTI test may result in moderation of the effects. Recent studies support similar approach in human subjects (LeWinn et al., 2014; Teicher et al., 2014).

5. Conclusion

We found $G \times E \times T$ interaction and emphasize the role of timing of the SLE exposure during development as well as genetic vulnerability, in determining the longer-term outcome. These results suggest that inconsistent findings in clinical studies of $G \times E$ interactions examining effects of childhood adversity on later onset major depression (Caspi et al., 2003; Eley et al., 2004; Kaufman et al., 2004; Kendler et al., 2005; Zalsman et al., 2006a) could be due to the timing of childhood adversity.

Notes

Biolume (no longer in service) was founded by Tamar Blumenfeld-Katzir and Efrat Sasson. Their current affiliations are: TB-K, Imaging Q, Haifa, Israel and ES, Wiselume, Hod Hasharon, Israel.

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Contributors

GZ, LS, AG & AW designed the study. AG, LS & AW wrote the protocol. LS, AG & RR performed the experiments. LS, AG, JJM & AW undertook the statistical analysis. Interpretation of the pattern of results was discussed between all authors. GZ and LS wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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