Dynamic alterations in neural communication and neurogenesis in a rat model of depression

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ABSTRACT

Despite the prevalence of major depression (MD) and the large amount of research in the field, the underlying physiological mechanisms are yet far from being well understood. Several neurological mechanisms have been hypothesized to play key roles in the etiology of MD, including monoamine deficiencies, reduced neurogenesis and elevated hypothalamic-pituitary-adrenal (HPA) axis activity. More recent approaches view depression as a system-wide disorder, and several studies attempted to characterize MD-related changes in the functional connectivity of various brain networks.

In the current study we aimed to assess the temporal dynamics of changes in the serotonergic system, in hippocampal neurogenesis and in the resting-state functional connectivity (rsFC) in an animal model of depression.

To do so, we exposed Sprague-Dawley rats to five weeks of chronic mild stress (CMS) – a well established rodent model of depression. Serotonergic activity was assessed using manganese-enhanced M.R.I (MEMRI) in which manganese was injected to the median raphe. Neurogenesis in the dentate gyrus of the hippocampus was measured using BrdU and doublecortin staining. Resting-state functional connectivity was evaluated using BOLD contrast M.R.I. In addition, behavioral tests were administered, including the sucrose preference, the social exploration, the elevated plus maze and the novelty suppressed feeding tests.

At the end of 5 weeks of CMS, reductions were evident in all parameters, including the overall effective activity of the median raphe, neurogenesis levels and the global rsFC strengths, as well as in the behavioral tests. More specifically, the amygdaloid nucleus differed significantly between CMS and control in the MEMRI results, and changes in rsFC included an increase in the relative amount of bilateral connections and a reduction in the amount of rsFCs between cortical and sub-cortical areas.

The time-course of these changes proved worthy of attention. Both proliferation levels and the results in the social exploration test were elevated in the middle of the CMS session. Furthermore, an enhanced manganese signal in the habenula – a region highly involved in the processing of negative reward – was evident after 2.5 weeks of CMS, but not after five. These results demonstrate the complex and non-linear properties of the physiological modulations that underlay the development of depression.
INTRODUCTION

Major Depression

Background: According to the World Health Organization, Major Depression (MD) is the leading cause for years lived with disability (WHO 2001). It can present with a wide spectrum of symptoms, including emotional (depressed mood, loss of interest or pleasure), cognitive (diminished concentration) and neuro-vegetative or psychomotor (disturbed sleep or appetite, psychomotor agitation or retardation etc.) (DSM-IV).

Etiology and neurophysiology: Although clinical depression is commonly viewed as having a physical-neurological basis, the underlying pathogenesis remains far from being well understood. Several physiological parameters have been suggested to underlie the development of depression, including hypofunctional monoamine neurotransmission, reduced neurogenesis, impaired hypothalamus-pituitary-adrenal (HPA) axis activity and dysregulated inflammatory responses in the brain. Evidence supporting the monoamine-deficiency hypothesis includes, among other phenomena, reduction in 5-HT$_{1A}$ receptor binding and decreased availability of 5-HT reuptake sites in patients with major depression, the ability of tryptophan (a 5-HT precursor) depletion to evoke depressive symptoms, as well as some beneficial effects various monoamine-reuptake-inhibitor antidepressants achieve (Sharp & Cowen, 2011).

In support of the relation between neurogenesis and depression the following findings stand out: a reduction in the volume of the hippocampal dentate gyrus (a major neurogenesis site in the brain) found in MD patients, a reduction in neurogenesis in animal models of depression and the ability of the disruption of antidepressant-induced neurogenesis to block the behavioral responses to antidepressants (Lucassen, Stumpel, Wang, & Aronica, 2010; Santarelli et al., 2003)

Rather than any of the above physiological parameters accounting for major depression alone, it is reasonable to assume that the pathogenesis of MD relies on the combination of multiple impaired systems (Lanni, Govoni, Lucchelli, & Boselli, 2009; Prins, Olivier, & Korte, 2011).

The first goal of this study was to examine the time course of changes in serotonergic raphe effective connectivity and in dentate-gyrus neurogenesis in a chronic mild stress rat model of depression.
Viewing MD as a system-wide disorder characterized by alterations in brain function has also been gaining increasing support, the term 'brain function' referring to changes in both localized activity and network connectivity.

Various studies focused on regionally localized brain activity in depression using PET and fMRI in human patients (Fitzgerald, Laird, Maller, & Daskalakis, 2008; Monkul et al., 2012) and histological methods in animal models (Harro, Kanarik, Matrov, & Panksepp, 2011). PET and fMRI studies in humans report abnormal activity in the cortex (predominantly the prefrontal cortex), limbic and para-limbic structures as well as in sub-cortical areas. Although disagreement between studies is found, resulting primarily from the different methodologies and paradigms employed, the change observed in some areas is typically in the direction of hypoactivity (e.g. dorso-lateral PFC and insula) while others often demonstrate a typical hyperactivity (e.g. limbic regions, thalamus and basal ganglia). In some cases, the observed abnormality was reversed by the use of antidepressants or by other treatments such as ECT (Fitzgerald et al., 2008).

Other studies adopt a broader point of view and examine the aspect of network connectivity, which entails the temporally correlated activation patterns of multiple regions. Several M.R.I studies examined what has been termed resting-state functional connectivity (rsFC, described more in detail later in the introduction) and evaluated the characteristic changes in rsFC seen in patients suffering from MD (Greicius et al., 2007), as well as the influences of various antidepressant drugs on the rsFC of healthy humans (McCabe & Mishor, 2011) and of control animals (Schwarz, Gozzi, & Bifone, 2009). It has been shown that rsFC is evident also in anesthetized animals, and this connectivity has been suggested to correspond with functional networks evoked by specific stimulations and reflect functional organization which is independent of levels of consciousness (Vincent et al., 2007).

To the best of our knowledge, M.R.I-based measurements of rsFC have not yet been tested in animal models of depression. **The second goal of this study was to assess the characteristic alterations in brain resting-state-functional-connectivity in the rat CMS model of depression and their relation to depression-like behaviors.**

To accomplish both goals, two sets of 5-week chronic-mild-stress sessions were conducted at different times. MEMRI and neurogenesis were obtained from the first set, while rsFC measurements were obtained from the second.
**Animal models and Chronic Mild Stress**

The diversity of findings regarding the pathogenesis of MD reflects both the complexity of mood disorders and the breadth of conditions that currently fit the definition of ‘depression’—which most likely encompass a variety of seemingly similar, but in fact distinct, types of depression. Put together with the limited ability to employ clinical therapeutic studies on human patients, the need for reliable animal models of depression which enable the study of homogenic cohorts and exploration of new clinical treatments for MD becomes clear.

Various risk factors for depression, mainly genetic and environmental, have been identified. Stress-related factors are the most prominent among the environmental risk factors and stressful life events have been shown to be a significant predictor of depression (Colman & Ataullahjan, 2010). Hence, the majority of animal models used for the study of depression are stress-based. The Chronic Mild Stress model (CMS) is a widely used animal model of depression and is based on introducing the animals to a variety of uncontrollable mild stressors over the course of several weeks. CMS has been shown to cause behavioral changes in rodents that presumably parallel symptoms of depression in humans (Willner, 2005).

**Manganese-Enhanced-M.R.I**

The manganese ion (Mn$^{+2}$) has long been used in biological research as an indicator of Ca$^{+2}$ influx in conjunction with fluorescent microscopy, as Mn$^{+2}$ is known to enter cells via L-type voltage gated calcium channels (Merritt, Jacob, & Hallam, 1989; Simpson, Challiss, & Nahorski, 1995). Though the processes that occur after the manganese enters the cell are not yet fully understood, current models outline three main stages: accumulation of the manganese in the endoplasmic reticulum (ER), packaging into vesicles and transportation along microtubules to the synaptic cleft where it is released and can be taken up by the next neuron in the circuit (Pautler, Mongeau, & Jacobs, 2003; Sloot & Gramsbergen, 1994).

Another property of Mn$^{+2}$ ion is that it is paramagnetic and effectively shortens the spin-lattice relaxation time constant (T1 and T2) in tissues where it accumulates, causing positive contrast enhancement in T1-weighted MRI images in those areas (Cory, Schwartzentruber, & Mock, 1987). The proportional relationship between the
water R1 and R2 relaxation rates and the concentration of Mn$^{+2}$ provides the basis for manganese-enhanced M.R imaging. Several uses of MnCl$_2$ for MEMRI have been developed, including use as a whole-brain contrast agent after systemic administration and as a surrogate marker for calcium influx into excitable cells. The ability of paramagnetic Mn$^{+2}$ to enter cells via Ca$^{+2}$ channels in conjunction with its microtubule-based, function-dependant, axonal transport features make MnCl$_2$ an attractive candidate for use as an MRI detectable in-vivo neuronal tract tracer of specific neuronal connections. It should be stressed that the accumulation of MnCl$_2$ in a region distant from the injection site provides information on the effective connectivity between the injection site and the other region, which depends on the joint activity of both regions rather than the individual activation level of any of the regions alone.

Previous studies in our lab demonstrated that a peak in MnCl$_2$ signal enhancement in regions distant from the injection site is detected 24-48 hours after injection (varying according to different animal models, site of injection and measured region).

In order to examine the neuronal propagation from the raphe nuclei in the CMS rat model of depression, MnCl$_2$ was stereotactically injected into the median raphe nucleus and its neuronal trace was measured 28 hours after injection.

**Resting-State Functional Connectivity**

In recent years it has become clear that a full understanding of brain-cognition and brain-emotion relationships cannot rely on localized structure-function relationships alone. An additional examination of the context in which regional activity is seen must be considered. The co-occurring activity, and the spatiotemporal and interactivity patterns seen throughout the brain, are essential to the understanding of the functional relevance of any local activation (McIntosh, 2000). To gain such an understanding, one must also relate to the patterns of brain 'connectivity'. The term

\[ R_{1,2}(\text{[Mn}^{2+}\text{]}) = R_{1,2} + X_{1,2}\text{[Mn}^{2+}\text{]} \]

Where R1 and R2= 1/T1 and 1/T2 respectively; [Mn$^{+2}$]= Mn$^{+2}$ concentration (mM); R1,2([Mn$^{+2}$])= mean water R1 and R2 relaxation rates (1/s) at a given [Mn$^{+2}$]; X1,2=T1 and T2 relaxivity (s$^{-1}$ mM$^{-1}$) of manganese respectively.
connectivity can refer either to structural or functional connectivity. While 'structural' connectivity relates to the physical anatomical connections between regions, the term functional connectivity (FC) refers to the relationship between the activity in two or more neural sources, and usually to the temporal correlation between the different sources (Deco, Jirsa, & McIntosh, 2011). Though functional connectivity is in many cases constrained by structural connections, it does not necessarily reflect the existence of such direct anatomical connections (Honey et al., 2009). The use of MRI for obtaining measures of FC has become prominent over the years. Functional-MRI (fMRI) typically uses sequences sensitive to changes in blood-oxygenation-level-dependent (BOLD) signal, which presumably reflect changes in neuronal activation levels. Quantification of FC between regions is obtained by examining the temporal correlation between the fluctuations of the BOLD signals in the different regions (Rogers, Morgan, Newton, & Gore, 2007).

Low frequency fluctuations in BOLD signal were suggested to result from spontaneous neural activity (Fox & Raichle, 2007; Lu et al., 2007). Measuring FC on the basis of these spontaneous fluctuations of the BOLD signal during rest (i.e. in the absence of external stimulation) has come to be known as 'resting-state functional connectivity' (rsFC). The examination of rsFC has lead to the identification of several networks which are consistently found in healthy subjects and may exhibit characteristic alterations in various mental abnormalities (Rosazza & Minati, 2011).

In order to assess the alterations in brain rsFC in the CMS rat model of depression, M.R.I scans were obtained before and after a 5-week CMS session.

**Radial Correlation Contrast (RCC):** The RCC measurement is based on the same BOLD data from which rsFC analysis is obtained. Contrary to the rsFC in which the temporal correlation between the BOLD signal fluctuations of two separate ROIs are examined, the RCC focuses on the local behavior within a given ROI. The RCC quantifies the average temporal correlation between the BOLD signal fluctuations of each voxel in the ROI and all its neighboring voxels. It is hypothesized to reflect the level of BOLD synchronization within the ROI. Under the common assumption that BOLD signals are proportional to local neural activity, this regional BOLD synchronization can conceivably be related to the general efficiency of the regions activity. It is further hypothesized that greater synchronization of neural activity
within a region optimizes the regions functional efficiency, thus reducing its energy demands. Indeed, a preliminary study in our lab demonstrated an inverse correlation between PET measures of glucose metabolism and the RCC measure in rat brains.

In the present study we made use of the BOLD data obtained for rsFC analysis to assess the effects of chronic mild stress on the local RCC measures of the ROIs under examination.

**METHODS**

**Animals:** Sprague-Dawley male rats (Harlan Laboratories, Jerusalem, 260-300g at beginning of experiment) were housed in pairs, held in a 12h light/dark schedule, and provided food and water ad libitum (when not under CMS stressor of food or water deprivation). Three manganese and two control groups were used in the first CMS set: a manganese-injected group subjected to five weeks of CMS ("Mn-full-CMS", n=8), a manganese-injected group subjected to 2.5 weeks of CMS ("Mnmid-CMS", n=6), a five-week CMS group that was not injected with manganese ("full-CMS", n=8), a control manganese group ("Mn-5-week-Control", n=6) and a control group that was not injected with manganese and was not subjected to CMS ("control", n=8). Four rats were used in each time-point for histochemical measurements. In addition, three rats were used to examine the effects of manganese diffusion through the CSF. The second CMS set comprised of two groups: a 5-week CMS group (n=10) and a control group (n=8). Four pups were used in each session of the social exploration test.

All animal handling and procedures were approved and conducted according to the Animal Care and Use committee of the Hebrew University of Jerusalem.

**Chronic Mild Stress:** For both experiments we used a modified version of the basic model described by Willner (Willner, Muscat, & Papp, 1992). Rats were subjected to 1-2 of the following stressors daily: food deprivation overnight, water deprivation overnight, cage tilted to a 45° angle, stroboscopic light (4-8 Hz), white noise (70-80 dB), soiled cages (300 ml of water per cage), and lights on during dark time phase. Stressors were designed in a weekly schedule (Table 1a) and administered for 5 weeks. Since we aimed in the first CMS set to examine the behavioral and physiological changes also before the full scale of changes become apparent, a milder
protocol was used, comprised of the same stressors though administered for shorter time periods (Table 1b).

Table 1a

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<th>Tuesday</th>
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<tr>
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<td>White noise</td>
<td>Strobe light</td>
<td>Cage tilt</td>
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<tr>
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<td>Soiled cages</td>
<td>Water deprivation</td>
<td>Strobe light</td>
<td>Lights on</td>
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Table 1b

<table>
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<tr>
<th></th>
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<th>Water deprivation</th>
<th>Tilted cage</th>
<th>Stroboscopic light</th>
<th>White noise</th>
<th>Soiled cage</th>
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<td>3-5 hours</td>
<td>6-8 hours</td>
<td>overnight</td>
<td>10 hours</td>
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**Behavior tests**

All behavior tests were performed in the dark phase of the light cycle, in a dimly lit room at least 12 hours after the last stressor. Due to learning effects from which most behavioral tests suffer, only two of the four tests used in this study (the sucrose preference and the social exploration test) could be administered multiple times allowing an examination not only of the end result of the CMS but also of the dynamics of the behavioral changes.

**Sucrose preference test:** This experiment is designed to assess the influence of CMS on the preference for a palatable solution, and is proposed to be a measure of anhedonic behavior – a core symptom of depression (Yirmiya, 1996). Rats were given two drinking bottles, one containing water and the other containing water sweetened with sucrose. Bottles were placed adjacent to each other on the left side of the cage.
(opposite to the usual location of the drinking bottle). Preliminary tests were conducted and the relative locations of the water and sucrose bottles were counterbalanced throughout the experiment in order to rule out existence of side preference. During the three days prior to the first baseline test, rats were given a bottle with sweetened water for several hours a day in order to accustom them to the sweet solution and reach a stable base-line. The drinking bottles were handed to the rats right before the beginning of the dark phase and were left in the cages for 3 hours. Bottles were then weighed and sucrose vs. water consumption was compared. "Sucrose preference" was defined as sucrose consumption divided by the total consumption (sucrose + water). In the first experiment, a solution of 1% sucrose was used (the percentage used in most published experiments). After noticing what seemed to be a ceiling effect (rats drank over 90% from the sweetened water throughout the experiment), sucrose concentration was lowered in the second experiment to 0.65%.

**Juvenile social exploration test:** A decrease in social interaction in this test putatively reflects an anxiogenic effect (File & Seth, 2003). During the first half of the dark phase, rats were transferred to a dimly lit room and placed individually in novel cages containing only wood bedding. Each rat was given 14-18 minutes for acclimation before a juvenile rat (P24-P35) was introduced to the cage for a period of 3 min. During that time the adult rat was monitored for the total time in which he expressed exploratory behaviors (i.e. interaction initiated by the adult rat, including behaviors such as anogenital sniffing, grooming, following and pinning).

**Elevated plus maze:** This test is widely used for screening anxiolytic effects (Rodgers & Dalvi, 1997). The apparatus consisted of two open and two closed arms extending from an open square center (arms: 42cm x 11cm, walls on sides of closed arms: 30cm, center: 11cm x 11cm, elevation from ground: 50cm). Rats were placed individually on the center of the maze, always facing the same arm, and were given five minutes to explore the maze freely. Sessions were videotaped and analyzed later for the number of entries to each type of arm (counted only when all four paws were in the arm), total amount of time spent in the opened arms, and number of head-dippings outside the maze.
**Novelty Suppressed Feeding test:** Like the well-known Open Field test, this test also attempts to evaluate the animal's reaction and behavior in a novel environment, and is considered to be a measure of depression-like behavior. The main modification here is that the rats are deprived of food 28 hours before being tested, and several food pellets are placed in the center of the arena during the test. This introduces a motivational factor—wanting to reach the food in the center of the field—that can counter the basic fear of novel open space (Stedenfeld et al., 2011). After the initial food deprivation, rats were individually placed in an open arena (opaque black floor: 100cm x 100cm, surrounding walls: 40cm high), always in the same corner and facing the wall. Sessions were videotaped and analyzed later for time elapsed until commencement of eating (defined as taking the first actual bite and not merely sniffing or playing with the food), and time spent immobile in the first three minutes of the session. Rats were given 10 minutes in the arena and were moved back to their home cage either as soon as they began to eat or at the end of the time limit.

**Histology**

**BrdU injection:** BrdU (Sigma-Aldrich, Rehovot, Israel) was dissolved in 0.9% saline (20mg/ml), by sonication in a 33c-37c water bath. Solution was placed in aliquots and kept in -20c. Aliquots were always defrosted and re-sonicated the day of injection. Rats were injected with BrdU twice a day for two consecutive days (100 mg/kg, i.p.) and perfused the morning following the last injection.

**Perfusion and tissue preparation:** Rats were deeply anesthetized with pentobarbitone sodium and perfused transcardially with PBS followed by 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for one day, then transferred to 30% sucrose in PBS and kept at 4 °C until they sank (3-4 days). Coronal sections (8µm) were obtained using a freezing microtome. Sections were stored in a cryoprotectant storage solution at −20 °C until later use.

**Immunohystochemistry- BrdU:** 8 µm frozen brain sections were fixated in 50% formamide/ 2Xssc (0.3M NaCl and 0.03M sodium citrate) for 2 hours at 65°C. After 1 wash with 2XSCC, sections were incubated in 2N HCl for 30 minutes at 37°C. Sections were than rinsed in 0.1M boric acid, pH 8.5 and washed 3 times in PBS. Sections were incubated in 3% normal goat serum in 1% BSA, 0.1% Triton for 30
minutes at room temperature and then incubated with anti-BrdU (1:200, AbD serotec) 48 hours at 4°C. Sections were then incubated with a secondary antibody (1:200 goat anti rat IgG, conjugated to Alexa 555, Invitrogen) for two hours at room temperature. Counterstaining was done with DAPI (Sigma, Israel).

**Immunohistochemistry- Doublecortin (DCX):** 8μm frozen brain sections were fixed in cold methanol for 20 min at -20. After 3 PBS washes sections were incubated in 1%BSA and 0.5% triton in PBS for one hour at room temperature, they were then incubated with the primary antibody (guinea pig anti-doublecortin 1:500, Millipore) for 24 hours at 4º. Sections were subsequently washed three times with PBS and incubated with the secondary antibody (biotinylated goat anti guinea pig, 1:100, Enco) for 1 hour at room temperature and visualized using extravidin-tritc (Enco).

**M.R.I**

**Manganese injection:** Three groups were used for MEMRI measurements. All groups underwent base-line injections, and were given 10 days to recover before the beginning of the experiment. Two groups were injected a second time five weeks later, one after five weeks of CMS ("Mn-full-CMS", n=8) and one control group ("Mn-5-week control", n=6). The third group was tested a second time after 2.5 weeks of CMS ("Mn-mid-CMS", n=6).

Rats were anesthetized using a mixture of Ketamine (90 mg/Mk) and Xylazine (5 mg/Kg) and were fixated to a stereotactic device. The hair above the skull was shaved and the skin was sterilized with alcohol pads before a single incision was made to reveal the skull. Head orientation was fine-tuned by assuring the Bregma and Lambda were aligned on the same horizontal plain. The injection trajectory was planned so that it would not pass through the ventricle located above the injection target (Figure 1). Using a micro-drill, a 0.7mm hole was made (coordinates: A/P= -0.76, M/L=-0.33) and a 1μl Hamilton microsyringe fitted with a 26 G needle was inserted in a 22º angle to a depth of 0.88 cm, reaching the Median Raphe (A/P= -0.76, M/L= 0, D/V= -0.86). Manganese Chloride (0.12M, 0.3μl) was injected at a rate of 0.1μl/min. The syringe was left in place for an additional five minutes in order to minimize leakage of the substance. It was then slowly drawn out of the brain, the hole in the skull was sealed with bone wax and the skin was sewed. Animals were removed from the stereotactic
device and were placed in a preheated cage where recovery from surgery was monitored.

Rats were injected with Rymadil for pain relief 20 min before and twice daily for 2 days after surgery (Rimadyl, 5 mg/Kg, IP).

M.R.I scans were obtained 28 hours after injection.

Fig. 1 – MnCl2 injection to the Median Raphe. T1-wighted MRI slice (bregma -7.4) 28 hours after manganese injection, with the rat brain atlas overlaid. For illustration, the green arrow shows the entry point of the needle and the dashed line shows the injection trajectory. The black circle indicates the final injection location. Contrary to the more distant areas to which the manganese arrives through axonal transportation, the very high levels of MnCl2 obtained in the injection site cause a decrease in signal strength, expressed by the darker area in the image.

The post-injection scans intend to detect accumulation of manganese in ROIs distant from the injection site. In order to assure that the manganese reached the ROI via axonal transportation from the injection site and not by diffusion through the CSF, MnCl2 was injected directly to the ventricle located above the median raphe in three control rats (using the same amount and rate as with all other rats). One-hour and 28-hour post-injection scans were then obtained.

**MRI measurements for manganese:** Each time-point was comprised of two scans—a pre-injection scan taken 1-3 days before MnCl2 injection (for normalizing purposes in later analysis) and a post-injection scan taken 28 hours after MnCl2 injection (Figure 5). Scans were obtained using a horizontal 4.7T BioSpec Bruker scanner. Rats were anaesthetized with isoflurane (1.5-2.5% with a mixture of ~30:70 O2:N2O) and placed in a custom-made holder with the head held in the center of a 38mm Doty transmit-receive quadrature rat head-dedicated volume coil. Respiration was
monitored and held at a range of 50-60 breaths/minute by adjusting anesthesia level. Rats were heated while in the scanner using a homemade water bed.

For anatomical purposes, T2-weighted control images were obtained for the same slices as the functional imaging using a spin-echo sequence (TR=2300ms, TE=40ms, matrix=256x192, FOV=3cm, slice thickness=1mm, averages=8). For MEMRI measurements, 2D high spatial resolution T1 weighted images were obtained using a gradient-echo sequence (TR=58ms, TE=3.1ms, averages=50, matrix=256x192, FOV=3cm, slice thickness=1mm). T1-weighted rather than T1 measurements were used to minimize acquisition length, keeping the experiment as short as possible in order to prevent mortality from long repeatable anesthesia sessions.

**MRI measurements for resting-state Functional Connectivity:** Anesthesia, positioning and anatomical scans were obtained in the same manner as described above for the manganese scans. Anatomical and BOLD contrast scans were obtained for 15 slices, covering most of the brain. The BOLD contrast scans were collected in three sequential scans of 10 minutes each (EPI-FID, TR=2000ms, TE=20ms, repetitions=300, matrix= 128x64x15, FOV=3x3cm$^2$, 1mm slice width) enabling exclusion of non-stable data in later analysis.

**Data analysis:** Data was analyzed using IDL (Interactive Data Language, Research system inc., Boulder, CO, USA) custom self-written software. All images were linearly realigned to their appropriate slice in the Paxinos rat brain atlas (Paxinos and Watson, 6th edition, 2007). T2-weighted images were used to confirm alignment and anatomical locations.

**MEMRI analysis:** The process of defining ROIs for analysis is essential. Two main approaches can be found in literature– a data driven approach and an anatomy-based approach. Each of the above has its limitations; data-driven ROIs fail at times to correspond with well-defined anatomical regions, causing clinical interpretations to be elusive. On the other hand, regions that are well defined in the atlas are many times comprised of functionally distinct sub-units and using the region as a whole can introduce extra noise to the analysis. The process of ROI selection in this study combined data-driven information together with anatomical knowledge, enabling us to limit the analysis to regions in which manganese accumulates in baseline
conditions (Figure 2). All scan slices were smoothed using a five-point kernel and normalized by the pre-injection scan. The signal strength distribution across the brain was obtained. Without signal enhancements introduced by the manganese, a near-gaussian distribution (centered around one) can be expected, reflecting normal signal fluctuations. The extended positive tail (Fig. 2a) is assumed to reflect areas in which manganese has accumulated, resulting in a higher signal than in the pre-injection scan. In order to focus on those voxels, a cutoff was defined as the gaussian mean + FWHM (full width at half maximum) and voxels passing the cutoff were marked. This procedure was repeated for several animals, the resulting images were superimposed and the voxels that didn't pass the cutoff in over 50% of the animals were excluded. An M.R.I image was displayed with an overlaying atlas figure and the voxels that passed the cutoff were marked in a pink color scale. The resulting image was used to guide the manual choice of anatomically-defined ROIs that best fit the areas with signal enhancements. This process enabled to define 40 ROIs on the basis of data driven information, yet relying on atlas-defined anatomical structures in the final stage (Appendix 1).

To evaluate the changes in the median raphes' connectivity before and after CMS, two measures were obtained: 1) a global measure of the median raphe's effective activity (using a paired t-test across all ROIs in the different time-points). 2) specific ROI measures (using a group t-test between the different time-points, with a Dunn-Sidak correction for multiple comparisons), examining whether individual connections are compromised more than others, regardless of global changes in the median raphe's connectivity.

**rsFC analysis:** analysis included spatial smoothing by a Gaussian 3-point kernel, a time-shift correction and a band-pass filtering (0.01<->0.1 Hz). 33 ROIs were pre-selected in the atlas, the majority of which belonging to the extended limbic system (Appendix 1). Linear Pearson correlations between the mean ROI time-courses were calculated and converted to normal distribution by Fischer's z transformation.
Radial Correlation Contrast: RCC data was analyzed using the same BOLD contrast data from which the rsFC data was analyzed and the same set of predefined ROIs. The data was spatially smoothed using a Gaussian 3-point kernel. The average correlation between each voxel in the ROI with all of its neighboring voxels (within two voxels away) was calculated. The RCC values of all voxels within an ROI were averaged to obtain an RCC measure of the ROI.

Fig. 2 – The process of defining ROIs for the manganese analysis. Images were first aligned to the appropriate atlas figure and then smoothed and normalized by the pre-injection scan. For each individual we obtain: (A) The distribution of all voxels in the brain excluding ventricles and blood vessels (white solid line) and its gaussian fit (dashed yellow line). Without signal enhancements introduced by the manganese, a normal distribution centered around 1 can be expected. The extended positive tail (green arrow) is assumed to reflect areas in which manganese has accumulated, resulting in a higher signal than in the pre-injection scan. A cutoff (dashed green perpendicular line) is defined as mean+ FWHM (full width at half maximum). (B) A coronal T1-wighted M.R.I image (bregma -3.3) with the atlas figure overlaid. Voxels that pass the cutoff are marked in a pink color scale. Such can be seen for example in hypothalamic areas as well as in the habenula (yellow arrow) and in a small region of the cingulate cortex. Images from several animals were superimposed and voxels that didn’t overlap over in 50% of the animals were excluded. The resulting image was used to direct the manual choice of anatomical, atlas-based, ROIs. (C) An example of the final ROI definition: After an enhanced signal was shown to be present in the habenula, a final habenula ROI was defined based on the borders of the habenula in the atlas.
RESULTS

CMS set #1:

Behavior results:

No significant differences were found between the full-CMS and the Mn-full-CMS groups; therefore, they were combined into one CMS group in the analysis of CMS vs. control.

Sucrose preference test – The average sucrose preference stayed close to 90% throughout the experiment, resulting in no significant differences between groups.

Social exploration – Due to the variations in the ages of the puppies used at each time-point, which can potentially influence the typical interaction between the rats, all scores were normalized to the mean score of the control group at each time-point. Two weeks after initiation of stress, the CMS rats presented an increase in their social exploration compared to the control group. From the third week onwards, rats showed a steady decrease in social exploration times, which became lower than the control group by the end of the five-week stress session (Figure 3a). The final score each rat received was the slope of the linear regression of the rat's performance along the stress regime (Figure 3b). The slopes of the CMS group were more negative than those of the control group (p=0.03).

Elevated plus maze – The total number of times the rats passed between arms was the only measure found to be significantly different between the CMS and control groups (p=0.002). The final score assigned to each rat was a weighted average of all parameters tested (Figure 3b).

Novelty suppressed feeding – The time spent immobile during the first three minutes was significantly lower in the CMS group compared to control (p=0.03). Time elapsed to first bite did not prove to be significantly different. The final score each rat received was the average of both parameters and was slightly lower in the CMS group, though not statistically significant (Figure 3b).
Histology: Both BrdU and doublecortin levels were significantly lower in the CMS group compared to the control group at the end of the CMS session (Figure 4). BrdU levels were slightly elevated in the CMS group after three weeks of CMS, though not statistically significant. The doublecortin level in the baseline group was significantly higher than in all other groups. This may well be due to the erroneous use of relatively young rats for this group (7 weeks), and the interpretation of their result with respect to those attained from the rest of the experiment is problematic.

Fig. 4- Levels of proliferation and neurogenesis in the hippocampal dentate gyrus throughout CMS implementation, as measured by (A) BrdU, and (B) Doublecortin staining. Data are presented as mean±SEM. By the end of the week 5 CMS the levels of BrdU and doublecortin expression in the CMS group were significantly lower compared to the control group (p=0.005, p=0.03 respectively). After 3 weeks of CMS the average BrdU levels were a bit elevated in the CMS group, though not statistically significantly. The significantly higher levels of doublecortin in the base-line group may be attributed to the erroneous use of young rats (7 weeks old).
MEMRI:

Manganese levels were always quantified in terms of the fraction of signal enhancement in the post-injection scan relative to the pre-injection one (Figure 5).

Injection to the CSF: while one hour after the MnCl$_2$ injection, the manganese was strongly visible in the ventricles posterior to the injection site, no substantial signal enhancements were seen 28 hours after injection. This indicates that residual MnCl$_2$ that may diffuse to the CSF cannot account for the signal enhancements seen 28 hours after manganese injection to the median raphe.

Global changes: at the end of CMS week #5 the global level of signal enhancement across ROIs in the Mn-full-CMS group was 9.6% lower than that seen at base-line scans prior to CMS implementation (paired t-test across all ROIs, p=0.023), indicating a general reduction in the effective connectivity of the median raphe. Similar, yet milder and not significant reductions were seen also in the Mn-mid-CMS group tested after 2.5 weeks of CMS, as well as in the Mn-5-week-Control group (8.2%, p=0.32 and 6.2%, p=0.43 respectively). The mechanisms that can plausibly underlie such changes will be discussed later on.

Fig. 5 – MEMRI scans before (top row) and 28 hours after (bottom row) MnCl$_2$ injection. An enhanced signal compared to base-line is obtained from areas in which the manganese has accumulated. This can be seen in many regions, two of which are notated by the black arrows (the posterior hypothalamic nucleus in the left column and the habenula in the middle column).
Specific ROIs: Whereas the CMS group tested after 5 weeks of CMS was the only one in which a significant global reduction in signal enhancement was apparent, several individual regions differed between the baseline and the second injection in all three groups. These include the CPu in which manganese levels noticeably declined in all three groups (p=0.007, p=0.015 and p=0.032 in the Mn-mid-CMS, the Mn-full-CMS and the Mn-5-week-Control groups, respectively), and the posterior hypothalamic nucleus in which manganese levels were elevated in all groups (p=0.006, p=0.007 and p=0.017 in the Mn-mid-CMS, the Mn-full-CMS and the Mn-5-week-Control groups, respectively).

Following a multiple comparisons correction (Dunn-Sidak correction), the right amygdaloid nucleus differed significantly between the Mn-full-CMS and the Mn-5-week-Control groups (0.5% and 4.5% signal enhancement compared to pre-injection scan, respectively, p<0.001).

Signal enhancement in the habenula was uniquely elevated in the mid-CMS group, showing a 7% stronger enhancement than baseline (p=0.032). This was not the case in the full-CMS or in the control groups.

CMS set #2:

Resting-state Functional Connectivity: The global distribution across all possible rsFCs was narrower in the CMS group and with a smaller variance than that of the control group (standard deviation of 0.115 and 0.141 respectively, p<0.001) (Figure 6). This reflects a general reduction in the functional connectivity strengths in the CMS group.

Fig. 6 – Global distributions of correlation strengths of the CMS and control groups. Though the centers of both distributions are similar (0.064 and 0.056 for CMS and control groups respectively), the correlation distribution of the CMS group is significantly narrower than that of the control group (standard deviation of 0.115 and 0.141 respectively, p<0.0001), reflecting a general reduction in rsFC strengths in the CMS group.
Maps of the strongest rsFCs in each group were generated using two different cutoffs (Figure 7). Each map is presented in three orthogonal projections with a schematic atlas figure in the background. Several common characteristics across groups and cutoffs can be pointed out: (i) The number of bi-lateral (e.g. left and right dentate gyrus) connections that passed the cutoffs was always significantly higher than their percentage in the population. This most likely reflects the fundamental functional symmetry of the brain, and the relatively high synchronization between bi-lateral structures. (ii) There were noticeably more connections within either sub-cortical or frontal-cortical areas than there were between them.

Comparing the maps of the two groups reveals the following: 1. Fewer connections pass either cutoff in the CMS group, agreeing with the narrower rsFC distribution of the CMS presented earlier. 2. Several regions appear to have strong functional connections with multiple regions (>2 non bi-lateral) in the control, but not in the CMS group. These include the prelimbic area, the anterior cingulate, the dorsal striatum (CPu) and the habenula. 3. The mammillary bodies and the raphe are the only two regions from which, non-bilateral connections pass the cutoff in the CMS but not in the control group. 4. Though relatively few connections cross between the frontal-cortical and the subcortical areas even in the control group, none at all pass the cutoff in the CMS group.

T-tests were performed to directly examine the group effect on specific rsFCs. After correction for multiple comparisons, no single rsFC differed significantly between groups with a certainty level of $\alpha=0.05$. When a non-corrected $\alpha$ was allowed to be used, several rsFCs showed increased rsFC strengths in the CMS group, while others showed decreased strengths (Figure 8). Among the rsFCs that were weaker in the CMS group (orange lines) more were related to the right hemisphere than to the left one. Furthermore, two of the eight rsFCs that decline in the CMS group connect frontal with subcortical regions (the prelimbic area$\leftrightarrow$CPu and cingulate$\leftrightarrow$globus pallidus). Both of the above results agree with the two trends mentioned earlier regarding the drop in right-hemisphere dominance and in cross frontal-subcortical connections in the CMS group. The mammillary bodies stand out as the region with the largest amount of rsFCs that increase in strength in the CMS group.
**Behavior results:** Technical problems in the administration of the elevated plus maze and the novelty suppressed feeding tests rendered the results unreliable.

Even after lowering the sucrose level in the sucrose preference test to 0.65%, there was a clear ceiling effect. Almost all rats kept drinking very high percentages of the sweetened water throughout the experiment, preventing from differences between groups to be detected.

In the social exploration test a trend towards larger decreases in exploration times at the end of the stress was seen in the CMS group (p=0.060, figure 9a). If one assumes a monotonic, yet non-linear, relationship between stress levels and reduction in exploration times, it is logical to convert the scores of all rats to a rank scale of 1-18 (total number of animals in this experiment). The difference between the average ranks in both groups was significant (p=0.03).

**Relation between social exploration results and rsFC:**

The strongest correlations between specific rsFCs and the social exploration scores were found for the bilateral CPu (r=0.80, p<0.01) (Figure 10a) and the prelhcinc(rl)→cingulate(rl) rsFCs (r=0.67, p<0.04). Positive correlations infer a decrease in rsFC strength being in line with a decrease in behavior results. Interestingly, both the CPu and the prelimbic regions were pointed out earlier as being related to the seemingly lower frontal-to-subcortical connectivity in the CMS group.

To check if there is a consistent relation between changes in rsFC strengths and behavior in the social exploration test, we divided all rsFCs that differed between groups (with p<0.05) into those that increased and those that decreased in the CMS group. Since behavior results are generally lower in the CMS group, we hypothesized that the rsFCs that decreased in the CMS group will typically correlate positively with behavior (lower behavior results being in line with weaker rsFCs) while those that increased will correlate negatively with behavior (lower behavior results being in line with stronger rsFCs). Indeed, though not high on average, all 7 rsFCs that decreased in the CMS group had a positive correlation with behavior, while 11 out of the 13 rsFCs that increased in the CMS group had a negative correlation with behavior. The difference between the correlation directions of the increasing and decreasing rsFCs with behavior was very significant (p<0.0001) (Figure 10b), demonstrating the intrinsic relation between rsFCs and behavior.
Fig. 7 – Resting-state Functional Connectivity (rsFC) maps obtained for control and CMS groups individually. Two thresholds were used enabling relatively few (A) or many (B) rsFCs to be presented. Each row presents a single data set, illustrated in three orthogonal projections (coronal, axial and sagittal projections in the left, middle and right columns respectively).

Two common features between groups stand out: 1. There is a relatively high proportion of bilateral connections (e.g. left and right CPu), most likely reflecting the high functional synchronization between bilateral structures. 2. There are more connections within the frontal-cortical or the sub-cortical regions than between them.

Several differences between groups are noticeable: 1. Fewer connections pass any given cutoff in the CMS group compared to the control group. Connections that appear in the control, but not in the CMS group are all non-bilateral connections. 2. Regions exhibiting multiple non-bilateral connections (>2) in the control group, but not in the CMS group, include the prelimbic area, the cingulate, the CPu and the habenula. 3. Two regions exhibit non bilateral connections in the CMS, but not in the control group— the raphe and the mammillary bodies (with the dentate gyrus and the hypothalamus respectively). 4. No connections connecting frontal and subcortical regions appear in the CMS group. In the control group, such connections include the bilateral prelimbic area→CPu, the right anterior cingulate→CPu and the left anterior cingulate→dentate gyrus rsFCs.
Fig. 8 – Resting-state Functional Connections that differ most between groups. Depicted are all rsFCs that differ between groups with a significance level of p<0.05 (uncorrected for multiple comparisons). They can be either weaker (orange) or stronger (black) in the CMS group compared to control. The line width indicates the effect size.

The following points stand out: 1. The majority of connections that are weaker in the CMS group are related more to the right hemisphere than to the left one. 2. the prelimbic area $\leftrightarrow$ CPu and the cingulate $\leftrightarrow$ Globus pallidus rsFC decrease in strength.

These differences between groups are in concordance with the results described earlier in respect to the general loss of right hemisphere connections as well as the loss of rsFCs connecting frontal with subcortical areas.

Also worth noting are the mammillary bodies from which eight connections show an increase in the CMS group.

Fig. 9 – Results of the social behavior (SE) test. Decrease in exploration time at the end of the CMS in terms of (A) absolute seconds spent exploring the juvenile rat, and of (B) ranking compared to all other animals in the experiment. Rats from the CMS show a higher average decrease in exploration times at the end of the CMS (p=0.06) which is significant when measured in terms of rank (p=0.027).
Radial correlation Contrast: One means of testing the validity of the RCC measure was to examine the degree of symmetry between RCC values obtained from the two hemispheres. All ROIs, but the amygdala, showed a high level of symmetry between hemispheres (r=0.74 and r=0.90 including or excluding the amygdala, respectively), reinforcing the RCC's validity (Figure 11).

Fig. 11 – Symmetry of Radial Correlations Contrast values between left and right hemispheres of the brain. The scatter plot presents all group means of right vs. left hemisphere RCC values. The outlier ROI, denoted by the arrow, is the amygdala. The high correlation between hemispheres (r=0.94 or r=0.74 with or without excluding the amygdala) can account for the reliability of the RCC measure.

Fig. 10 – Correlation between social exploration and rsFC. (A) Scatter plot of scores in the social exploration test vs. the bilateral CPu rsFC for all animals. This rsFC exhibited the highest correlation with the social exploration results (r=0.80). (B) Average correlations between behavior results in the social exploration test and the resting-state functional connections that decreased (blue bar) or increased (red bar) in the CMS group compared to control (with significance level of p<0.05,uncorrected for multiple comparisons). Control rats received on average higher scores in the behavior test. Connections that are significantly higher in the CMS group can be expected to exhibit a negative correlation with behavior (i.e. stronger connections will correlate with lower behavioral results) and vice versa. The average correlations between behavior results and rsFC are indeed negative for rsFCs that increase in CMS and positive for those that decrease in CMS (p<0.001).
The RCC levels across ROIs in the CMS group was significantly lower than in the control group: 30 out of 33 ROIs showed decreased RCC levels relative to the control, dropping by an average of 6% (p<0.0001). The anterior cingulate had the highest correlation with behavior results (0.90 and 0.64 for left and right anterior cingulate, respectively)

DISCUSSION

Despite the prevalence of major depression (MD) in the population and the vast amount of research in the field, the underlying physiological mechanisms have not yet been fully deciphered. According to one of the earliest hypotheses– the monoamine deficiency hypothesis– alterations in the activity of monoaminergic neuromodulator systems in the brain is the core reason for MD (Haenisch & Bonisch, 2011). Indeed, most anti-depressants currently prescribed for treating MD are base on elevating serotonin availability in the brain. An additional hypothesis postulates that a reduction in neurogenesis levels in the dentate gyrus of the hippocampus is imminently related to the development and maintenance of depression (Lucassen, Meerlo et al., 2010; Samuels & Hen, 2011). More recent approaches view MD as a system-wide disorder, involving multiple brain regions. Measuring the resting-state functional connectivity (rsFC) patterns in the brain is one means in which such system-wide alterations can be assessed. A variety of studies employed M.R.I rsFC analysis to define functional networks in the human brain as well as to examine the changes in rsFC which characterize various psychopathologies (Fox & Raichle, 2007), including MD (Carballedo et al., 2011). To the best of our knowledge, no study explored the modifications in rsFC patterns in a rodent model of depression.

In the current study, we aimed to evaluate the temporal dynamics of the changes in the above-mentioned neurological parameters, in an animal model of depression. To do so we subjected rats to chronic mild stress (CMS) – a highly validated rodent model of depression (Willner, 2005) which is based on exposing the animals to a series of unpredictable mild stressor over a time course of several weeks. Neurological measures were obtained by employing two M.R.I methodologies and
histochemical staining, and behavioral tests were administered to assess the development of depression-like behaviors.

Two sets of CMS experiments, each with control rats, were conducted at different times. In the first set, we focused on the changes in the effective activity of the median raphe (the main serotonin provider in the brain) and in neurogenesis levels. For that, manganese-enhanced-MRI (MEMRI) was used to evaluate the median raphes' effective connectivity, and BrdU (for general proliferation) and doublecortin (found in 'newly born' neurons) staining in the dentate gyrus of the hippocampus was used to evaluate levels of neurogenesis. In the second CMS set, BOLD contrast M.R.I was obtained in order to evaluate the modifications in the resting-state functional connectivity patterns that characterize chronic mild stress. On the basis of the same data, we examined the changes in the relatively novel radial-correlation-contrast (RCC) measurement. The RCC is hypothesized to reflect the level of BOLD synchronization within an ROI, which in turn may be related to the general efficiency of the regions’ activity. Behavioral tests were applied in both experimental sets in order to validate the CMS model, as well as to correlate between the changes in the neurological parameters and the development of depression-like behaviors. These tests included the sucrose preference, the elevated plus maze, the novelty suppressed feeding and the social exploration tests.

Many behavior tests suffer from substantial learning effects and consequently can be administered only once. Few tests – the social exploration and the sucrose preference tests among them – can be applied multiple times, qualifying them for use in assessing the temporal dynamics of behavioral changes.

**The validity and interpretation of the procedures and measurements used:**

In nearly all parameters, both behavioral and physiological, significant changes were noticeable after the implementation of chronic mild stress, thus validating the CMS protocols used.

**Interpretation of MEMRI:** The detectable passage of manganese from the injection site to distant nuclei via axonal transportation is presumed to reflect the effective connectivity between the two regions. While it can easily be assumed that substantial pre-synaptic neural activity (in the current study referring to the median raphe) is
needed for manganese to be transported to distant loci, the role of the postsynaptic neuron is often less intuitive. Like that of Ca^{2+}, accumulation of manganese in a postsynaptic cell will depend also on the matched activity of the postsynaptic neuron (Pautler et al., 2003). The mere firing of action potentials alone will not be sufficient for manganese to accumulate in a different region. If the neurotransmitters that reach the synaptic cleft do not elicit a postsynaptic response, the accumulation of manganese in the postsynaptic cell will be hampered. Alteration in manganese uptake in distant ROIs can reflect a range of modifications including changes in the median raphes’ activity (as can be measured for example by electrophysiology), changes in the distant ROIs' activity as well as changes in the neurotransmitter efficiency that can result, for example, from a reduction in appropriate receptors on the postsynaptic cell. For these reasons, manganese accumulation levels in distant ROIs can reflect only the effective connectivity between the regions, which can be influenced by any of the abovementioned factors (Doron & Goelman, 2010; Revital, Hagai, & Gadi, 2008).

rsFC and RCC measurements: the rsFC maps obtained lend strong evidence to the assumption that rsFC measures quantify a genuine physiological trait of brain regions. This can be seen by the overall symmetric patterns of the rsFC maps, as well as by the very high proportion of bilateral connections that pass high cutoffs (reaching on average nearly five times more than their percentage in the rsFC population examined). These findings can be taken to reflect the fundamental functional symmetry of both hemispheres. Similarly, the very high symmetry between the RCC values of the two hemispheres reinforces the assumption that the RCC quantifies an inherent functional property of local brain regions.

Understanding the results:

By the end of five weeks of CMS in the first CMS set (but not after 2.5 weeks) both neurogenesis, as indicated by BrdU and doublecortin levels, and the global effective activity of the median raphe were reduced significantly compared to baseline. These results are in line with various studies that report reduced neurogenesis and impaired serotonergic systems in depression (Jans, Riedel, Markus, & Blokland, 2007; Sharp & Cowen, 2011). Specifically, the amygdaloid nucleus had a significantly lower signal enhancement in the CMS group compared to control. The amygdala is one of the core
limbic structures and has extensively been linked to mood disorders (Carletti, Corsi, Melotto, & Caberlotto, 2005; Hajek et al., 2009). This finding agrees with the hypothesis that the amygdala and serotonergic raphe neurons are an integral part of a neural system that modulates anxiety states (Spiga, Lightman, Shekhar, & Lowry, 2006).

The time-course of the physiological changes proved worthy of attention. Proliferation levels, as measured by BrdU staining, showed a transient (though not statistically significant) elevation in the CMS group after three weeks of CMS, before reaching the lower-than-control level at the end of the CMS session. Initial proliferation of neural progenitor cells (NPCs) should be distinguished from the later and more specific stage of differentiation into specific cell types. Indeed, a study examining the effect of stimulated astrocytes on NPCs demonstrated increased proliferation, but not neurogenesis, levels (Go et al., 2009). A similar elevation in the mid-term CMS time-point was also seen in the behavior results of the social exploration test. Furthermore, the manganese levels in the mid-CMS group were not always mid way between the control and the full-CMS groups. The habenula, had elevated manganese levels compared to baseline after 2.5 weeks (p=0.032), but not after five weeks of stress. The habenula is intrinsically related to the serotonergic and dopaminergic systems and has been shown to play a key role in the response to negative rewards (Geisler & Trimble, 2008). Interactions between the habenula and the raphe have been suggested to contribute to the development of depressive symptoms (Hikosaka, 2010).

The above results lend further support to the understanding that the processes leading to depressive behaviors as a result of chronic stress are complex and far from linear. One possible explanation for these findings may be related to the fact that CMS levels in this experiment were kept relatively low in an attempt to induce changes slow enough to make the temporal evaluation of physiological and behavioral parameters possible. In this respect, it is plausible that the mild stressors induce an initial state of enhanced arousal in response to the negative stimuli, manifesting itself in elevated behavior and proliferation levels, as well as in elevated activity in regions such as the habenula. In a later stage, these processes may give in to the more chronic depression-like processes which lead to the end-results we obtained.
Resting-state Functional connectivity and RCC: There was an overall global decrease in both rsFC strengths and in RCC measures of the CMS group compared to control. The decline in rsFC across ROIs is in line with human studies reporting similar findings in various psychopathologies, including MD (Veer et al., 2011). Furthermore, the bilateral rsFCs tended to be more dominant in the CMS group maps. This may reflect a similar phenomenon to that described in several pathology-related fMRI studies. Increased functional bilaterality was reported, for example, in one study using language tasks in autistic populations (Anderson et al., 2010) and in another study using memory tasks in traumatic brain injury populations (Russell, Arenth, Scanlon, Kessler, & Ricker, 2011). It should be noted though, that the ROI selection was limited in this study mainly to the extended limbic system, so that any general conclusion should be limited to this system.

CMS-induced modifications in specific rsFCs were also found. The prelimbic cortex, habenula, superior colliculus, mammillary bodies, raphe, CPu and the cingulate cortex were all found do have different rsFC profiles between CMS and control groups. Most of these regions were linked with MD in many other imaging studies (Jans et al., 2007; Li et al., 2011; Price & Drevets, 2010). The changes in the rsFC pattern of the superior colliculus is not clear to us at the moment.

Additionally, in the CMS group there were fewer rsFCs connecting frontal-cortical regions (such as the prelimbic area and the anterior cingulate) with sub-cortical regions. This observation agrees with other studies that report a functional disconnection between frontal and limbic areas in MD patients (Carballedo et al., 2011). One such connection was the CPu-prelimbic rsFC which was significantly lower in CMS group compared to control, and indeed appeared in the map of the control, but not in that of the CMS group. The bilateral CPu rsFC was also the rsFC most highly correlated with the behavior results in the social exploration test. Put together, these results may point at the dorsal striatum as a key player in understanding the stress-mediated changes in rsFC.

Also worth noting are the mammillary bodies from which a large number of rsFCs increased in strength in the CMS group. The mammillary bodies are known to receive substantial inputs from the amygdala and the hippocampus and are part of the limbic system. Although this makes the region a natural candidate for being involved in
emotional processing, its main known function in humans relates to memory 
processes (more specifically, recollection type recognition) (Aggleton et al., 2010).

Perhaps the most intriguing result obtained is that showing the relationship between 
the directions in which rsFCs change (becoming either weaker or stronger) and the 
correlation with behavior. Indeed, rsFCs that decreased in strength in the CMS group 
had a typical positive correlation with behavior results (i.e. weak rsFC correlating 
with depressive-like behavior), while rsFCs that increased in strength were typically 
inversely correlated with behavior (i.e. strong rsFC correlating with depressive-like 
behavior). This finding strengthens the hypothesis that rsFC characteristics can 
reflect, at least partially, the emotional/ behavioral state of the animal.

**Reservations and limitations:**

The sucrose preference test: Though very commonly used, the sucrose preference test 
is highly labile (Strekalova et al., 2011), and a bias may well exist towards 
publishation of results that show a decrease in sucrose preference after CMS, reflecting 
a tendency not to publish results that contradict popular expectation. That said, a 
genuine methodological flaw in test administration may account for the lack of 
differences between groups. Throughout the CMS, all rats generally drank a very high 
amount of sweetened compared to plain water (∼ 90%, also after lowering sucrose 
level to 0.65% in the second experiment). This may introduce a ceiling effect causing 
our results to be much above the sensitivity level where changes in behavior can be 
detected. This can be evaluated in future studies by starting with lower levels of 
sucrose (even as low as 0.2%) and elevating the sucrose level only until a maximum 
base-line level of 80% sucrose consumption is reached.

General limitations: A major limitation of this study was the small number of animals 
used in most of the groups, making definite conclusions elusive.

Due attention should also be given to the results of the manganese control group. The 
examination of individual changes in specific ROIs revealed several connections that 
differed significantly between baseline and the second time-point in all three groups 
(Mn-mid-CMS, Mn-full-CMS and Mn-5-week-Control). These include the dorsal 
striatum (CPu) (p=0.006, p=0.031 and p=0.032 for the Mn-mid-CMS, Mn-full-CMS 
and Mn-5-week-Control groups, respectively) in which decreased signal
enhancements were evident, and the posterior hypothalamic nucleus in which increased signal enhancements were apparent in all groups (p=0.006, p=0.007 and p=0.017 for the Mn-mid-CMS, Mn-full-CMS and Mn-5-week-Control groups, respectively). This draws attention to the adverse effects the surgery itself may cause in both physical (direct damage to the tissue and the resulting inflammatory responses) and emotional levels. The stressogenic effects of surgery may introduce individual changes in the effective connectivity of the median raphe with specific regions, making it hard to differentiate between pure CMS and interaction effects between the CMS and the operation-induced stress. That said, some of the major results were unique to the CMS groups, namely- the global reduction in median raphe activity at the end of the CMS, as well as the significant differences between the signal enhancements in the amygdaloid nucleus and the habenula in the Mn-full-CMS and the Mn-mid-CMS groups, respectively.

Another word of precaution should be added regarding the use of anesthesia and its effect on rsFC. Though several studies suggested that rsFC obtained from anaesthetized animals accurately reflect functional networks, the exact relationship between the results obtained under anesthesia and those obtained from resting, yet fully awake, subjects is not fully clear.

Concluding remarks:

The above limitations notwithstanding, the results presented in this study demonstrate the validity of the M.R.I methodologies implemented, and express the relevance of such methodologies in the study of the neurological basis of psychopathologies using animal models.

In addition to the end-point results in which significant CMS-mediated changes were evident in nearly all parameters examined, our results demonstrate a complex non-linear time course these changes follow. It may be postulated that these non-linear patterns reflect, among other things, a shift from a more acute, stress-mediated arousal phase, to a more chronic and passive depression-like phase.

Furthermore, our results emphasize the need to move beyond the traditional attempt to relate symptomatic changes in behavior to alterations in localized brain regions. Instead, the functional and effective interactions between multiple regions, as well as system-wide changes, should gain increasing attention.
References


## Appendix 1

**ROIs in MEMRI analysis**

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<th>Structure Name</th>
<th>side</th>
<th>size # of pixels</th>
<th>size mm³</th>
<th>Coordinates (from bregma)</th>
<th>M/L</th>
<th>A/P</th>
<th>D/V</th>
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### ROIs in MEMRI analysis

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Appendix 2

תקציר

على אוס丰胸 어떤 התנהגות של דיכאון מוגדרי באוכלוסיות המ CHK מחקרו הורב תחת, התלךם
ויסלון המודרני ובם התנהגות דיכאון טרימבון שים. מﺳר הנהגות מתכון
על מנוגנים זיהויים וסוכנים לקחון שלק משמעויות באתוליגות של דיכאון. המורכבים של
מנוגנים אלה כלים ייחודי באקטיציון של מום- דルドכיונון, הפרש במרט
ויזורולט של זיהוייםlia ואריזנוס (ויזוינוי) הביא כמפוס הפיתוח לתל עליוון דלקוס.
הפרופיון-אריזנוס. נ kaç אל דיכאון מוגדר או הפרעה בר-מערבית
مؤسسات מהקריות בהן התקניי פורק וחקירת האפקטיבית של שיתוף של בזק מהאמפיני
דיקאון מוגדר.

ב막חר הנקבה מבושל לחץ את האפקטיות התחלשות של שוניי בעילית המערבות
הטטרוגונית, בנוכוון הספקולרים (فكر範ית בקוברויט-פרסטרווטיל במענה
המפרשים במודל של דיקאון בከת.

הפה חלקים משを行う של סטרורסומי קלים Sprague-Dawley.
שהפיפון חלוד שלסוסטושショן הראשון (stress- CMS)
שחקוןüm המודל מבוסס של דיקאון במקומם. מה פפליות האפקטיות של המפרשים
הטטרוגונית התכונה בفى ממצאות המנוגנים לתרשים manganese-enhanced-MRI
ה sharedPreferences המר Assyila. רומ רנווןו-כ-3 שבחפפפורט קמדוד על ידי ביעית
היסטוקומית של נורטילין doublecortin and BrdU
באמצע המיגון MRI ב BOLD. ברנס, בין מסר נוכוון התחלשות לאמר
הנסו, בבלל-6 מבזק העפרה סוכר, מבזק הקור הרורית, מבזק המנוב של הבצל-ר.
 veggies suppression feeding

בתוכה חתימה מבושל של שוניי בצל הפרוסים המנוגנים שמדים, בבלל
CMS, הרפ קרנייה בצל הפרוסים המנוגנים שמדימים, בבלל
ירידת וגלוות בפשיטת האפקטיבות של גורן הזר, ירידת בברת הנוון הפרסטרויט
ירידת וגלוות ברמות של פורק מאפקטיבות בזק, ירידת בברת התחלשות
המזוררות בזק של שוניי אלא עליה סורוסטרויט. רומ הפרסטרויט, ירידת בברת התחלשות
-CMS, (negative reward)
בנסף, בנובה של התקנה-אוזר הקורר ביצוע של גורן שויי שילות
CMS אלו אחת הפישוניים שב=img עם
בצפת עליה בסיון המגרים את מבצעים יוזי של התאום, שיזמה התחלשות
האופטימיסמש הצעות את והברת בתן, נсмер ראשיאית, של התחלשות והוריות
הunidad מש��ס הת蝰וש דיקאון.