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Effects of single and repeated electroconvulsive shocks on brain C-Fos activity and LPS-induced behavioral changes.

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While electroconvulsive shocks (ECS) have long been used as an effective treatment for major depressive disorder (MDD), its underlying mechanisms remain largely unknown. Recent studies suggest that changes in the brain immune system which result from repeated ECS may contribute to the effectiveness of the treatment. The current project seeks to clarify two aspects of this potential underlying mechanism. Firstly, it tests whether repeated ECS treatments have a different effect on brain C-Fos activity in mice, compared to single treatments. Secondly, it tests whether repeated ECS can protect mice against LPS induced depression-like behavior. Both single and repeated ECS significantly increased C-Fos activity short term, but not long term. In the second experiment, LPS failed to induce significant depression-like behavior. Therefore, we cannot draw definitive conclusions regarding the possible protective effects of repeated ECS on LPS-induced behavioral changes.

Contents

Introduction

Depression and the immune system

Major Depressive Disorder (MDD) is a mental disorder characterized by a pervasive and persistent low mood. Symptoms include decreased interest in the (social) environment or activities, low self-esteem, increased sensitivity to pain, insomnia/hypersomnia and a significant change in appetite (NIMH, 2016) . The onset of MDD is most common between 20 and 30 years old, and is slightly more common in women than in men (NIHM, 2016). Treatment is generally a combination of antidepressant medication and counseling (including cognitive behavioral therapy, interpersonal therapy and problem-solving therapy). A small subset of patients (unaffected by the medication or otherwise non-treatable) may receive other types of treatment like electroconvulsive therapy or sleep deprivation therapy (Lisanby, 2007; Benedetti et al., 2005).

The etiology of MDD is still largely unclear. Current research suggests various combinations of genetic, physiological, psychological and environmental factors. (Kendler et al., 1995; Wankerl et al., 2013). Mono-amine imbalance is one of the more well-known factors associated with this complicated disorder. In depressed patients, levels of serotonin, norepinephrine and dopamine (among other mono-amines) have frequently been found to be decreased (Meyer et al., 2006). Selective serotonin reuptake inhibitors (SSRIs) and many other types of anti-depressant medications aim to increase the availability of these mono-amines. However, to date the mechanisms underlying these imbalances remain unknown.

Increased activity of the (brain) immune system may constitute another biological factor linked to the etiology of depression. Although there are currently no biomarkers for MDD, depression is associated with increased levels of inflammatory cytokines, both peripheral and intracranial (Schiepers et al., 2005; Dowlati et al., 2010). Chronic inflammatory diseases, or diseases associated with chronic inflammation have also been associated with an increased risk for MDD (Moussavi et al., 2007; Van Manen et al., 2002)

During systemic inflammation, peripheral cytokines can activate brain pro-inflammatory cytokine signaling. These pathways can induce several behavioral changes, similar to the typical symptoms of depression (including anhedonia, reduced appetite, and increased sensitivity to pain). Under normal circumstances these behavioral changes are a typical part of the illness (they are suggested to improve recovery) and disappear when the pathogen is cleared. However, when combined with other risk factors and a more chronic inflammatory reaction, these new behaviors may be more similar to clinical depression (figure 1, Dantzer et al., 2008).

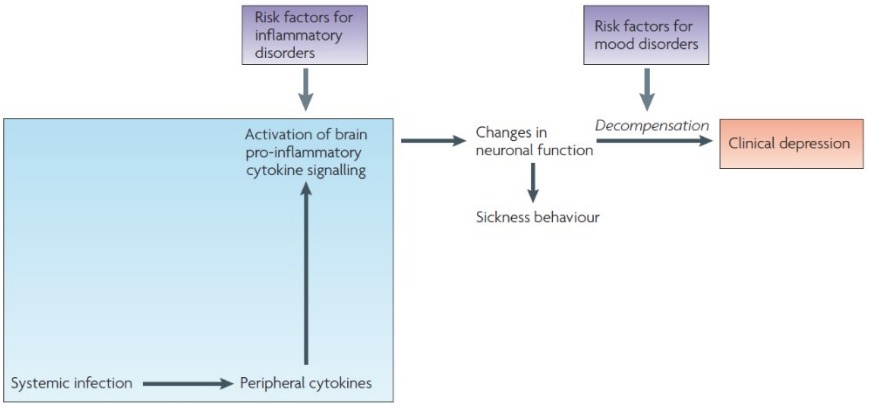

FIGURE 1: SIMPLIFIED OVERVIEW OF THE INFLAMMATION BACKGROUND PROPOSED IN

In rodent models, bacterial lipopolysaccharide (LPS, used to instigate an inflammatory reaction) activates monocytes, dendritic cells, macrophages and B cells, and promotes secretion of pro-inflammatory cytokines (e.g., IL-1B and TNF-a) (Yirmiya et al., 2001) Injections with LPS also induce many of the behavioral changes mentioned earlier (Yirmiya et al., 2001) Although its effects disappears after a few of days, LPS is a widely used method to study the relationships between neuroinflammation, behavior and brain chemistry. Several studies have shown

that inhibition of cytokine function may result in fewer or less pronounced behavioral changes following LPS injections (Maes et al., 1999).

Electroconvulsive Therapy

Although Electroconvulsive Therapy (ECT, or electroconvulsive shocks, ECS in animal studies) has developed a bad reputation as a result of widespread misuse, negative portrayal in media, and potentially severe side effects (retrograde amnesia, and general side effects and risks of anesthesia), it is currently used as a last option for treatment of MDD, schizophrenia, mania and catatonia (American Psychiatric Association, 2008). In those MDD patients that do not respond to medication, ECT may provide rapid and significant improvements (UK ECT Review Group, 2003; Heijnen et al., 2010).

Under general anesthesia, epi-cranial nodes induce unilateral or bilateral seizures in the brain. The placement of the electrodes as well as the electrical waveform and frequency of the treatment are vital for determining the effects of the treatment (Dukart et al., 2014). With regard to possible side-effects of ECT, these effects vary (depending on the variables mentioned above) and may include confusion, memory loss, muscle spasms, and learning difficulties (Dukart et al., 2014). In general ECT is carried out two or three times per week, until the symptoms of depression are alleviated.

The possible neurobiological mechanisms underlying the efficacy of ECT in treating symptoms of depression have been frequently studied over the past decades, both in human and in animal studies. Many of these studies found strong stimulating effects on neurogenesis, especially in the hippocampus. This is an interesting finding given that MDD is associated with impaired neurogenesis (Miller and Hen, 2015). Human MRI studies show a decrease in hippocampal volume in MDD patients (Sheline et al., 2003). Animal studies show that the behavioral effects of anti-depressant medication correlate with their ability to stimulate hippocampal neurogenesis. (Santarelli et al., 2003). Furthermore, chronic ECT treatment is also associated with a rapid and strong increase in hippocampal granule cells (Malberg et al., 2000).

An increase in neurotrophic factor levels was also among the more consistent findings in ECT research (Malberg et al., 2000; Segi-Nishida et al., 2008; Angelucci et al., 2002; Haghighi et al., 2013). For instance, in rat studies, ECS resulted in increased levels of brain-derived neurotropic factor (BDNF) in the hippocampus. Nerve growth factor (NGF), another important regulator of neurogenesis, was found in elevated levels in the frontal cortex, after ECT (Angelucci et al., 2002). Furthermore, expression of trkB (the BDNF receptor) was found to be increased (Nibuya et al., 1995).

Vascularedothelial growth factor (VEGF) may constitute another important factor in ECS-induced neurogenesis. In animal studies, VEGF was found to stimulate neuronal proliferation and angiogenesis, in various brain areas. In the hippocampus, VEGF infusion directly increased the number of neuronal progenitor cells (Jin et al., 2002). In human studies, ECS treatment correlated with increased levels serum VEGF (Minelli et al., 2011). Moreover, rodent studies showed that inhibiting VEGF signaling blocks ECS induced neurogenesis (Segi-Nishida et al., 2008).

There is ample evidence that the inflammatory responses may constitute a crucial factor in the pathology of depression. MDD is associated with increased levels of proinflammatory cytokines, as well as other immunity related factors. Furthermore, in animal models, a strong stimulation of the immune response (e.g., an injection of LPS) resulted in behavioral changes similar to symptoms of depression (Dantzer, 2001)

Recent studies show that ECS also has a strong and lasting effect on the brain immune system. For instance, Lehtimäki et al. (2008) showed that a single ECS session induced a short ,30 min, increase in the expression of pro-inflammatory cytokines (TNF-a, IL-1B and IL-6). Another study by Fluitman et al. (2011) found that a single ECS session increased sensitivity of peripheral blood monocytes to proliferating stimuli (for instance LPS). These short increases in sensitivity were also found after the fifth and eleventh ECS session, suggesting that it is a typical aspect of the session and does not disappear with repetitive treatments (Fluitman et al., 2011). However, although a single ECS treatment increases TNF-a levels, repeated treatments result in an overall decrease in TNF-a levels

(Hestad, 2003). It is currently not clear whether this reduction or normalization of TNF-a levels suggest a suppressing effect of ECS on the immune system, or whether they are a side-effect of general clinical remission.

Experimental setup and main questions

Recent literature suggests that ECS rapidly increases the levels of proinflammatory cytokines (Lehtimäki et al., 2008). Repeated treatments, however, eventually result in an overall decrease of proinflammatory cytokine levels (hestad et al., 2003). In healthy mice, it was shown that ECT acutely activated microglia, inducing a short term increase of immune activity. In the long run however, this treatment had no effect on microglial activity. Thus, while a single shock may acutely induce neuroinflammation, this does not seem to occur after repeated shocks and there may even be a reduction of inflammatory cytokines. This may resemble a resilience effect. The two experiments in the current project were designed to test whether repeated ECS induces a state of resiliency in the brain, making it less vulnerable to neuroinflammation.

One of the mechanisms underlying this resilience effect may involve restructuring of brain networks, such as the hippocampus and prefrontal cortex, which may explain the reduced neuronal activity after repeated ECS. ECS is known to be a powerful inducer of neurogenesis (Madsen, 2000). It is also known to increase proliferation of glial cell types (Orzi et al., 1990). Furthermore, ECS induces the formation of new synapses (Chen et al., 2009). Together, this may lead to an alteration of neuronal networks. In the present project we set out to answer the following questions: How does repeated ECS affect neuronal activity as a function of time? And does ECS induce resilience against neuroinflammation and the accompanying symptoms of depression?

Our first experiment (A) aims at studying the short term and long term effects of ECS on neuronal activity and neurogenesis in healthy mice. Healthy mice received repeated ECS (1, 5 or 10 shocks), and were sacrificed after 2 hours, 6 hours, or 24 hours. Brain chemistry was analyzed using immunohistochemical stainings to estimate both neuronal activity (C-Fos) and the number of macrophages in various brain regions.

We hypothesized that multiple ECS sessions would result in a less pronounced increase in C-Fos activity compared to single ECS treatments.

In our second experiment (B), healthy Mice received daily ECS for 7 days, followed by an LPS injection in the brain. Changes in anhedonia (a symptom of MDD, Willner et al., 1992) were estimated by a long lasting sucrose preference test. An open field test was performed to estimate exploratory behavior. Immunohistochemical stainings were used to measure neurogenesis (BRDU) and microglial activity (IBA-1).

We expected LPS to induce symptoms of depression and to induce an immune response in the brain, resulting in an increased proliferation and activity of microglial cells and decreased neurogenesis. We also expected ECS to induce a state of resilience, thereby diminishing symptoms of depression and the immune response in the brain, compared to the control group. Furthermore, we expected to see an increase in neurogenesis following ECS treatment, compared to LPS-treated control animals.

Method

Experiment A: C-Fos activity following repeated ECS

Subjects

Forty male C57Bl/6 mice (a subsample of the total experimental 72 animals, ordered from Janvier) were housed in single units under standardized conditions ((temperature 21 ± 2 °C, humidity 50–60 %, 12:12 h light/dark cycle), with access to ad lib food and water. After a weeklong habituation period, the animals were randomly assigned to one of six groups (table 1).

Sacrificed after:	**2 hours**	**6 hours**	**24 hours**	**total**
1x ECS	3	4	4	11
1x Sham	1	1	1	3
5x ECS	7		7	14
5x Sham	2		2	4
10x ECS	3		3	6
10x Sham	1		1	2

TABLE 1: EXPERIMENTAL GROUPS IN EXPERIMENT A: ANIMALS RECEIVED 1, 5 OR 10 ECS OR SHAM TREATMENTS, AND WERE SACRIFICED AT 2, 6 OR 24 HOURS AFTER THE LAST TREATMENT.

Procedures and timeline (fig. 2)

The experiment started with a one week habituation period prior to the start of the experiment. Next, animals were randomly assigned to their groups. Animals in the 1x ECS or 1x sham groups received a single treatment, and were sacrificed after 2, 6 or 24 hours. Animals in the 5x or 10x ECS/Sham groups received daily ECS or sham treatments for 5 or 10 consecutive days. These animals were sacrificed 2 or 24 hours after their last treatment.

After the animals were sacrificed, tissue and blood samples were collected via perfusion (with 4% paraformaldehyde).

ECS

The animals were anaesthetized with sevoflurane prior to ECS. The shocks (1 second, at 50 - 100 mA, frequency 50 Hz, pulse width 0.5 ms) were administered via earclips placed bilaterally. After the ECS or sham procedure, the animals were returned to their cage in a dark recovery room and monitored.

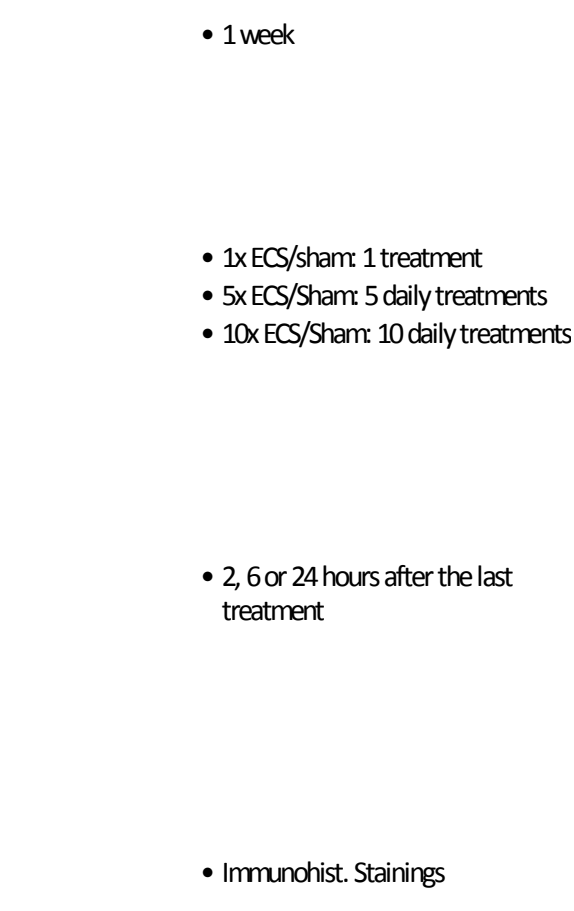

FIGURE 2: TIMELINE EXPERIMENT A

Immunohistochemical staining

Three mice per group were transcardially perfused with a saline solution containing 0.5 % heparin, followed by a 4 % paraformaldehyde (PFA) solution in 0.1 M phosphate buffer. Brains were removed and postfixated for 24 hrs in 4 % PFA in 0.1 M PBS, followed by 18 hrs in a 30 % sucrose solution for cryoprotection. Next, the brains were frozen using liquid nitrogen and cut into sections of 20 µm.

Sections containing the hippocampus, prefrontal cortex, and piriform cortex were stained for ChAT (Choline Acetyltransferase), C-Fos and PSA-NCAM (Polysialylated-neural cell adhesion molecule). Several pilots were done for each type of staining to ensure optimal staining and contrast with background.

The analysis of C-Fos was done using Image-Pro software (Image-Pro Plus 6.0.0.26, Media Cybernetics, Inc. Rockville, USA). Photographs were taken at ×100 magnification. In the hippocampus the hylus, Dentata gyrus, ca1 and ca3 regions were selected. The piriform cortex was also selected. Intensity and size thresholds were adjusted to ensure that only the C-Fos positive cells were measured. The software then estimated the number of cells. Finally, the cell bodies to total coverage ratio was calculated. This ratio was expressed as a percentage of the value of the control group. Since the control groups in the current experiment often consisted of a single mouse, 0 values were replaced by an average of the overall group (1x sham, 5x sham or 10x sham). Due to time constraints, samples stained for ChAt and PSA-NCAM were not analyzed.

Stainings

ChAT is an enzyme responsible for the synthesis of acetylcholine. It is expressed in cholinergic neurons in different regions of the brain, spinal cord, retina and PNS. ChAT antibody staining is frequently used to study cholinergic cell populations. Cholinergic neurons participate in learning, movement and memory (Oda, 1999).

PSA NCAM is a homophilic binding glycoprotein expressed on the surface neurons and is involved in cell–cell adhesion, neurite outgrowth, synaptic plasticity, and learning and memory (Bonfanti, 2006, Rutishauser et al., 1988). PSA-NCAM is often used as a marker of developing and migrating neurons and of synaptogenesis (e.g., in the developing brain, or in the adult hippocampus).

C-Fos is an immediate early gene that codes for a transcription factor. C-Fos is considered to mediate long-term changes in neural functioning. Once formed, the c-Fos protein interacts with the factor jun to create a transcription factor that regulates the expression of “downstream” late response genes. Variation in the expression of c-Fos has been used to identify and study brain areas involved in cytokine-induced depression-like behavior (Dantzer, 2008).

Experiment B: Effects of ECS on LPS induced depression-like behavior

Subjects

Sixteen male C57Bl/6 mice (a subsample of a larger project involving 32 mice, ordered from Janvier) were housed in single units under standardized conditions ((temperature 21 ± 2 °C, humidity 50–60 %, 12:12 h light/dark cycle), with access to ad lib food and water. After 2 weeks of habituation, the mice were semi-randomly assigned to one of the following four groups:

- ECS treatment and an LPS injection (4 mice, ECS+LPS)
- ECS treatment and a Phosphate-buffered saline (PBS) injection (4 mice, ECS+PBS)
- Sham treatment and an LPS injection (4 mice, Sham+LPS)
- Sham treatment and in PBS injection (4 mice, Sham+PBS)

Average starting weight and base sucrose preference were similar in all four groups.

Procedures and timeline (fig. 3)

The experiment started with a 2 week habituation period. During the second week of habituation (on day -7) the sucrose preference test was started.

Following habituation, animals were assigned to different groups and received either daily ECS or daily sham treatments for 10 consecutive days (0-10). Immediately following the ECS treatments, the animals received either an LPS or a PBS injection (days 11 and 12). One day later (days 13 and 14), the animals were injected with BrdU (10 ug/g) or saline. On day 15 all animals were tested in the open field exploration test. All animals were sacrificed afterwards, and tissue and blood samples were collected via perfusion (half of the mice with 4% paraformaldehyde, the other half with saline).

Brain slices containing the hippocampus were analyzed with immunohistochemical staining for IBA-1 and BRDU.

ECS

The animals were anaesthetized with sevoflurane prior to ECS. The electroshocks (1 second, at 50 - 100 mA, frequency 50 Hz, pulse width 0.5 ms) were administered via earclips placed bilaterally. After the ECS or sham procedure, the animals were returned to their cage in a dark recovery room.

LPS and PBS injections

On day 11 and 12, the mice were given an intracerebroventricular injection with either LPS or PBS. First, the mice were anesthetized with sevoflurane and fixated in a stereotact. Then, a subcutaneous injection of 2.5 mg/kg finadyne was administered prior to the LPS/PBS injection, to provide extra analgesics prior to the LPS/PBS injections. Small holes were drilled perpendicularly to the skull, using a small needle. The mice were then injected with 1 µl PBS or 1 µl 5 mg/ml LPS (l-6529, serotype 055:B5, Sigma-Aldrich) in PBS at the following

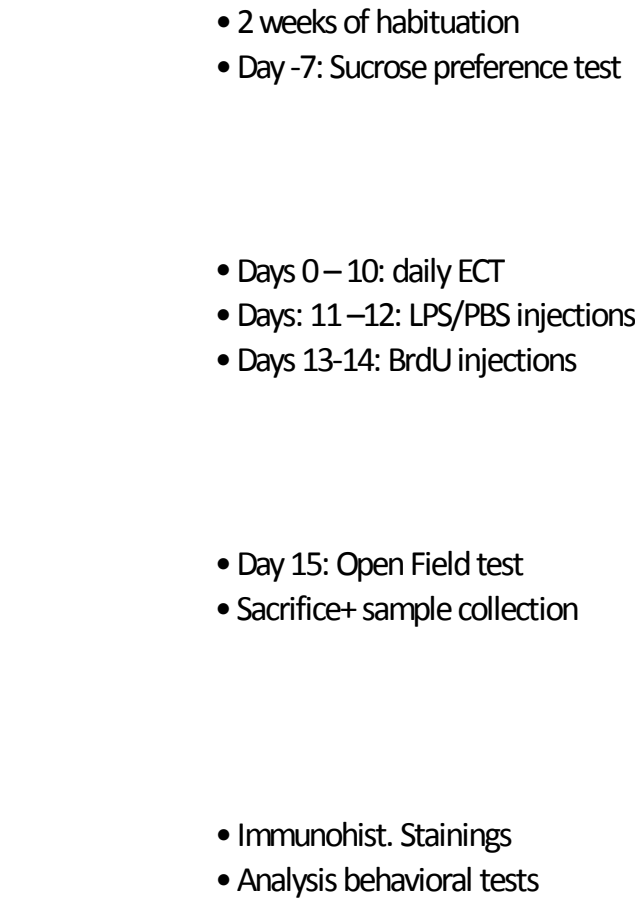

FIGURE 3: TIMELINE EXPERIMENT B

coordinates: −2.5 mm dorsal/ventral, −1.0 mm lateral, and −0.5 mm anterior/posterior from bregma. LPS/PBS was administered at a constant rate of 0.3 µl/min using a syringe pump (TSE Systems, Bad Homburg, Germany) in combination with a 25-µl Hamilton syringe connected to a 1-µl Hamilton needle (cat. nr.170431, Omnilabo). After removal of the pump, the perforation was closed with dental cement and the incision was closed with sutures.

Behavioral tests

Sucrose preference test (Willner et al., 1987)

The sucrose preference started during the second week of the habituation period and was meant to assess the mice's bias towards sweetened food. The loss of interest in pleasure (anhedonia) is a characteristic of depression and sickness behavior both in humans and in animal models. Both chronic stress and inflammation caused by LPS injections induce decreased preference for sucrose solution over water. This effect is significantly reduced or even entirely removed by antidepressants, (Nielsen et al. 2000). The sucrose preference test can be carried out as a standalone test, and may be repeated to show changes in preference as a function of treatment.

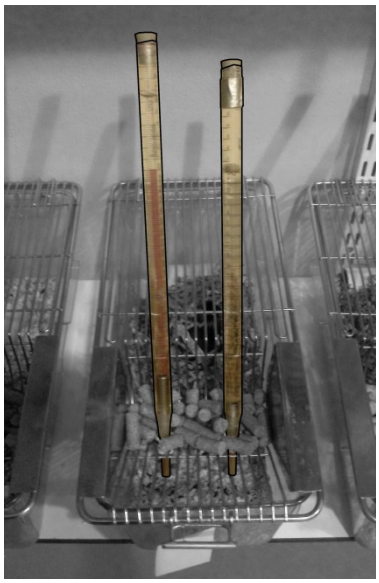

Figure 4: Experimental setup sucrose preference test. Two pipets (outlined) were placed in the cage.

Pilot experiments were done to optimize the repeated sucrose preference test. The animals did not show any significant changes in preference in the absence of outside experimental factors (stressors etc.). However, the animals were had a tendency to develop a side-bias when pipets remained in the same position for extended periods of time. Periodically switching the pipets removed this bias. The relatively low sucrose solution and daily cleaning schedule also prevent fungus and bacterial infections of the pipets (which could result in an immediate and long lasting refusal to drink sucrose solution).

The sucrose preference tests started 1 week prior to the daily ECT treatments to establish a base preference necessary to ultimately compare the effects of ECS/Sham versus LPS/PBS injections. All mice had free access to two pipets (made from a 50 ml lab pipet, a cork and a standard steel drinking tab). One pipet was filled with water, the other with a sucrose-solution (10%). The sucrose solution was made by dissolving sucrose in drinking water, the same drinking water was used for the control pipet. Pipets were read, cleaned (standard industrial dishwasher and soap used for daily cleaning of all animal water bottles), switched (to reduce any artifacts produced by side bias) and refilled daily. All pipets had been tested for leakage prior to the experiment. The test comprised 3 weeks, starting 1 week prior to the ECT trials until the end of the experiment. This allowed the measurement of the change in sucrose preference following ECT/sham versus LPS/PBS injections. Sucrose preference was estimated as the percentage of sucrose-solution intake over the total volume of fluid intake per day. Averages of 2 or 5 days were also estimated. A running average was used to remove artifacts.

Open field exploration test (Walsh & Cummins, 1976)

On day 15, all animals were tested in the open field exploration test. This test was used to measure locomotion, exploration and anxiety in an unfamiliar environment. The open field (OF) arena is an enclosed circular arena located in a brightly lit room. The surface was divided into 2 different zones (middle and border). Movement and location were recorded and tracked using Ethovision software (Noldus Information Technology, Wageningen, Netherlands). Behavior (grooming and rearing) was assessed using a behavior tracking program (ELINE, Rijksuniversiteit Groningen, Groningen, Netherlands).

Figure 5: Open Field exploration arena. The 2 outer zones form the border zone, the inner cirlce forms the center zone.

Distance moved, time spent in zones and overall behavior can give an indication of locomotion, exploratory drive and levels of anxiety. When untreated, animals tend to spend more time in the peripheral zones and only a small amount of time in the relatively open and well-lit center zones. This pattern can be altered through the administration of anxiogenic or anxiolytic drugs (Walsh &Cummings, 1976).

Animals that spend more time in the peripheral zone, or explored or moved less than control treated animals were considered to be more anxious. Although the effect has been found to be more pronounced in rats, a similar effect of anxiety on exploratory behavior can be found in most rodent species (including mice and hamsters) albeit less strong.

The open field exploration test was performed during the light-phase to minimize disruption of the circadian rhythm and to keep the entire experiment consistent (behavioral experiments in earlier trials were also performed during the day). The testing area was near the cage room, to minimize stress caused by transport.

The animals were placed in the center of the arena and were allowed to explore the environment for 3 minutes. Researchers exited the testing area after placing the animal in the arena. The animals' movements and behavior were recorded on the ethovision pc. After the tests the animals were returned to their cages and the arena was cleaned with soapy (non-smelling) water

Immunohistochemical staining

Iba1 and BrdU

Bromodeoxyuridine (BrdU) is a synthetic nucleoside (analog of thymidine) and commonly used to detect proliferating cells. It can be incorporated into newly synthesized DNA of replicating cells as a substitute of thymidine. By using an antibody specific to BrdU to stain samples, it is possible to estimate which cells were actively replicating when the animal was injected (day 13 or 14, one day after the last ECS/Sham treatment).

Iba1 is a 17-kDa EF hand protein (calcium binding proteins) that is specifically expressed in macrophages/ microglia and is upregulated during the activation of these cells. By staining samples for this protein, microglia can be made visible, allowing cell counting and morphology analysis (passive cells vs. activated cells).

Three mice per group were transcardially perfused with a saline solution containing 0.5% heparin followed by a 4% paraformaldehyde (PFA) solution in 0.1M phosphate buffer. Brains were removed and postfixated for 24 hours in 4% PFA in 0.1M PBS, followed by 18 hours in a 30% sucrose solution for cryoprotection. Next, the brains were frozen using liquid nitrogen and cut into sections of 20 µm. Sections containing the hippocampus were stained for Iba-1 and BrdU. Unfortunately due to time constrains the Iba-1 stainings were not analyzed in this project. The BrdU stainings were analyzed using Image-Pro software (Image-Pro Plus 6.0.0.26, Media Cybernetics, Inc. Rockville, USA). Photographs were taken at ×100 magnification. Hippocampal hilus area was selected for analysis. Intensity and size thresholds were adjusted to ensure that only the BrdU positive cells were measured. The software then estimated the number of cells. Finally, the cell count to total coverage ratio was calculated.

Results

Experiment A

Increase in C-Fos activity after single or repeated ECS, in the hippocampus and piriforme cortex (fig. 6).

C-Fos activity was measured as the number of C-Fos positive cells per μm^2 and is shown as a percentage of the control values. Effects of repetition (1x, 5x or 10x) and changes over time (2 hours, 6 hours and 24 hours) were compared.

Ca1 (fig. 6.A Neither ECS repetition nor time appeared to have a significant effect on C-Fos activity in the Ca1 region (ANOVA, P=0.2397, df= 29). The 1x or 5x repeated ECS conditions did seem to increase C-Fos activity short-term (2-6 hours), however this effect did not reach significance (1x: ANOVA, p=0.4975, df=9. 5; t-test, p=0.0607, df=12). 10x repeated ECS resulted in a much smaller increase in C-Fos activity, which did not change significantly as a function of time (t-test, p=0.80073, df=4).

Ca3 (fig. 6.B): In the Ca3 region, ECS repetition had a significant effect on C-Fos activity, but only in the longer term (after 24 hours), 10x repeated ECS resulted in a significantly lower increase in C-Fos activity compared to 1x repeated ECS and 5xECS (ANOVA, p= 0.0333, df= 12). Although the short term differences appeared to be much larger, they were not significant (ANOVA, p=0.192, df=12). C-Fos activity showed a tendency to return to control values after 24 hours. However, this effect only reached significance in the 5x repeated ECS groups (two-tailed t-test, p=0.0327, df=12).

Hilus (fig. 6.C): No significant effects of ECS repetition were found short term (comparing 1x, 5x, and 10x repeated ECS after 2 hours, ANOVA, p=0.6280, df=12). However long term (24 hours), 10x repeated ECS significantly increased C-Fos activity compared to 5x repeated ECS (ANOVA, p=0.0383, df=12). C-Fos activity increased 2 hours after 5x repeated and 10x repeated ECS, and significantly dropped after 24 hours (t-tests, p= 0.0007, df=12; p= 0.161, df=4). Though the same effect was found after a 1x repeated ECS, it did not reach significance (ANOVA, p=0.1612. df=9).

Dentate Gyrus (fig. 6.D): Short term, no effects for repetition were found (ANOVA, p=0.8325, df=12). After 24 hours, the C-Fos levels in the 10x repeated ECS samples was significantly higher compared to 1x and 5x repeated ECS conditions (ANOVA, p=0.0030, df=12).

1x, 5x, and 10x repeated ECS all increased C-Fos activity short term (up to 15000%). Long term, C-Fos activity significantly decreased in all groups (t-tests, p=0.0136, df=12; p=0.0012, df=4; ANOVA, p=0.0139, df=9).

Piriform Cortex (Fig. 6.E): Short term: 2 hours after treatment, C-Fos activity increased in all three groups. This increase was significantly higher in the 10x repeated ECS group (ANOVA, p=0.0001, df=12). After 24 hours, C-Fos activity significantly decreased in all groups (1x: ANOVA, p=0.0001, df=9. 5x: t-test, p=0.0015, df=12. 10x: t-test, p=0.0448, df=4), and between group were no longer significant (ANOVA, p=0.0834, df=12).

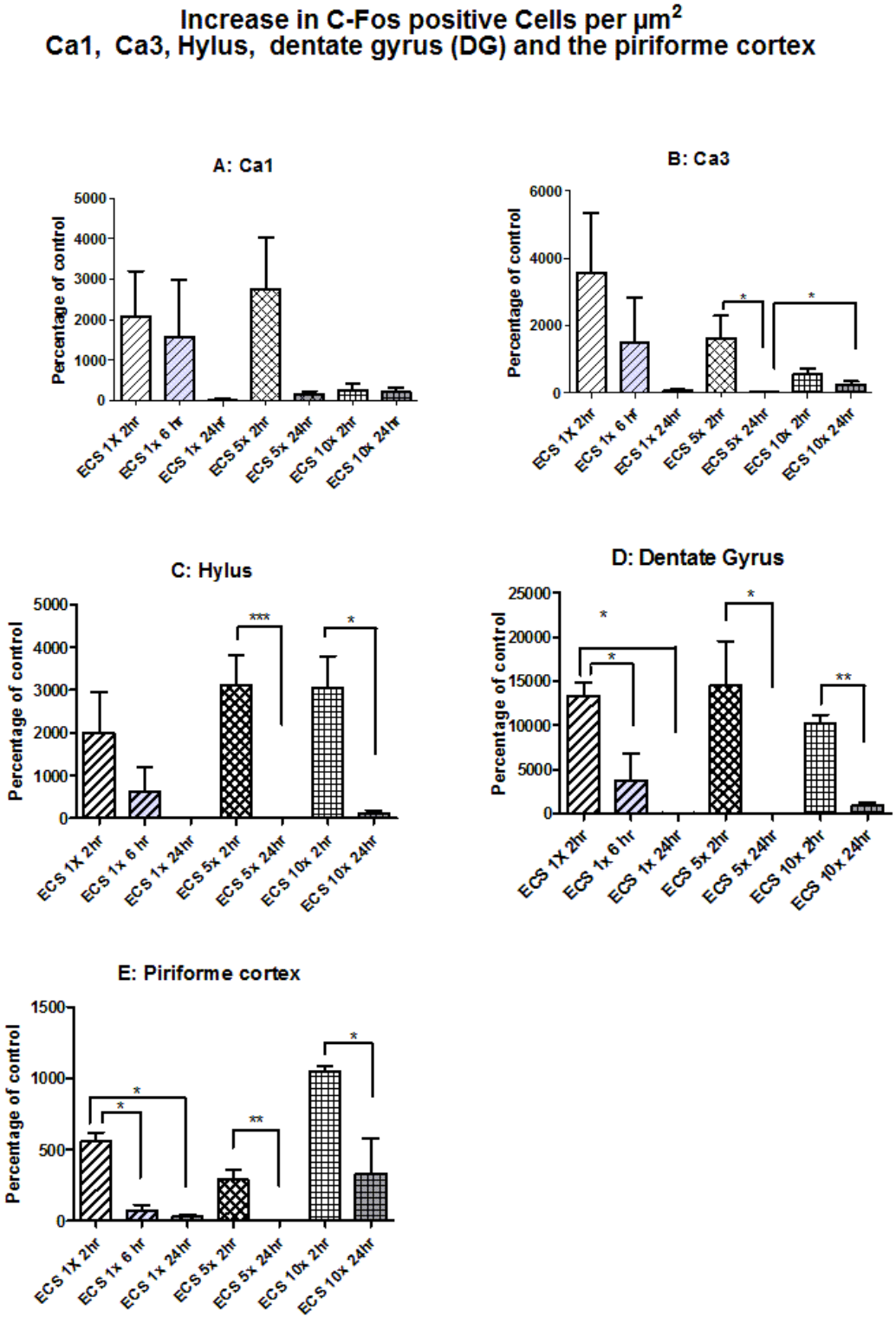

FIGURE 6 : INCREASE IN C-FOS POSITIVE CELLS PER MM2, IN THE HIPPOCAMPUS AND PIRIFORME CORTEX. **BARS INDICATE MEAN + SEM. * = P<0.05. ** = P<0.001. GROUP SIZES: ECS 1X 2HR = 3, ECS 1X 6 HR = 4, ECS 1X 24HR = 3, ECS 5X 2HR = 7, ECS 5X 24HR = 7, ECS 10 2HR = 3, ECS 10X 24 HR = 3.**

Experiment B

Open Field Exploration Test (fig. 7)

Exploration (fig.7.A)

Exploration is shown as a percentage of the total time (3 minutes) spent in the border or center zone of the arena. More time spent in the center indicates decreased levels of anxiety or depression-like behavior.

The animals spent the majority of time in the border zones (70-80%; T-tests, $P<0.0001$, df=18, df=16).

The ECS+PBS group spent significantly more time in the border zone (ANOVA, $P=0.0089$, df=38), and less significantly less time in center zone of the arena (ANOVA, $P=0.0033$, df=38). This effect was most pronounced for the comparison the ECS+PBS group versus the SHAM+PBS group (Tukey post-analysis). Although the ECS+LPS group also appeared to spent slightly more time in the border zone compared to the SHAM+LPS group, this effect was not significant (t-test, $p=0.2325$, df=17).

LPS treatment did not appear to affect exploration in neither the border nor the center zones. No significant differences were found for the comparison of the SHAM+LPS group versus the SHAM+PBS group (t-tests, $p=0.5121$, df=17), nor for the comparison of the ECS+LPS group versus the ECS+PBS group (t-test, $p=0.1872$, df=17).

Behavior (fig.7.B)

Time spent either exploring or grooming is shown as a percentage of the total time spent in the arena. A higher percentage of time spent grooming indicates decreased anxiety or depression-like behavior.

The animals (in all four groups) spent significantly more time on exploring the arena than on grooming (t-tests, $P<0.0001$, df=18, 16). A small significant difference was found between the SHAM+PBS group and the ECS+LPS group (ANOVA, $P=0.0347$, df=38). No significant between group effects were obtained for grooming (ANOVA, $p=0.0599$, df=38).

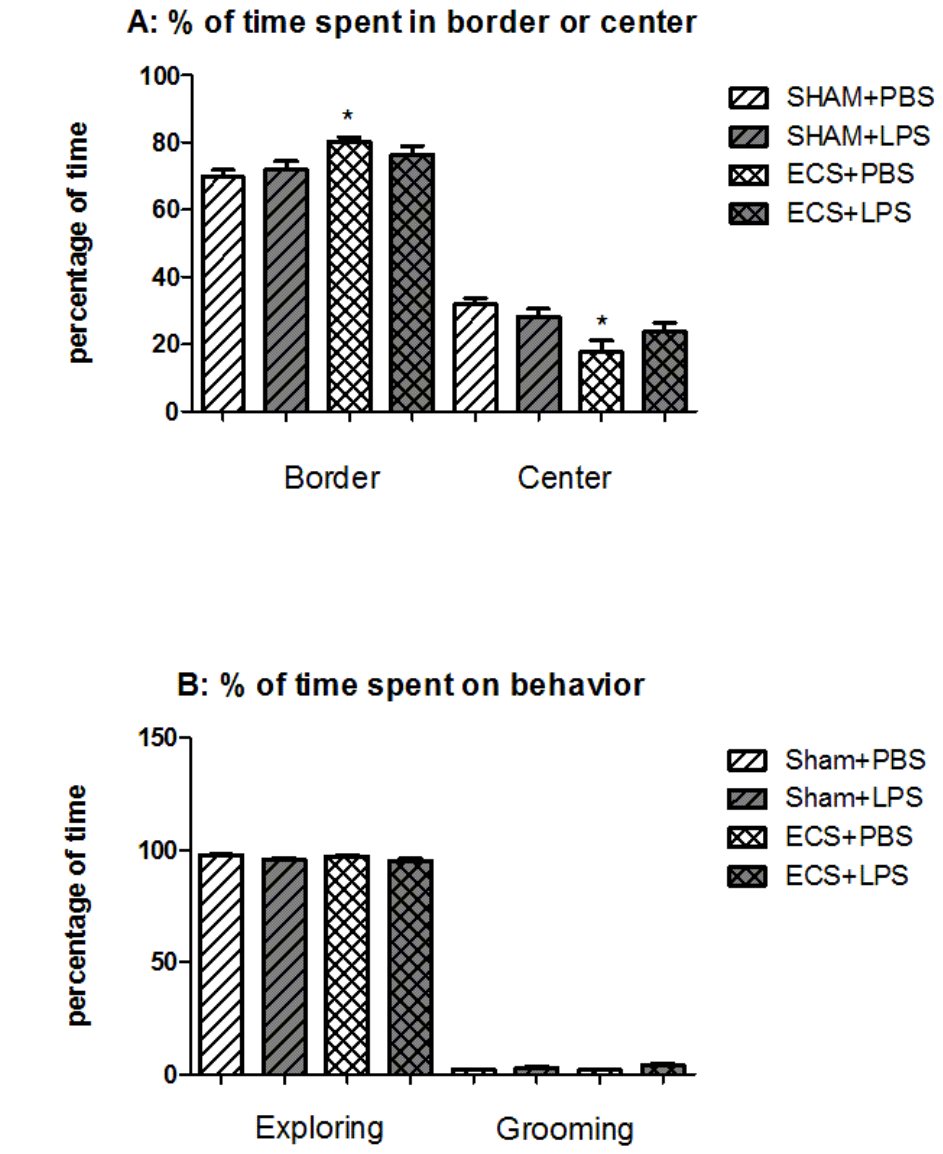

FIGURE 7: OPEN FIELD EXPLORATION TEST. TIME SPENT IN BORDER OR CENTER ZONE AND TIME SPENT ON BEHAVIOR. **BARS SHOW MEAN PERCENTAGES + SEM. * = P<0.05. GROUP SIZES: SHAM+PBS=10, SHAM+LPS=10, ECS+PBS=10, ECS+LPS=9.**

Sucrose Preference Test (Fig. 8)

Daily measurements of mL sucrose and mL water consumed, were used to calculate the sucrose/water ratio. These daily values were then standardized to day 5 and 6 (the last two days of the habituation duration). ECS/Sham treatments started on day 7, LPS/PBS injection was administered on day 17.

Overall, no significant effects for ECS were found (repeated ANOVA, $p=0.7651$, dfd=324). Time did significantly affect sucrose preference (repeated ANOVA, $p<0.001$, dfd=324). Although the differences on day 17 (after the LPS injection) appeared to be the largest, they did not reach significance (ANOVA, $p=0.0761$, df=39).

BrdU (fig. 9)

BrdU positive cells were counted in the Hippocampal hilus, and are shown as cells per μm^2. A higher BrdU cell count indicates an increase in cell division (neurons). Average values ranged from 0.001098 cells/µm2 (Sham+PBS) to 0.001796 cells/µm2 (Sham+LPS).

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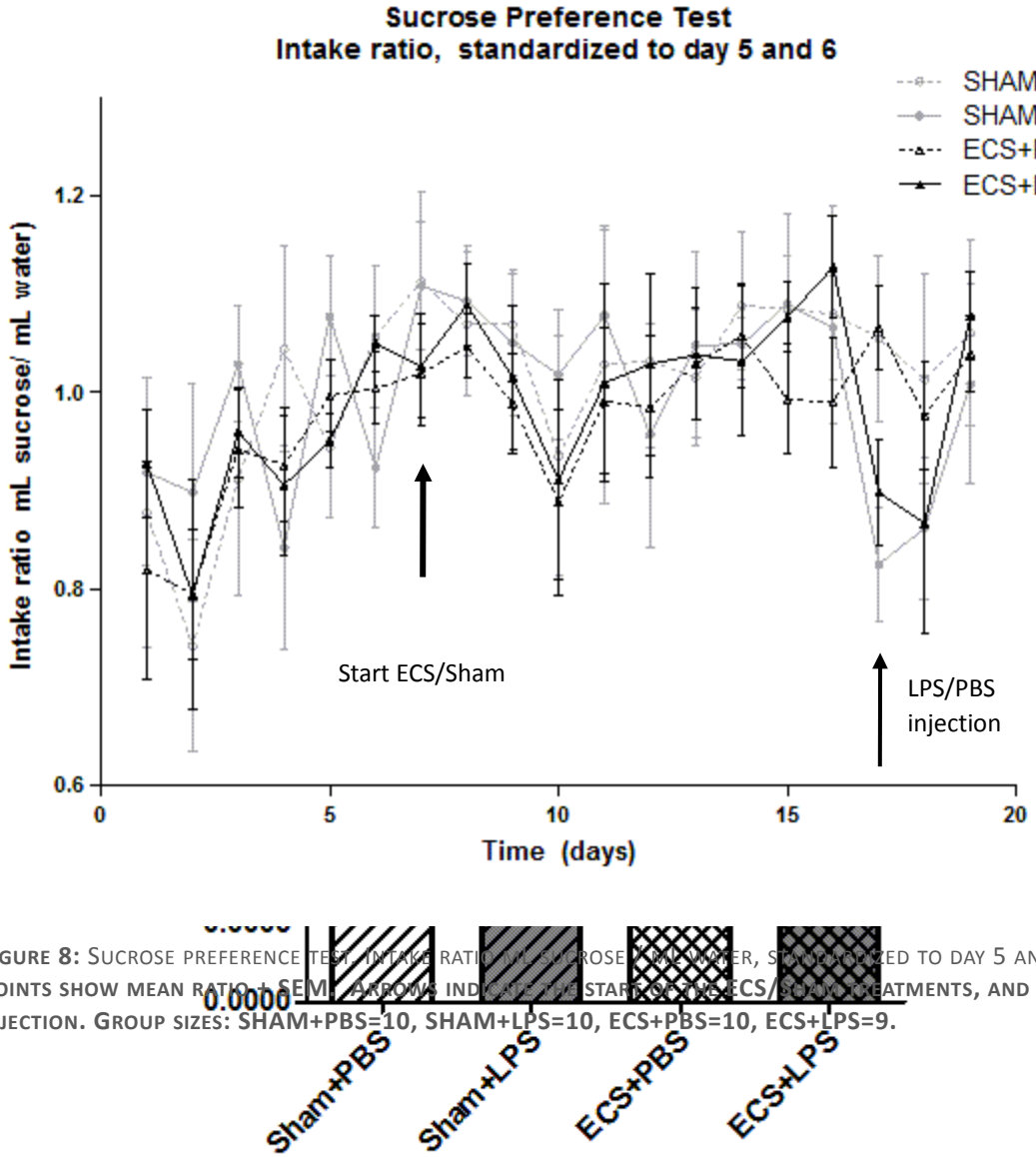

FIGURE 8: SUCROSE PREFERENCE TEST. INTAKE RATIO OF SUCROSE AND WATER, STANDARDIZED TO DAY 5 AND 6. POINTS SHOW MEAN RATIO + SEM. ARROWS INDICATE THE START OF THE ECS/SHAM TREATMENTS, AND THE LPS INJECTION. GROUP SIZES: SHAM+PBS=10, SHAM+LPS=10, ECS+PBS=10, ECS+LPS=9.

FIGURE 9: BRDU POSITIVE CELL COUNT IN THE HIPPOCAMPUS HYLUS. **BARS INDICATE MEAN CELL COUNT PER MM^2 + SEM. SHAM+PBS (MEAN= 0.001098 ± 0.0001541, N=6); SHAM+LPS (MEAN= 0.001796 ± 0.0002092, N=7); ECS+PBS (MEAN= 0.001233 ± 0.0001972, N=6); ECS+LPS (MEAN= 0.001013 ± 0.0002233, N=5)**

Discussion

Earlier studies have shown electroconvulsive shocks (ECS) to be a potent antidepressant treatment (Heijnen et al. 2010). In animal studies, ECS resulted in both increased brain activity and increased neurogenesis, as well as an altered immune response (Santarelli et al., 2003). Interestingly, the results of repeated ECS sessions differ from those of single ECS treatments, in terms of neurogenesis, immune reaction markers and activity. Single ECS sessions were shown to increase brain activity and immune reaction markers (Fluitman et al., 2011; Hestad et al., 2003). In contrast, repeated ECS treatments showed overall decreased levels of brain activity and immune reaction markers, as well as an increase in neurogenesis (Hope et al., 1994; Malberg et al., 2000; Winston et al., 1990). To date it remains unclear whether the different effects on neurogenesis and brain activity triggered by repeated ECS treatments can reduce the symptoms of neuroinflammation-related depression. The current project was designed to test two aspects of ECS. Firstly, whether repeated ECS has stronger and/or longer lasting effects compared to single ECS treatments (experiment A). Secondly, whether repeated ECS induces a state of resilience against neuroinflammation-related depression (experiment B).

Experiment A: C-Fos activity following repeated ECS

Based on earlier findings in the literature (see above) and earlier pilot experiments, we hypothesized that single ECS sessions would result in a larger increase in brain activity (as measured in C-Fos expression) compared to repeated (5x or 10x) ECS. Our results showed that C-Fos activation increased substantially short term (2 hours) in nearly all tested brain regions in the 1x ECS, 5x ECS and 10x ECS conditions. Long term (24 hours) C-Fos activity returned to control levels in all three ECS conditions.

In the hippocampal areas, the 1x and 5x ECS conditions seemed to result in a higher increase in C-Fos expression compared to the 10x ECS condition. However, this short term effect of repetition only reached significance in the Ca3 region. Long term - after 24 hours – C-Fos levels in the 1x and 5x ECS conditions returned close to control levels. However, the 10x ECS condition consistently resulted in higher long term C-Fos activity compared to the 1x and 5x ECS conditions, which could indicate an increased baseline c-Fos activity following 10x repeated ECS. The 10x repeated ECS treatment could therefore result in a more pronounced and long-lasting effect on brain chemistry compared to 1x and 5x repeated ECS. The consistent decrease in all brain areas might suggest that initial increase in C-Fos activation is a short term effect of ECS. This decrease in C-Fos activity is partly confirmed by the 1x ECS 6 hours results, which were consistently lower than the 2 hour values, but higher compared to the 24 hour measurements.

The piriform cortex results deviated from the hippocampal results, in that the 10x ECS condition resulted in a much larger increase in C-Fos activity compared to the other repetition conditions (1x and 5x). The larger increase in C-Fos activity in the ECS 10x condition persisted, resulting in higher C-Fos activity 24 hours compared to other ECS conditions.

Experiment B: Effects of ECS on LPS induced depression-like behavior.

BRDU

We hypothesized that Lipopolysaccharide (LPS) injections would induce an immune response in the brain (including an increased proliferation, an increased activity of microglial cells, and decreased neurogenesis). This immune response was also expected to trigger depression-like behavior. We expected repeated ECS to reduce these symptoms by inducing a state of resilience.

In contrast to our hypothesis, we found that the BrdU positive cell count was significantly higher in the Sham+LPS groups compared to the other groups (Sham+PBS, ECS+LPS, and ECS+PBS). This indicates that the effect of LPS on the BrdU positive cell count in the Sham group was absent in the ECS treated group. However, the increase in the number of BrdU positive cells following LPS was in contrast to what was expected.

One possible explanation for this finding could be that repeated ECS inhibited the LPS effect on neurogenesis. Another explanation could be that the type of staining used did not exclude the possibility of the presence of dividing immune cells (also caused by LPS). During earlier pilot experiments, IBA-1 staining results showed very few microglia in the GCL (granule cell layer) even during massive microglia activation. Although this does not rule out the presence of other cell types, it suggests that the majority of BrdU positive cells are dividing neuronal cells. A double-staining (IBA-1 and BrdU) could potentially be used to determine whether dividing immune cells were present in the analyzed regions, however, early attempts at such a staining were unsuccessful.

Open field exploration test and sucrose preference test

In the open field exploration test and the sucrose preference test, we expected LPS to result in increased depression-like behavior. In the open field exploration test, the mice in the Sham+LPS group were expected to spend more time in the border zone and less time in the center zone of the arena, compared to the Sham+PBS group. In the sucrose preference test, we hypothesized that the Sham+LPS treatment would result in a decreased sucrose to water ratio following LPS-injection. It was, therefore, hypothesized that mice treated with ECS prior to the LPS injection would not show depression-like behavior. Instead, we expected animals in the ECS+LPS group to show behavior more similar to the Sham+PBS group.

We found no significant effects of LPS on exploration behavior in the arena, nor was there a significant effect on sucrose-preference. In the open field exploration test, ECS+PBS treatment resulted in a slight, but significant, increase in time spent in the center zone, and a decrease in time spent in the border zone, compared with the Sham+PBS treatment. This suggests that in the absence of LPS, ECS can decrease anxiety-like behavior during exploration. No significant effects of ECS or LPS were found on other behavior in the arena (i.e., grooming), or on sucrose-preference.

Since LPS did not appear to alter behavior as measured in our behavioral tests, it was not possible to reject or confirm our hypothesis on the effects of ECS on depression-like behavior. Although the LPS injections (method, amount, and timing) were carried according to a protocol used in earlier (successful) experiments, it is possible that in this experiment, the LPS injection simply did not have a large enough inflammatory effect to result in behavioral changes.

Implications

The current results suggest that repeated ECS increases C-Fos activity in the short term, but not in the long term. There are some indications that 10x repeated ECS has a more long lasting effect on C-Fos activity than 1x or 5x repeated ECS. We also found some indications that 10x repeated ECS had a more long-lasting increased C-Fos activity compared to the 1x and 5x repeated treatments, further suggesting that repeated ECS can result in lasting alterations to brain activity. How exactly these changes in neuronal activity relate to the anti-depressant effects of ECS remains mostly speculative. The altered long term effects of chronic ECS might indicate a more structural change to neuronal networks, as a result of chronic ECS. Chen et al. 2008 found that repeated ECS resulted in an increase in synapses in the hippocampal area. An increase in synapses could be related to an increase in neuronal activity in the same area. The change in neuronal activity could also be related or even a result of the increase in neurogenesis following chronic ECS, found in other studies (Malberg et al., 2000). However, due to the limited sample size and number of analyzed areas, no definitive conclusions can be drawn from the current results.

In experiment B, ECS inhibited the effect of LPS on the BRDU positive cell count in the hippocampus. Although it remains unclear whether these BRDU positive cells were neuronal stem cells or immune cells, these results show a significant effect of ECS on LPS induced cell proliferation. These results directly contradicted our expectations, since earlier studies indicated that LPS has a detrimental effect on hippocampal neurogenesis (Ekdahl et al., 2003). Chronic ECS treatment on the other hand, has been found to significantly increase neurogenesis (Malberg et al., 2000). Furthermore, we found no significant effects of LPS were found in the behavioral tests. Therefore, we cannot draw definitive conclusions regarding the possible protective effects of repeated ECS on LPS-induced behavioral

changes. What caused these surprising results is unclear. Both the BrdU+ and the LPS protocols were used in earlier experiments and found to be effective.

In both experiments, there are still many samples to be analyzed. IBA-1, CHAT and PSA-NCAM stainings of various brain regions, as well as brain tissue samples from earlier experiments could potentially increase group sizes and, therefore, may give a more precise and complete picture of the effects of repeated ECS. It is also possible to re-analyze the BRDU samples with a double-staining protocol (BRDU + IBA-1), to check whether the cells counted were neuronal stem cells or immune cells.

In experiment B, ECS treatments and the LPS injection could not be administered during the same time-period. We could, therefore, only test the effects of repeated ECS on depression-like behavior induced at a later stage in the experiment. By using a different method of inducing depression-like behavior (e.g., a chronic environmental stressor) that does not interfere with ECS treatment, the direct effects of ECS may potentially be measured. If applicable, this method could also be used to more accurately compare the effects of ECS prior to depression-like behavior versus the effects of ECS during depression-like behavior.

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