Gender differences in rat brain serotonin transporter levels after exposure to prenatal stress and/or chronic mild stress in early adulthood

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Acknowledgements 2

Abstract 3

Dutch abstract 4

1. Introduction 5

1.1 Stress 5

1.2 Serotonergic system 9

1.3 Prenatal stress 11

1.4 Predictive Adaptive Responses (PARs) 13

1.5 Aim of the study 14

2. Methods and Materials 15

2.1 Animals 15

2.2 Immunohistochemistry 16

2.3 Statistics 16

3. Results 17

3.1 Outer DG 17

3.2 Inner CA3 18

4. Discussion 19

Conclusion 20

References 21

Appendix 23

Results of the behavioral tests 23

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Abstract

Prenatal stress and chronic mild stress are great risk factors for developing mood disorders. When the stress response cannot cope with the amount of stress, the brain will undergo long-term changes. These changes can lead to several diseases associated with chronic stress. In these diseases the serotonergic system is a main player, especially in mood disorders. Serotonin is taken up by the serotonin transporter, which is presynaptically localized. In case of depression, serotonin levels are to low. Most commercial anti-depressants act by inhibiting the reuptake of serotonin, thus increasing serotonin levels again.

In this investigation we focused on the serotonin transporter in rats which received prenatal stress and/or chronic mild stress in early adulthood. Earlier behavioural investigations with these animals revealed that depression-like behavior was increased after prenatal and/or chronic mild stress. Our goal is to link behavioural changes with the possible changes is serotonin transporter in the brain. We used immunohistochemistry to quantify the amount of serotonin transporter in the dorsal hippocampus, more specifically in the projection regions of the CA3 and dentate gyrus.

In both brain regions we observed a significant increase in the amount of serotonin transporter in male rats which had been chronically stressed, compared with the control rats. Prenatal stress itself was not effective but it appeared to reduce the effect of chronic stress. The females had no significant differences between groups, but with one exception, in the outer DG, i.e. the group which received both prenatal stress and chronic mild stress had significant more serotonin transporters when compared with the group which received only prenatal stress.

Dutch abstract

Prenatale stress en chronisch milde stress in het latere leven vormen grote risicofactoren voor de ontwikkeling van psychologische stoornissen. Wanneer de normale stressrespons de hoeveelheid stress niet meer aankan, zullen de hersenen structurele veranderingen ondergaan. Deze veranderingen kunnen leiden tot verschillende ziektes,vaak geassocieerd met chronische stress. In deze aandoeningen heeft het serotonine systeem een grote rol, vooral in psychologische aandoeningen. Serotonine wordt na signaaltransductie presynaptisch terug opgenomen door de serotonine transporter. In het geval van depressie zijn de serotonine levels te laag, dit verklaart waarom de meeste antidepressiva werken door de inhibitie van de serotonine heropname.

In deze studie lag de focus op de serotonine transporter in ratten die prenataal gestresst werden en/of chronisch milde stress ondergingen. Eerdere onderzoeken testten deze ratten in gedragstaken. Deze testen toonden verhoogd depressiegedrag in gestresste ratten.

Ons doel in dit onderzoek is om dit gedrag te linken met de serotonine transporter in de hersenen. We maakten gebruik van immunohistochemie om de hoeveelheid serotonine transporter te bepalen in de dorsale hippocampus, meer specifiek in de projectiegebieden van de CA3 en de dentate gyrus.

Onze resultaten toonden een significante verhoging in het aantal serotonine transporters in mannelijke ratten die chronisch mild gestresst waren. De vrouwtjes vertoonden geen significante verschillen tussen de groepen, met uitzondering van de dentate gyrus, waar de groep met zowel prenatale als chronische milde stress significant meer serotonine transporter vertoonde in vergelijking met de groep die enkel prenataal gestresst was.

1. Introduction

1.1 Stress

1.1.1 What is stress?

Stress is defined as a state of (perceived) threatened homeostasis and represents both the subjective experience induced by a stressor, as well as the adaptive neurochemical and behavioural response to it, in order to preserve homeostasis.(1).
A stress response results in activation of the appropriate central and peripheral nervous systems to prepare the individual for the right response.(2) The stress reaction leads to the activation of the sympathetic part of the autonomic nervous system (ANS) and is characterized by avoidance behavior and release of cortisol from the adrenal gland. The homeostatic responses to stress are necessary to provide an immediate survival advantage. In general, the stress response is designed to be of a limited duration, and the homeostatic changes are rapidly restored to normal. However, in some cases, the individuals response is not sufficient and the stress becomes chronic. (2) Chronic stress is defined as the abnormal ongoing physiological response to the continuing perception of unresolvable major threats or demands. The concept of chronic stress implies a functional reorganization of parts of the brain in a way that cannot be quickly or easily reversed.
In case of depression, the definition of stress must be changed to reflect a perception of 'loss', i.e. a feeling of emptiness, rather than a perception of 'major threats or demands'. These two perceptions however are closely related. (3)

1.1.2 Response of the autonomic nervous system

The autonomic nervous system (ANS) is part of the central nervous system. The ANS is divided in the sympathetic and parasympathetic nervous system. In case of stress, the sympathetic system will be activated, and a fight-or-flight response will be generated. This direct response includes an increase in heart rate, cardiac contractility, blood pressure and ventilation of the lungs. Glucose is set free in the bloodstream to provide energy to the body. The sympathetic nervous system also triggers the release of epinephrine and norepinephrine, wich cause similar effects to the direct stimulation, but is slower in action. (4)

1.1.3 Hormonal response to stress

The fastest hormonal response to stress consists of the hypothalamo-pituitary-adrenal (HPA) axis. It is an integrated neural and endocrine system designed to trigger an appropriate response to every form of stress. Every physical or physiological stressor will activate the HPA-axis. When the HPA-axis is activated, it will respond by releasing corticotrophin-releasing factor (CRF) from the paraventricular nucleus (PVN). This CRF is set free in the hypophyseal portal, where it stimulates the production and the release of adreno-corticotropic hormone (ACTH) from the anterior pituitary into the circulation. Both CRF and ACTH control the synthesis and secretion of glucocorticoids (cortisol, corticosterone in rodents) and catecholamines from the adrenal gland. All these factors are important to give the appropriate behavioural, vascular and immune responses of the body in the case of stress. It is important to point out that arginine vasopressin (AVP), which is also produced by the PVN, is a potent synergistic factor of CRF.

Under normal conditions the HPA-axis has a negative feedback mechanism The hippocampal activation suppresses the CRF rather then stimulating it. In the hippocampus, there are numerous glucocorticoid receptors that respond to the released cortisol. When the stressor is beyond adaptive capabilities, this may result in the hyperactivation of the HPA-axis. This impaired feedback can be caused by glucocorticoid-induced damage to the hippocampus or a reduced function of the glucocorticoid receptor (GR) at the level of the PVN, pituitary or hippocampus. This hyperactivation of the HPA-axis can contribute to the development of stress-related pathologies. (5) (6) (7)

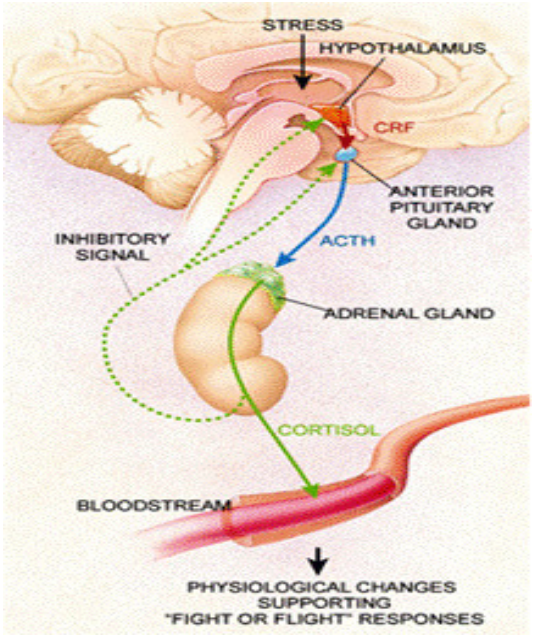

Fig 1: Overview of the HPA-axis. Stress triggers the release of corticotrophin-releasing factor (CRF) from the paraventricular nucleus (PVN). This in turn activates the anterior pituitary gland to release adreno-corticotrophic hormone (ACTH). ACTH and CRF control the release of cortisol into the bloodstream by the adrenal gland. As seen in the picture, CRF is responsible for a negative feedback system on the anterior pituitary as well as on the hypothalamus. (www.lumc.nl)

1.1.4 Chronic stress

Chronic stress is a normal physiological process, but it can also be the abnormal ongoing physiological response to the continuing perception of unresolvable major threats or demands. The concept of chronic stress implies a functional reorganization of parts of the brain in a way that cannot be quickly or easily reversed. (3) Diseases that may result from chronic stress include migraine, essential hypertension, depression and the metabolic syndrome (3)
The definitions of chronic stress-related diseases are not informative, but they all imply the existence of a set-point. This set-point is the normal physiological compromise caused by all the feedback loops which control the parameter in question. The existence of a set-point implies that there also may be a locus for control of the set-point, presumably in the limbic system. The limbic system consists of the hippocampus (in this case defined as the dentate gyrus and CA regions), hypothalamus and the structures to which they are connected.
In this hypothesis they state that there is a basic circuit of emotion which runs from the hippocampus to the amygdala, and from there to serotonergic pacemaker cells in the dorsal raphe nucleus (DRN). From the DRN the serotonergic cells project back to the dentate gyrus. This projection happens in two ways: first, there is a direct route without a stop, second, there is an indirect route which involves the pacemaker cells in the entorhinal cortex. The goal of the direct route is to promote neurogenesis in the subgranular zone of the dentate gyrus (DG) of the hippocampus. The indirect route has 2 purposes: first, to imprint the ongoing moments

of consciousness onto new dentate cells, second, to provide a negative feedback loop for regulation of the whole process.

The hippocampus, amygdala and DRN all project to the hypothalamus, a structure central in the hypothalamo-pituitary-adrenal-axis. When the DRN goes in pathological overdrive in times of stress, it causes overdrive of the entorhinal cortex, where fibers to the dentate gyrus (DG) of the hippocampus have their roots. This leads to atrophy of the hippocampus, which is related to neuropsychiatric disorders. When the negative feedback loop is badly regulated, the amygdala and DRN are no longer inhibited and are free to orchestrate the syndromes of chronic stress. (3) (8)

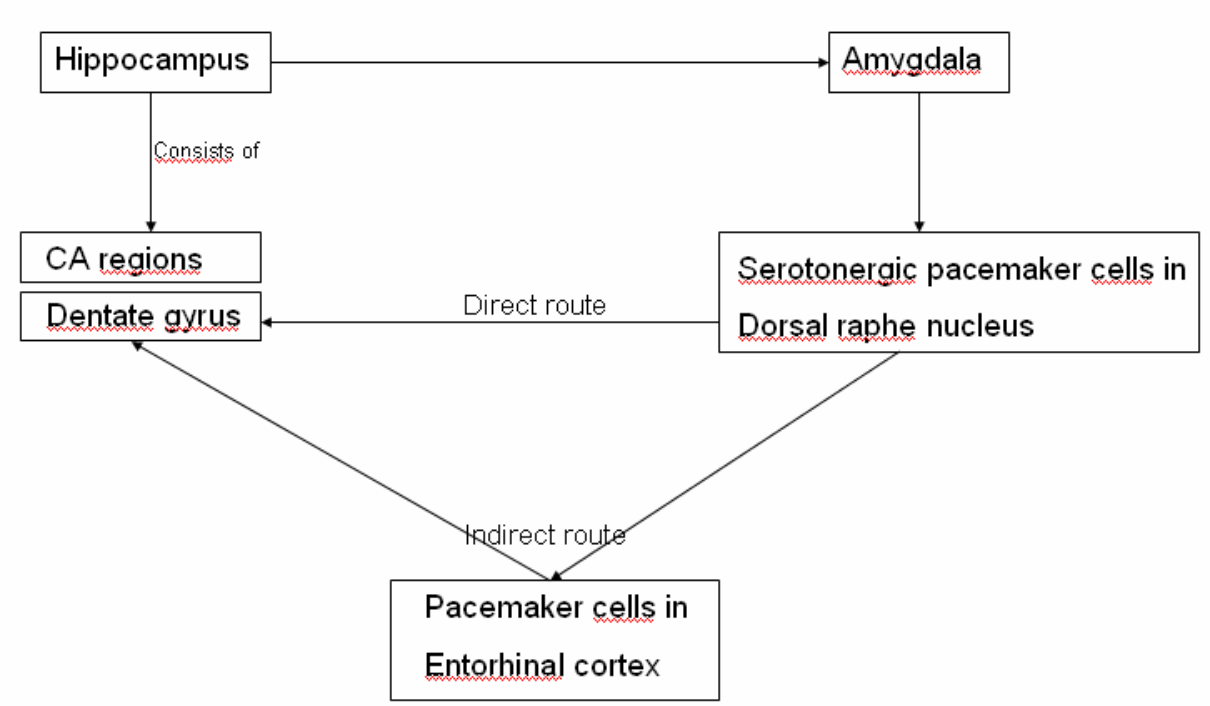

Fig 2: Schematic overview of the direction of the circuit of emotion as stated in the hypothesis of Eggers. The CA regions and the dentate gyrus are very important in the emotional circuit. Fibers from the hippocampus project to the amygdala. From here, the signal is conducted to the dorsal raphe, in particular the serotonergic pacemaker cells. From these cells, the signal takes two routes, a direct route to the dentate gyrus. Or an indirect route to the dentate gyrus via the pacemaker cells in the entorhinal cortex.

1.2 Serotonergic system

Serotonin(5-hydroxytryptamine, 5-HT) is an amine neurotransmitter, derived from the amino acid tryptophan. It is synthesised by serotonergic neurons in the central nervous system and by enterochromaffin cells in the gastro-intestinal tract. The synthesis of serotonin occurs in two steps. First, tryptophan is converted to an intermediary, 5-hydroxytryptophan (5-HTP), using the enzyme tryptophan hydroxylase (TPH). Second, this 5-HTP is converted to 5-HT by the enzyme 5-HTP dexarboxylase. The TPH exists in 2 forms: TPH1, found in several tissues and the brain specific TPH2. The 5-HT synthesis seems to be limited by the availability of tryptophan in the extracellular fluid around the neurons.

Following release from the axon into the synaptic cleft, 5-HT is removed by reuptake through serotonin transporters (5-HTT). (6) (9) When 5-HT is released from the synaptic vesicles into the synaptic cleft, it can bind to several pre- and post-synaptic 5-HT receptors. These receptors can induce or repress several signal transduction cascades, thus changing the functional status of the target neurons, and inducing long-term structural changes by regulation of gene expression. The signal transduction is stopped by reuptake of 5-HT by presynaptic 5-HT transporters (5-HTT) The availability of 5-HT and hence the signalling potential of it, is regulated only by 5-HTT.(10)

The primary source of 5-HT in the brain, are the neurons in the DRN. It is believed that 5-HT is released from synaptic serotonergic varicosities along the axon, instead from synaptic terminal buttons at the end of the axon. From here, the 5-HT is free to diffuse over a large region of space and activate 5-HT receptors on dendrites, cell bodies and presynaptic terminals of nearby neurons. (6)

5-HT is believed to be a major player in the process of the developing brain. Though all the monoamine neurotransmitter systems are present early in the developmental brain, serotonin is presented the earliest in the most terminal regions. (11)It is involved in the development of the CNS, where it works as a differentiation signal for progenitor cells developing along the serotonergic pathways. (12)

1.2.1 5-HTT

The 5-HTT is located primarily on presynaptic serotonergic neurons. It's function is the regulation of the concentration of 5-HT in the synaps. The 5-HTT has 12 transmembrane domains, with both the amino- and carboxy-termini located intracellular.
The 5-HTT makes sure that 5-HT is taken up again in the presynaptic neuron. This reuptake system is saturable and has high affinity. The reuptake of 5-HT from the synaptic cleft is an active process. This process is dependable on temperature and external Na^+ and Cl^-. The reuptake process is inhibited by metabolic inhibitors as well as inhibitors of the Na^+/K ATPase activity. The great demand for Na^+ implies that the energy requirement for the 5-HT reuptake is not directly connected to the transport of 5-HT, but rather to the maintenance of the Na^+ gradient across the plasma membrane.
The transport model has one Na^+, one Cl^- and one protonated 5-HT binding to the transporter to form a quaternary complex. This complex will lead to conformational changes in the transporter so it can release the ions and 5-HT in the cytoplasm of the cell. In the cell, K^+ associates with 5-HTT to promote the reorientation of the transporter to get ready for a new cycle of reuptake. (13)
Many agents can inhibit the reuptake, including MDMA, amphetamines and cocaine, but inhibition is also accomplished by anti-depressants, the group of the selective serotonin reuptake inhibitors (SSRI) with Prozac as a well-known example, and the group of tricyclic antidepressants (TCA) with imipramine as an example.

1.3 Prenatal stress

Because of its rapid growth, the fetus in particulary is vulnerable to insults and changes in the hormonal milieu. The rate of growth in its turn is a predictor for the developmental outcome. (2) (5) During pregnancy, the regulation of the HPA-axis is somewhat different.

In humans, the placenta forms an additional source of CRF (placental CRF, pCRF). This leads to an increase in the levels of plasma CRF during gestation. pCRF influences maternal HPA-axis as well as the fetal HPA-axis, resulting in increased levels of fetal ACTH and cortisol. Although normally cortisol in the brain has a negative feedback system, pCRF release is stimulated by cortisol through positive feedback loops. Gestational stress activates maternal HPA-axis, and increases the release of CRF of the placenta(14). This release is under influence of catecholamines and cortisol, as well as fetal hypoxia.

CRF could play a role in the development of anxiety disorders in adulthood caused by prenatal and early life stress. The CRF neurons are found in the neocortex and in the central nucleus of the amygdala. These neurons also innervate brainstem serotonin and noradrenalin nuclei that project to the forebrain and influence mood and the HPA-axis. (7)

The fetus is protected from these high levels of CRF by corticotrophin-releasing factor binding protein (CRF-BP), which is present only in humans and monkeys. This protein binds and inactivates the circulating CRF. Levels of CRF in the fetal blood do not increase during gestation. Although CRF crosses the placenta from the maternal blood to the fetus, about 80% is metabolized to cortisone. Levels of CRF-BP drop towards the end of gestation, stating the hypothesis that changes in maternal blood levels of CRF are implicated in the timing of parturition. The levels of ACTH and cortisol are increasing at the end of gestation.

Taking all this together, pregnancy can be seen as a state of mild sustained hypercortisolism. The fetuses protect themselves against these high levels of cortisol using the placental enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2). This enzyme converts the cortisol into bio-inactive 11-keto forms. Although there is protection, fetal cortisol levels are linearly related to the maternal cortisol levels. This can have important implications in stressful situations. (2)

Chronic or repeated stress during human brain development is associated with various behavioral and/or mental disorders in later life (7) (5).Prenatal stress also leads to preterm delivery and low birth weight. In preterm deliveries, an increased CRF was found in maternal and umbilical cord blood, when compared to gestational-matched subjects (2).As an example for the consequences of prenatal stress in humans behavioral assessments were made on children until 15 years of age. These children showed a delay in normal development, such as walking, speech and toilet training. In addition hyperactivity attention deficit disorder (ADHD) is associated with prenatal stress, but also with complications during delivery, such as prolonged labor or forceps delivery. These observations point out a possible association between prenatal stress and subsequent psychopathology and behavioral aberrations in children (7).

In rodent studies, prenatal stress (PS) is associated with disturbances in the HPA-axis, anxiety and depressive-like behavior. It has also been pointed out that PS has an influence on the serotonergic system in the hippocampus. (15)

Under normal conditions, the brain is formed according to a spatio-temporal blueprint of the central nervous system. During critical phases of neurogenesis, a various numbers of neurons are produced in each neurogenic region, followed by migration to their designated site. On this location, the neurons grow axons and dendrites. Knowing this, the damage caused by prenatal stress and its behavioral outcome depend on the timing of the occurrence of the prenatal stress. (7)

When pregnant rats are stressed, their adult offspring will develop abnormal behavioral and physiological responses to fear-provoking stimuli., but also abnormalities in emotional responses and even memory. In prenatally stressed rats, higher basic levels of corticosterone were found. There is also a difference in the HPA-axis response to stress in normal and PS rats. Some researchers found that PS rats had adrenal hypertrophy, which could be a result from chronic overstimulation of the adrenal gland by ACTH.(16) In behavioral testing, corticosterone levels went back to normal when the animals were repeatedly exposured to the same environment, but only in the normal rats. PS rats continued to release high levels of corticosterone. This may indicate that prenatal stress interfered with the normal adaptive process to mild stress. (16)

The HPA response should be rapid in onset and quick to terminate to be effective. When there is a failure to restore the glucocorticoids levels back to basal, the risk for a permanent change in the HPA-axis' feedback system increases.

1.4 Predictive Adaptive Responses (PARs)

When the homeostatic responses act during developmental plasticity (e.g. prenatal), they might lead to long-term consequences.

An interesting hypothesis about the responses to prenatal stress is the predictive adaptive responses (PARs). These PARs are not for the immediate reaction, but they predict changes that might be necessary in the future. They also induce irreversible changes in structure and function. (17) The key factor for appropriate PARs is the degree of match between the prenatal and postnatal environment (fig.1)

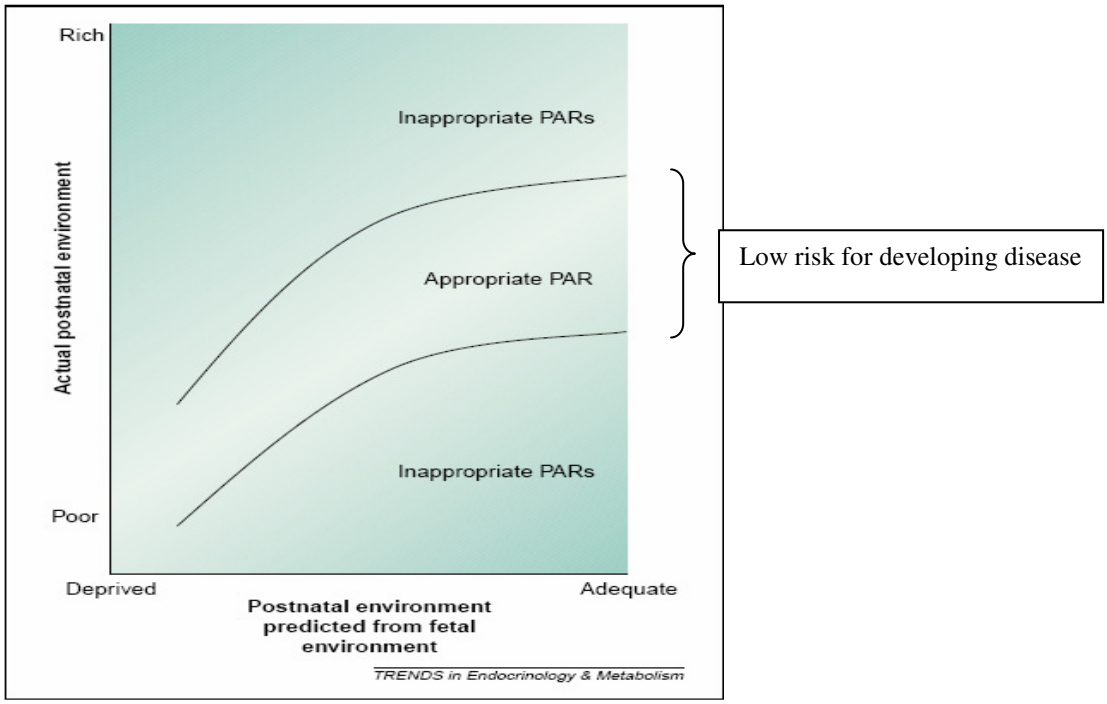

Fig 3 : PAR- hypothesis. The nature of the predictive adaptive response (PAR) is believed to be determined by the predicted and actual postnatal environment. As seen in the figure, when the acute postnatal environment does not fit the predicted postnatal environment, the PARs will be inappropriate. When both environments are equal, the PAR is appropriate and the risk for developing disease is lower. (Gluckman and Hanson, 2004)

1.5 Aim of the study

The aim of the present study is to check if prenatal stress, as well as chronic mild stress in adulthood, has an influence on the 5-HTT. In this investigation we particularly focussed on the serotonin transporter, which is responsible for the reuptake of serotonin. The regions studied in this research are the hippocampus, with interest to the inner region of CA3 and the outer region of the DG, and the medial prefrontal cortex. These regions were picked as targets because of their important role in the regulation of mood and cognition.
The choice for investigating the 5-HT is because of its great role in signalling in the brain and regulation of mood, cognition and memory. In this part of the study, our interest was focused at the 5-HTT, the main regulator of serotonin after its release from the synaptic vesicles.

2. Methods and Materials

2.1 Animals

All experiments were approve by the animal ethics board of the university of Maastricht. Pregnant Sprague-Dawley rats (Charles River, The Netherlands) were housed individually in a temperature-controlled environment (21± 1°C). The rats had a 1:1 light:dark cycle (lights on at 7.00 am) and had free access to food and water.

The rats were divided into 4 groups: One group did not receive prenatal stress, nor chronic mild stress after they were born, this is the C/C group. The second group is the prenatal stress group, the PS/C group. The third group only got chronic mild stress, the C/CMS group. The last group is the PS/CMS group. These animals were prenatally stressed and chronically stressed after birth.

The application of the stress was with the restraint technique. The rats were restraint during the last week of pregnancy (embryonic day 14-21). The pregnant rats were restraint in transparent plastic cylinders simultaneously with exposure to bright light, three times a day for 45 minutes. Pregnant rats from the control group were left in their cage.

An hour after the birth of the last pup of a litter, pups were labelled by toe cut, gender and body weight. This study only included litters of 10 or more pups, if necessary the litters were reduced to 10 pups.

At postnatal day 21, the pups were weaned and housed together (2 males or 2 females/cage) From this point on, animals were kept at a reversed day-night rhythm (lights on from 17.00 – 5.00 h)

At postnatal day 77, half of the rats were subjected to chronic variable mild stress for the coming 3 weeks. The stressors were housing in mice cage, tilt cage (45° angle), housing in a cage without sawdust, wet bedding in the cage or low intensity (2.5 Hz) flashing light during the dark phase. These stressors were applied at random, 2 times each day, each lasting 3 hours.

Body weights of the offspring were measured at birth (postnatal day 0), weaning (postnatal day 21), and postnatal days 77, 84, 91 and 98. From postnatal day 100, anxiety- and depression-related behavior was analyzed. These analyzes were done using the elevated zero maze test, home cage emergency test, forced swim test and sucrose intake test.

At postnatal day 120, the rats were anesthetized using pentobarbital (Nembutal® 60 mg/kg i.p.) and perfused transcardially with a tyrode solution followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were removed and post-fixed overnight with the same fixative. Hereafter the brains were subsequently immersed in respectively 10%, 20% and 30 % sucrose in 0.1M phosphate buffer.

2.2 Immunohistochemistry

Immunohistochemistry was performed using rabbit polyclonal serotonin transporter (SERT/5-HTT). The SERT antibody (calbiochem, 1:1500) was incubated for 2 nights at 4°C. The sections were washed and incubated with the secondary antibody (Donkey anti-rabbit biotin, Jackson, 1:800) for 1 hour at room temperature (RT). In the next step, ABC-kit (1:800) was administrated to the sections, incubating for 1 hour at RT. The sections were finally stained using a DAB solution; containing DAB, Tris-HCl and H_2O_2, incubating for 10 minutes. The sections were washed and mounted onto gelatine–coated glass slides, dehydrated and sealed using PERTEX.
The mounted sections were analysed using a SIS microscope (Olympus Provis). The regions of interest were the inner site of the dorsal CA3 and DG. The pictures were taken at bregma level: -3.3. For each region, 3 pictures were taken and the mean grey value was computed using the computer programme ImageJ. The first investigated region was the DG, where fibers from the entorhinal cortex enter the hippocampus. The second region of interest was the CA3 region, were fibers coming from the DG end. The regions of interest were chosen based upon the fact that in those regions there is a lot of signalling by 5-HT and thus a lot of 5-HTT on the presynaptic neurons.

2.3 Statistics

Serotonin transporters were analyzed with rank-scores using a two-way ANOVA (prenatal stress x chronic mild stress). If the ANOVA showed a significant difference; a more detailed LSD-test was done for comparison between groups. Males and females were analysed independently because of the gender differences.(18)

3. Results

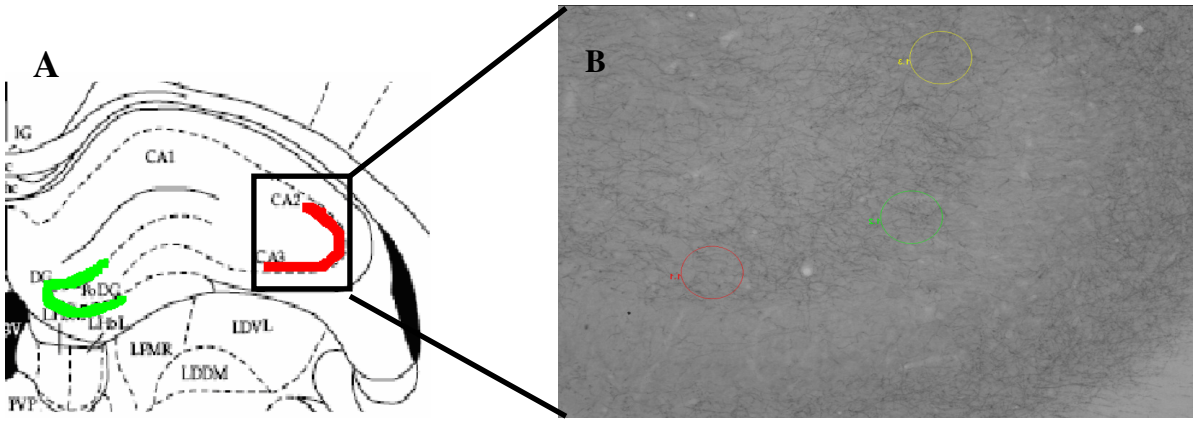

Fig 4: A: schematic overview of the regions of interest of the dorsal hippocampus. B: Microscopic picture of the inner CA3 of the dorsal hippocampus.

Figure 4A shows a schematic overview of the regions of interest, at bregma -3.3. Figure 4B gives an example of the pictures taken with the SIS microscope.

3.1 Outer DG

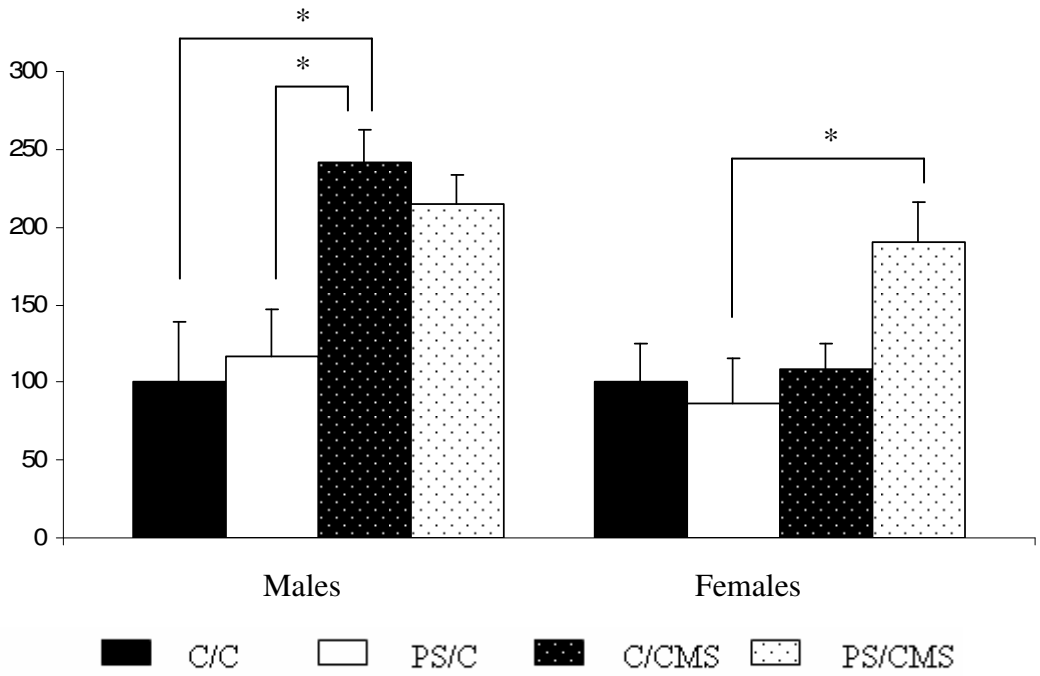

Fig 5: Analysis showed a significant difference in the male group when comparing the C/CMS group with the C/C group and the PS/C group. The female group showed a significant difference between the PS/C and the PS/CMS groups.

*p<0.05

When analysing the outer DG, a gender specific result is observed. In the group of male rats, significant differences were observed between the C/C group and the C/CMS group. The comparison of the PS/C group with the C/CMS group, also showed a significant increase in the amount of 5-HTT. A trend was observed in the comparison of the C/C group with the PS/CMS group (p=0.065)
In the female group, a significant difference in 5-HTT presence is found when comparing the prenatally stressed group to the group who received only chronic mild stress.

3.2 Inner CA3

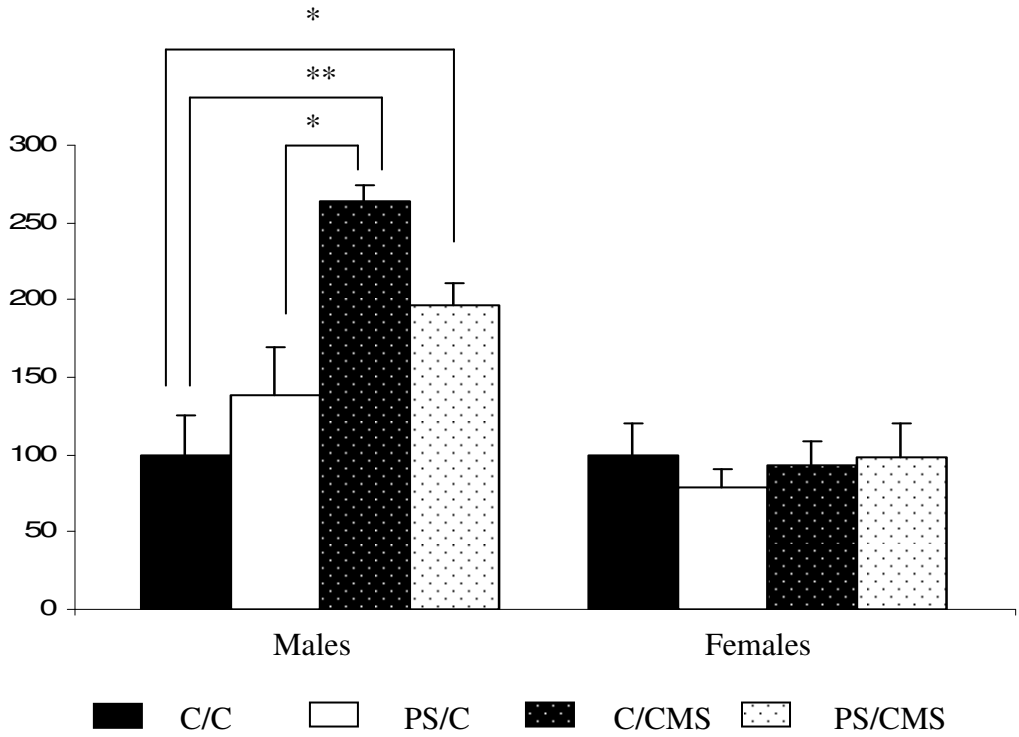

Fig: Analysis showed a significant difference in the presence of 5-HTT between the groups, but only in the male part of the group. The C/C group differs significantly with both the CMS groups, showing an overall effect of CMS on 5-HTT ($F(1,19) = 10.835$, $p< 0.005$). There is also a significant increase in the amount of 5-HTT when comparing the PS/C group to the C/CMS group. In the female group, no significant difference was observed.
* $p<0.05$ ** $p<0.005$

In the inner region of CA3, a similar pattern as in the outer DG is found in the male group. Both chronic mild stress groups show a significant increase of 5-HTT when compared with the C/C group. The prenatal stress group shows significant less amounts of 5-HTT when comparing to the PS/C group. In the female group, no significant effects were found.

4. Discussion

As shown in the results, there were several significant differences in the presence of 5-HTT between groups. The biggest differences were observed in the male groups, female groups only showed a significant difference when analysing the outer DG. In the male group of the outer DG, there was a significant increase in the presence of 5-HTT when comparing the C/C group with the group who received only CMS. The PS group also showed a significant lower number of 5-HTT in the outer DG than the group with CMS. The PS/CMS group showed a trend in comparison with the C/C group. In the female group, a significant difference was seen when comparing the PS/C group with the PS/CMS group.
The analysis of the CA3 showed significant findings in the male group. In this analysis, an overall effect of CMS is observed. When comparing individual groups, there was a significant increase of 5-HTT in the C/CMS and the PS/CMS group to the C/C group. The PS/C group showed significant lower values for 5-HTT when compared to the PS/C group.

The results show an increase in 5-HTT in case of stress. This finding can be important, because it is believed that 5-HTT plays an important role in the generation of depression and possible other mood disorders. As stated earlier, 5-HTT is the only transporter responsible for the reuptake of 5-HT from the synaptic cleft and so to terminate the signal transduction. In several mood disorders, 5-HT is taken up to fast and can not do its function properly. The signal transduction is stopped too quickly and mood disorders can evolve. That is the reason that several commercial anti-depressants work by inhibiting the 5-HTT. (19)
In the case of the male group, we see that in the graphs, the PS/CMS shows a lower amount of 5-HTT compared to the C/CMS group. From this view, we could conduct that maybe PS has a protective function when subjects are exposed to CMS in the future. This would support the PAR hypothesis. But when we compare the statistical analysis of the staining with the analysis of the mood tests (see appendix) we see a different pattern in the mood tests. In the tests, the rats who received PS and CMS have a higher rate of depression like behavior than the group receiving only CMS. This could be an interesting point for future studies, seeing in how far the PAR hypothesis can be applied to the case of PS and/or CMS.

We see in both investigated regions a difference in response according to genders. Although not many studies in humans and rodents mention if there were any differences according to gender and several studies were only conducted in males, this result is also seen in previous studies. (20) (21)
Our group is one of the first to investigate the relationship between prenatal stress/chronic mild stress – mood disorders – 5-HTT in this way, but other groups reported a relationship between 5-HTT and evolution of mood disorders as well. (22) (23) (10)
If we can extrapolate these findings to humans, they suggest that persons who receive chronic mild stress during their life are more vulnerable to depression and other mood disorders that are related to the serotonergic system. Prenatal stress shows no significant effect in the immunohistochemistry, but in behavioral tests, these animals have significant depression-like behavior. This can be an interesting point of future studies, depression like behavior is related to the amount of 5-HTT in the hippocampus, but there are also other mechanisms involved.
In the future, other structures of the limbic system may be of interest like the prefrontal cortex, the ventral hippocampus or the dorsal raphe. Staining of other serotonin related molecules , such as TPH or serotonin itself can be an objective. It may also be interesting to investigate why the females show a very different response pattern compared with the male group.

Conclusion

In our investigation we showed a correlation between PS and/or CMS in the development of depression. We did this by behavioral testing and immunohistochemistry. Rats who received CMS have significantly more 5-HTT and so more 5-HT reuptake than other groups. Females show almost no effect on 5-HTT, only when analysing the outer DG, there was an effect when comparing the PS/C group with the PS/CMS group. Other mechanisms are thus involved in the female relationship between PS and/or CMS and depression-like behavior. This is a point for future studies.

References

(1) Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stres response. Annu Rev Physiol. 67, 259-294

(2) Weinstock, M., 2004. The potential influence of maternal stress hormones on development and mental health of the offspring. Brain Behav. Immun. 19, 296-308

(3) Eggers, AE., 2007. Redrawing Papez' circuit: a theory about how acute stress becomes chronic and causes disease. Med Hypotheses (in press)

(4) Guyton, AC., Hall, JE., Textbook of medical physiology (10th Ed.) , Philadelphia; Saunders company

(5) Egliston, K., McMahon, C., Austin, M., 2006. Stress in pregnancy and infant HPA-axis function: Conceptual and methodological issues relating to the use of salivary cortisol as an outcome measure. Psychoneuroendocrinology 32, 1-13

(6) Bear M.F., Connors B.W. and Paradiso M.A., 2001. Neuroscience. Exploring the brain (2nd Ed.). Philadelphia: Lippincott, Williams and Wilkins.

(7) Weinstock M., 2001. Alterations induced by gestational stress in brain morphology and behaviour of the offspring. Progress in neurobiology 65, 427-451

(8) Sapolsky, RM., 2000. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry 57, 925-935

(9) Owashi, T., et al, 2004. The distribution of serotonin transporter immunoreactivity in hippocampal formation in monkeys and rats. Brain Research 1010, 166-168

(10) Sibille, E., Lewis, DA., 2006. SERT-ainly involved in depression, but when? Am J Psychiatry 163:1

(11) Whitaker-Azmitia, PM., 2001. Serotonin and brain development: Role in human developmental diseases. Brain Research Bulletin 56, 479-485

(12) Lauder, JM., Krebs, H., 1978. Serotonin as a differentiation signal in early neurogenesis. Dev Neurosci. 1, 15-30

(13) Frazer A., Hensler, JG., Basic neurochemistry molecular, cellular and medical aspects. Lippincott, Williams and Wilkins

(14) Petraglia, E., et al, 1996. Peptide signalling in human placenta and membranes: autocrine, paracrine and endocrine mechanisms. Endocrin rev. 17,156-186

(15) Porter, RJ., et al, 2004. Corticosteroid-serotonin interactions in depression: a review of the human evidence. Psychopharmacology (Berl) 173, 1-17

(16) Ward, HE, et al, 2000. Effects of prenatal stress on defensive withdrawal behavior and corticotroping releasing factor systems in rat brain. Psychology & behavior 70,359-366

(17) Gluckman, PD., Hanson, MA, 2004. The developmental origins of the metabolic syndrome. Trends Endocrinol Metab. 15, 183-187

(18) Van den Hove, DLA., et al, 2006. Prenatal stress reduces S100B in the neonatal rat hippocampus in a sexually divergent manner. NeuroReport, submitted

(19) White, KJ., Walline, CC., Barker, EL, 2005. Serotonin transporters: implications for antidepressant drug development. AAPS Journal 7, 421-433

(20) Weinstock, M., 2007. Gender differences in the effects of prenatal stress on brain development and behaviour. Neurochem Res. In publication.

(21) Bowman, RE., et al, 2004. Sexually dimorphic effects of prenatal stress on cognition, hormonal responses, and central neurotransmitters. Endocrinology 145, 3778-3787

(22) Ansorge, MS, et al, 2004. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science 306, 879-881

(23) Owens, MJ., Nemeroff, CB., 1998. The serotonin transporter and depression. Depression and anxiety 8, 5-12

Appendix

Results of the behavioral tests

Anxiety- and depression-related behavior

Elevated zero maze

The percentage of time spent in the open arms of the elevated zero maze as well as distance covered in the elevated zero maze are depicted in Figure 1. Within males an overall effect of CMS was observed, with CMS animals spending relatively more time in the open arms of the maze ($P=0.014$) and covering more distance ($P<0.01$). Post-hoc analysis revealed that PS/- males both spent less time in the open arms of the maze and covered less distance as compared to all other male groups. Within females, PS animals spent less time in the open arms of the elevated zero maze ($P<0.01$) and tended to cover less distance ($P=0.066$) during the trial as compared to controls. Further, CMS females tended to spent more time in the open arms ($P=0.08$) and covered significantly more distance as compared to the corresponding controls ($P=0.033$). In addition, a significant PS x CMS interaction was observed ($P=0.011$). Post-hoc analysis showed that PS/- females both spent less time in the open arms of the maze and covered less distance as compared to all other female groups.

Home cage emergence

The average escape latencies in the home cage emergence test are shown in Figure 1. Within males, no overall effects were observed, though C/CMS animals showed a tendency towards an increased escape latency as compared to C/- animals ($P=0.075$; post-hoc LSD test). Within females, an overall CMS effect was found, with CMS animals showing increased escape latencies as compared to controls ($P=0.041$). PS/CMS females tended to have increased escape latencies as compared to C/- females ($P=0.083$; post-hoc LSD).

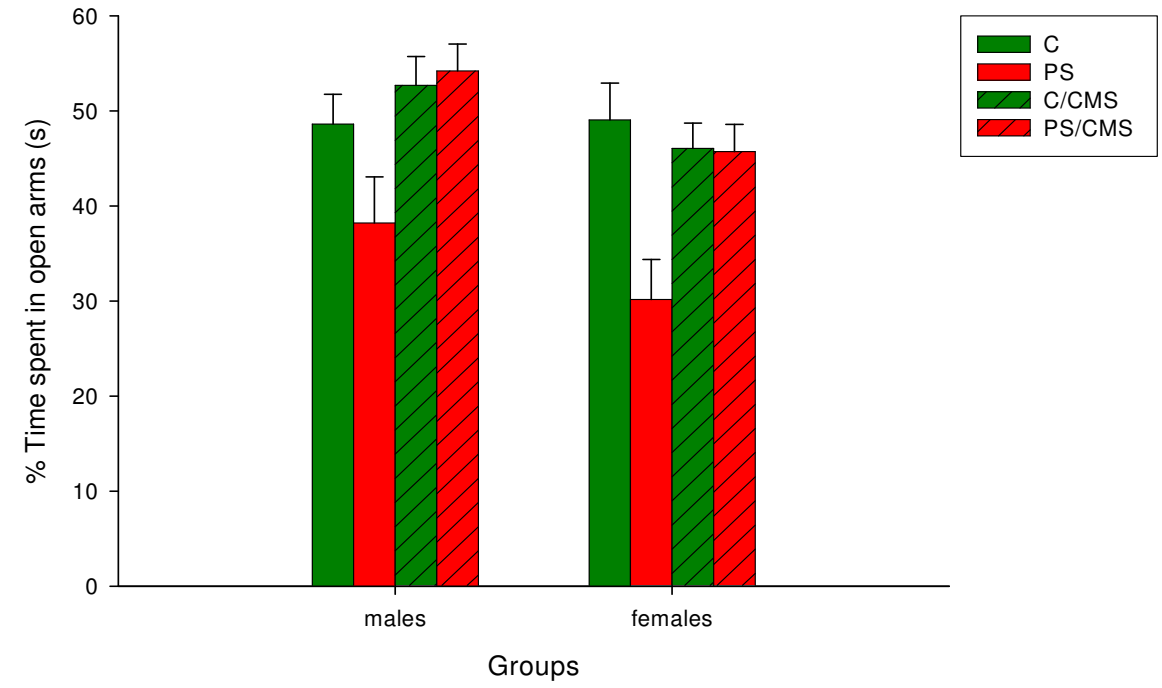

Figure 1. Time (s) spent in the open arms of the elevated zero maze test. Values represent means + S.E.M.

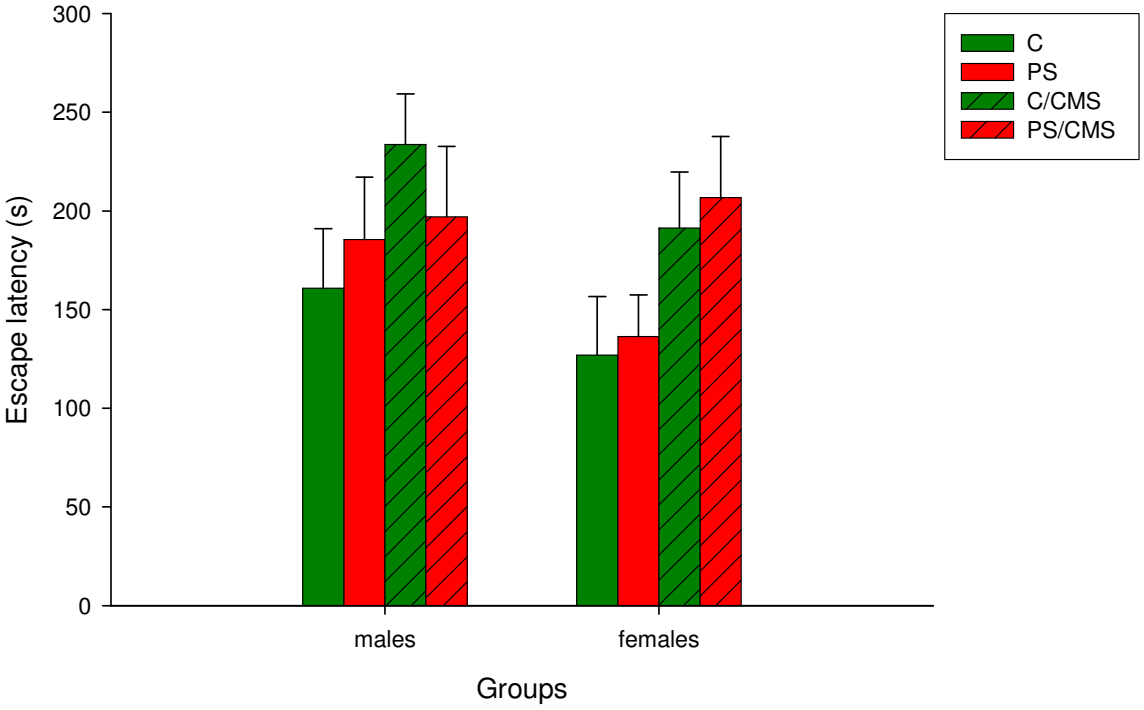

Figure 2. Average escape latencies (s) in the home cage emergence test. Values represent means + S.E.M. *P<0.05, #0.05<P<0.10 (ANOVA based on ranks).

Forced swim

Results from the forced swim test are shown in Figure 3.

Over the first 5 minutes, there was an overall effect of PS on the degree of 'mobility' within males, i.e., 'mobility' was reduced after PS (P=0.034). No overall effects were observed for 'immobility' and 'strong mobility'. Post-hoc analysis revealed that PS/- males tended to show more 'immobility' as compared to C/- males (P=0.077). Further, both PS/- and PS/CMS males showed a tendency towards less 'mobility' as compared to C/- males (post-hoc LSD; see Figure 3 for more details). There were no differences between female groups in any of the parameters over the first 5 minutes.

Over the second period of 5 minutes, a tendency towards an overall PS x CMS interaction was observed within males (P=0.064). Post-hoc analysis revealed that PS/CMS males tend to be more 'immobile' and less 'mobile' as compared to C/- males (P=0.080 and P=0.069, respectively). Further, PS/CMS males showed more 'immobility' (P=0.030) as well as a tendency towards less 'mobility' (P=0.073), and significantly less 'strong mobility' (P=0.033) as compared to C/CMS males (post-hoc LSD; see Figure 3 for more details). PS/CMS males also tended to show more 'immobility' and less strong mobility as compared to PS/- males….
There were no differences between female groups in any of the parameters over the last 5 minutes.

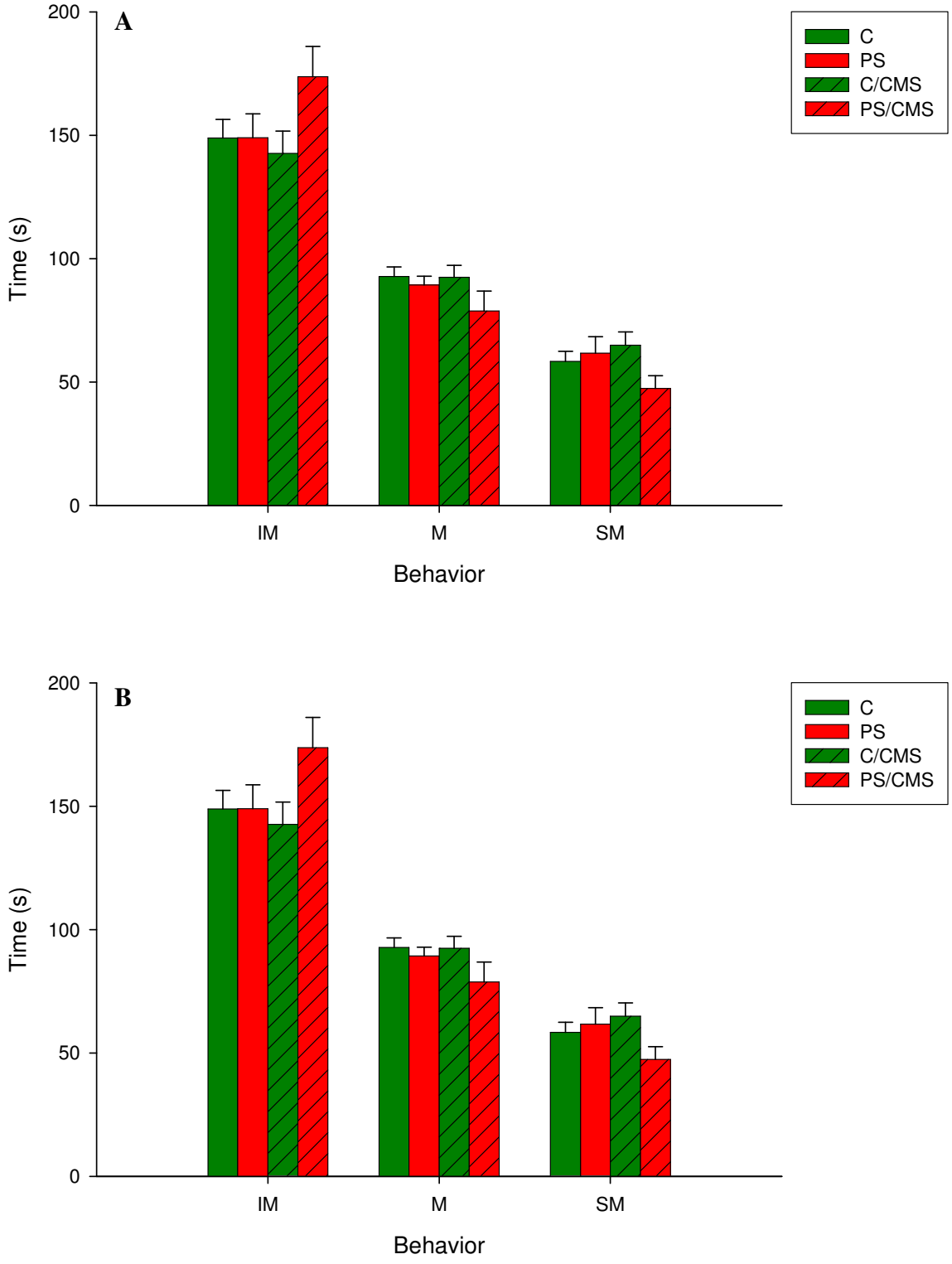

Figure 3. Immobility (IM) and strong mobility (SM) in the forced swim test; Values represent means (s) + S.E.M. A: 0-5 minutes B:5-10 minutes. Females show no effect (values not presented)

Sucrose intake

The effects of PS and CMS on sucrose intake are depicted in Table 3

Within both genders, a significant effect of age was observed, with relatively more sucrose intake at P98 as compared to P77 ($P<0.001$ in both cases).

Further, over time, PS males consumed more sucrose solution as compared to control males ($P=0.021$). This overall PS effect was most pronounced at P77 ($P<0.014$), whereas at P98 there was only a tendency towards a significant increase in sucrose intake after PS ($P=0.073$). Post-hoc analysis at P77 revealed that the PS effect could be attributed mainly to the difference between the PS/- and C/- groups ($P=0.026$; see Table … for more details).

Within females, over time, a significant PS x CMS interaction was observed, with PS females that were exposed to CMS drinking less sucrose solution as compared to females that were exposed to PS only ($P<0.036$). At P98 a tendency towards a similar interaction between PS and CMS was observed ($P=0.089$). Post-hoc analysis at this time point revealed that this effect could be attributed mainly to the difference between the PS/- and PS/CMS groups ($P=0.030$; see Table for more details).

Gender	Group	P77	P98
Males	C/-	0.095 ± 0.009	0.185 ± 0.018
	C/CMS	0.089 ± 0.006	0.194 ± 0.028
	PS/-	0.125 ± 0.012*,‡	0.238 ± 0.012
	PS/CMS	0.107 ± 0.006	0.212 ± 0.010
Females	C/-	0.150 ± 0.023	0.352 ± 0.082
	C/CMS	0.165 ± 0.028	0.375 ± 0.073
	PS/-	0.181 ± 0.025	0.446 ± 0.039
	PS/CMS	0.145 ± 0.009	0.264 ± 0.035†

Table 3. Sucrose intake (ml/kg body weight). Values represent means ± S.E.M. Abbreviations: C: control, PS: prenatal stress, CMS: chronic mild stress. Over time, PS males consumed more sucrose solution as compared to control males (P=0.021). This overall PS effect was most pronounced at P77 (P<0.014), whereas at P98 there was only a tendency towards a significant increase in sucrose intake after PS (P=0.073). Within females, over time, a significant PS x CMS interaction was observed, with PS females that were exposed to CMS drinking less sucrose solution as compared to females that were exposed to PS only (P<0.036). At P98 a tendency towards a similar interaction between PS and CMS was observed (P=0.089). *P<0.05 (post-hoc LSD, as compared to C/-); †P<0.05 (post-hoc LSD, as compared to PS/-); ‡P<0.05 (post-hoc LSD, as compared to C/CMS)

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