



## RESEARCH ARTICLE

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## Changes in Synaptic Terminal Structure in Adolescent Rat during Pregnancy; the Action Potential Propagation and Synaptic Transmission

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### ABSTRACT

Synaptic plasticity is a biological system of specific pattern of synaptic activity result in changes in synaptic strength. This influence puberty, pregnancy hormones, sensory experiences, and brain disorders. Long-term synaptic plasticity is accompanied by protein synthesis and trafficking, leading to structural changes of the synapse. Increasing evidence connects the terminal synaptic changes with potential propagation in adolescent and pregnancy. We investigate on the synaptic structural plasticity, which has mainly been studied with in vivo two-photon laser scanning microscopy. We also discuss how a different type of synapses, the multi-contact synapses associated with pregnancy.

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### Introduction

The term “synapse” represents the junction between nerve cells. There are two major types of synapses namely chemical and electrical. The electrical synapses are found in both neurons and glia. Cells connected through electrical synapses are directly linked by an intercellular specialization called gap junction. Gap junctions are intercellular channels composed of six protein units that allow electrical current to pass through, thus electrically coupling the connected cells and allowing bi-directional signal flow. In contrast, chemical synapses are composed of distinct pre- and post-synaptic elements, with a synaptic cleft in between. Depolarization of the presynaptic membrane induces the release of neurotransmitters from the presynaptic terminal, causing depolarization, in the case of an excitatory synapse, or hyperpolarization, in the case of an inhibitory synapse, of the postsynaptic membrane. The signal flow is thus unidirectional. In the central nervous system of vertebrate animals most synapses are chemical. Chemical synapses are composed of asymmetrical pre- and postsynaptic structures. Synaptic plasticity is a biological system of specific pattern of synaptic activity result in changes in synaptic strength and highly dynamic. The synaptic structure components of most excitatory synapses in the mammalian brain reside on the spiny protrusion and influence during pregnancy. Adolescent female brain profound changes in mammalian brain maturation which characterized by the emergence of executive function. Recent evidence from animal studies indicates numerous functional and structural synaptic changes in the female brain during pregnancy. Synaptic pruning of excitatory contacts is the signature morphologic event of late brain maturation during adolescence. Mounting evidence suggests that glutamate receptor-mediated synaptic plasticity, in particular long term

depression (LTD), is important for elimination of synaptic contacts in brain synaptic changes during pregnancy. Synaptic changes influenced differently by development, sensory experiences, and brain disorders. Long-term synaptic plasticity is accompanied by protein synthesis and trafficking, leading to structural changes of the synapse. Female Adolescence stage is a unique period of puberty development, marked by maturation of the prefrontal cortex and regulate maternal caregiving behavior such as licking and grooming. During this time, the human synaptic strength decreases in overall volume and thickness. Keyser-Marcus and colleagues shows in his experiment that synaptic changes replicated in virgin female rodents after a hormonal treatment mimicking pregnancy based on exogenous steroid hormones, stressing the implication of the hormonal surges of pregnancy and the observed gestational synaptic plasticity. Pregnancy hormones such as prolactin, oxytocin and glucocorticoid have been shown to change synaptic structures in maternal brain. Several studies revealed that female adolescent pregnancy mice, losses of neurons, dendrites, dendritic spines and neurotransmitter receptors have been documented within the adolescent pregnancy. However, changes in the number of synapses in pregnancy with action propagation during this time have not been investigated. In this study, we stereologically quantified the number of synaptophysin-immunoreactive boutons in female adolescent pregnancy rat across multiple time points deepen into action propagation and transmission. We investigate the adulthood rat with pregnancy (ARwP) and with other without pregnancy (ARwoP). In ARwP, there was a significant losses in synaptophysin boutons and symmetrical pattern of extensive gray matter volume decreases across pregnancy, majorly the onset of pregnancy. In ARwoP, there was no significant change on synaptophysin boutons; however, in ARwP, pregnancy onset

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was associated with significant synaptic losses but there is more decrease in size related to pregnancy in humans. These results suggest that adulthood pregnancy is a critical period for synaptic pruning within the rat, potentially contributing to maturation of adolescent executive function. There is evidence that gray matter may decrease due to synaptic pruning. In humans, an increase in synaptic density occurs across early childhood, peaking around the time of puberty, followed by a decrease across adolescent pregnancy [1]. Changes in synaptic terminal strength- often are layer-specific and may not reach adult levels until the third decade of life [2]. This synaptic pruning pattern has also been found in the cortex of nonhuman primates [3-5]. Currently, the existing literature only examines changes in synaptic changes in adolescent but no investigation during pregnancy, as opposed to the total number of synapses, though there are volumetric changes across adolescence. Additionally, current literature on synaptic changes during adolescence focuses primarily on female subjects, though work from our laboratory suggests differential trajectories in pregnancy. Synaptic changes occur in the rat during adolescence pregnancy. Studies show that synaptic terminal increases in rats without pregnancy and then decrease between adolescence and adulthood during pregnancy, however, only females pregnant have decreased synaptic structure adulthood. The measure of synaptic terminal structure on non pregnancy is at three inches more than pregnant female. Furthermore, the rise in ovarian hormones at puberty may have a role in synaptic losses. The timing and extent of synaptic changes by Willing and Juraska further supports a role for ovarian hormones as an ingredient that results in synaptic decreases [6]. Therefore as a start in exploring this, we will be examining the adulthood status and the number of synapses during the pregnancy will be stereologically calculated through the use of synaptophysin immunocytochemistry across adulthood ages in these rats.

## Material and Methods

This study was carried out in strict accordance with the recommendations in the guide for the animal laboratory care and use of Rosalind Neurodegenerative disease research institute, Jos. The protocol was approved by the committee on the ethics of animal experiments department, University of Jos (Permit Number: 2020:9543). All surgery carried out under anesthesia (urethane).

### Animals in Adulthood Pregnancy and Non Pregnancy

#### Subjects

Subjects were offspring of Long-Evans hooded rats (*M. natalensis*) obtained from Rosalind Laboratories, Jos and bred in the vivarium of the Rosalind Neurodegenerative Research Institute, Jos. Subjects were caged at 1 week of pregnancy and housed in a different cage apart from non pregnant rat. All the caged rats were in adulthood age include the non pregnant rat period through adulthood: ARwP is the pregnant rat; ARwoP are the non pregnant rat female at adulthood stage; and ARwP and ARwoP represent the pregnant adolescence and non pregnancy adulthood. The two groups contained 15 subjects per cage for a total of 30 animals. Within each age group, animals came from a minimum of five separate litters, and no age had more than two animals from one litter. Subjects were given ad libitum access to water and standard rat chow while kept on a 12:12 hour light-dark cycle throughout their life. All procedures adhered to the Research Institute of Animal guidelines on the ethical use of animals and were approved by the University of Jos, Nigeria Institutional Animal Care and Use

Committee. Pregnancy status was assessed using identifiable anatomical indicators and pregnancy test via ultrasonographic method by using a 12MHz linear transducer. Ultrasonographic evidence of pregnancy day 9 post coitum for all animals that reached adulthood stage and pregnancy prior to sacrifice. In pregnant subjects, pregnancy onset was marked by vaginal opening, which coincides with maturation of the hypothalamic-pituitary-ovarian axis, increases plasma LH and FSH levels as well as the appearance of the estrous cycle [7,8]. Pubertal onset was verified in female rats by preputial separation, or separation of the prepuce from the which is dependent upon the pubertal surge of endogenous androgen hormones [9].

#### Tissue Collection

Experimental subjects were deeply anesthetized with sodium pentobarbital. Subjects were then perfused intracardially with 0.1M phosphate buffered saline (PBS) with a pH of 7.4, followed by 4% paraformaldehyde fixative in PBS. Brains were removed, stored in the same fix solution for an additional 24 hours, and cryoprotected in a PBS solution with 30% sucrose for three days. All brains were coded at this time so that the experimenters were blind to the animal's group. Once the brain had sunk in sucrose solution, it was sliced with a freezing microtome into 40µm coronal sections. Every sixth slice section was then placed into 0.1M PBS and mounted on gelatin-coated slides. These slices were stained with methylene blue, a cell body stain previously used by our laboratory for volumetric estimations [10]. These sections were used to calculate synaptic terminal volume and neuron and glia number [6].

#### Volume Estimation

The area of the ventral cerebrum region on each mounted slice was parcellated and divided into six layers by using the same cytoarchitectural guides described by previous studies with StereoInvestigator software [11]. Each area was measured across each mounted slice, with the anterior edge of the marked by the emergence of the underlying white matter and the most posterior border of the cerebrum defined by the crossing of the genu of the corpus callosum. The thickness of the mounted cerebrum sections was determined by measuring the depth of the focal length from the top to the bottom of the tissue. This analysis was done using a motorized Scientific method, which measured the thickness of the z-plane across more than 50 sites for each subject. Average tissue thickness was then calculated. Total volume of the cerebrum was calculated using StereoInvestigator software by multiplying the area of the cerebrum on each mounted slice by the average thickness of the tissue and the distance between slices.

#### Immunocytochemistry

Every sixth section containing cerebrum was stained for synaptophysin, primary protein used as a marker for synapse [12]. Sections were first rinsed three times (5 minutes each) in Tris Buffered Saline (TBS, pH 7.6). Sections were then incubated according to Drzewiecki principle [13], in a blocking solution (20% normal goat serum, 1% bovine serum albumin, 1% hydrogen peroxide in TBS) for 35 minutes. Tissue was then put in primary antibody for 72 hours. Primary antibody dilution was made in tris-triton goat (TTG) solution (2% normal goat serum, 0.3% triton X-100 in TBS). After two days in primary antibody solution, slices were rinsed three times with TTG (5 minutes each) and incubated in a biotinylated secondary antibody (Anti-Mouse IgG Antibody,

5 µg/ml, Vector Laboratories) for 90 minutes at room temperature. Following rinses in TTG and TBS, sections were placed in avidin-biotin complex for one hour at a normal room temperature. Lastly, tissue was stained with diaminobenzidine for five minutes. The avidin-biotin + diaminobenzidine technique is an effective method of visualizing specific antibodies with high staining intensity [14]. After rinsing off diaminobenzidine with TBS, stained sections were mounted on gelatin-coated slides and cover-slipped with permount. To confirm the specificity of the primary synaptophysin antibody during adolescent pregnancy, a negative control staining protocol was conducted on each tissue slices without the anti-synaptophysin primary antibody. This resulted in no detectable immunolabeling, thus confirming the synaptic losses. Meanwhile, opposite is the case for non pregnancy.

### Stereology and Synapse Number Estimation

Stereological analysis of tissue stained for synaptophysin has been shown to be a reliable measure of synaptic numbers within the central nervous system [12]. The overall density of the labeled synaptophysin boutons within the brain was determined using the optical disector in Stereoinvestigator. Prior to counting, the experimenter manually parcellated six layers of the brain across the right and left hemispheres. The optical disector then imposes a grid (here 120 µm × 120 µm) across the tissue and places a counting frame (here 5 µm × 5 µm) in the corner of each grid square to ensure systematic uniform random sampling across the brain. The counting frame perimeter is made up of two "acceptance" edges and two "exclusion" edges. If a synaptophysin bouton falls entirely within the counting frame or it touches the "acceptance" edge, it is counted. All boutons that contact the "exclusion" end are omitted from analysis. The depth of the counting frame was kept to 6 µm with guard zones of 0.1 µm, and the Stereoinvestigator software tracks total bouton counts and the number of counting sites visited for each subject. The same technique has been reliably used by our laboratory to quantify the number of synapses [10]. Synaptophysin boutons were quantified separately in six layers of the rat ventral brain. For each animal without pregnancy, the recommended minimum of 200 boutons was counted within each layer (Gundersen and Jensen, 1987), resulting in an average of 211, 243, 294, 300, 314, 350 synaptic boutons per animal counted in the six layers respectively. For each animal with pregnancy, the recommended minimum of 50 boutons was counted within each layer (Gundersen and Jensen, 1987), resulting in an average of 17, 13, 14, 7, 12, 10 synaptic boutons per animal counted in the six layers respectively. The number of counted boutons was divided by the total volume of the counting sites to obtain the synaptic changes. Changes were then multiplied by the previously calculated volume of the brain to determine the total number of synaptophysin immunoreactive boutons within each layer of the six layers slice of cerebrum. This was subsequently added across all layers resulting in an estimation of the total number of synapses in the adulthood female.

### Statistical Analysis

Due to the same sex in used in these studies, different trajectories of synaptic decrease in both the females were hypothesized. Thus, female pregnant and non pregnant female subjects were analyzed separately. For the overall number of synapses, a one-way analysis of variance (ANOVA) with age as the factor was run with litter as a cofactor, combining counts from all layers. Separate ANOVAs were also conducted for each layer (layer A, layer B, layer C, layer D, layer E, layer F). When age was significant, four post hoc comparisons of Fisher's least square difference (LSD) were run that compared adjacent ages to each other (R21 vs 32 35 vs 45, 45 vs 60, 60 vs 90). In addition, the effect size was assessed using Cohen's d for all findings [15-18].

To specifically examine the synapse number (increase/decrease), a student's t-test was used to compare the number of synapses for ARwP and ARwoP, respectively that had or had not yet pregnancy. Subjects that had not yet pregnant were also compared to their next older age counterparts. In females, a t-test was run comparing all pregnant female to all non-pregnant females [19,20].

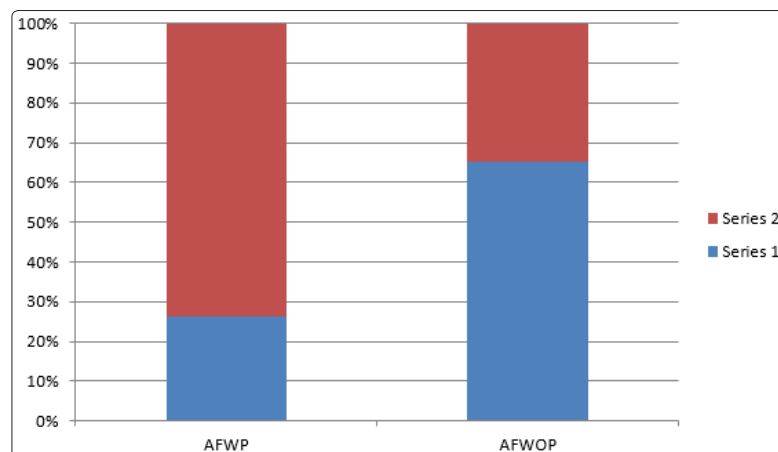
### Results

#### Adulthood and Synaptophysin Boutons

ARwP: A one-way ANOVA did not show a main effect of adulthood on the total number of synapses in female subjects  $F(4, 36) = 1.49$ ;  $p = 0.226$ . Effect sizes of  $d = 0.61$  and  $d = 0.73$  (moderate effects) were calculated respectively. There was not a main effect of age in layers A or B, C, E, F  $F(4,36) = 2.68$ ;  $p = 0.047$ . Post hoc tests revealed a significant decreases in synaptic boutons in pregnancy  $p < 0.05$ ;  $d = 1.20$  (above 1 is considered a large effect). To examine the effects of adulthood on the total number of synapses, a t-test was run for females at R34 comparing those that had reached adulthood to those who had not. Females that had reached adulthood by R34 had no significantly differences in synapses than females who had not except during pregnancy  $t(10) = 2.76$ ;  $p = 0.02$ ;  $d = 2.75$  [21-23].

ARwoP: the one-way ANOVA revealed a main effect of adulthood on total numbers of synapses  $F(4, 30) = 4.25$ ;  $p = 0.01$ . Post hoc analysis indicated a significant increase in synapses [ $p < 0.05$ ;  $d = 1.11$ ], as well as a decrease in synapse numbers between P35 and P45 [ $p < 0.05$ ;  $d = 1.31$ ]. There was no significant difference between any of the other adjacent ages [24-26]. Analyses of layers found a significant main effect of age on the number of synapses in layer I  $F(4, 30) = 5.315$ ;  $p < 0.01$ . Post hoc tests show significantly higher numbers of synapses at R30 compared to both R40 [ $p < 0.05$ ;  $d = 1.42$ ] and P45 [ $p < 0.05$ ;  $d = 1.33$ ]. There was no main effect of age in layers. There were no significant differences in synapse number at R34 between females who had or had not gone through adulthood  $t(9) = 0.75$ ;  $p = 0.47$ ;  $d = 0.50$  [27-30].

| Van Eden CG (1989)<br>Development of connections between the mediodorsal nucleus of the thalamus and the prefrontal cortex in the rat. J Comp Neurol 244: 349-359. | R1         | R2         | R3         | R4         | R5        | R6         |
|--|------------|------------|------------|------------|-----------|------------|
| ARWP   |            |            |            |            |           |            |
| Layer A  | 4.03±.26   | 4.03 ±.26  | 4.03 ±.26  | 4.03 ±.26  | 4.03 ±.26 | 4.03 ±.26  |
| Layer B  | 3.87± 28   | 3.87± 28   | 3.87± 28   | 3.87± 28   | 3.87± 28  | 3.87± 28   |
| Layer C  | 24.03 ±.26 | 24.03 ±.56 | 4.03 ±.36  | 4.03 ±.46  | 17.3 ±.21 | 14.03 ±.23 |
| Layer D  | 23.87± 12  | 34.87± 16  | 33.87± 22  | 23.87± 24  | 43.87± 27 | 26.87± 26  |
| Layer E  | 24.13 ±.26 | 26.03 ±.24 | 32.03 ±.16 | 12.03 ±.21 | 34.3 ±.32 | 4.03 ±.25  |
| Layer F  | 43.07± 28  | 37.87± 28  | 54.87± 28  | 34.87± 28  | 43.87± 28 | 45.87± 28  |
| ARWOP  |            |            |            |            |           |            |
| Layer A  | 4.03 ±.23  | 4.03 ±.26  | 4.03 ±.21  | 4.03 ±.32  | 4.03 ±.26 | 4.03 ±.16  |
| Layer B  | 7.87± 25   | 4.87± 18   | 4.81± 23   | 6.87± 28   | 5.87± 28  | 4.87± 78   |
| Layer C  | 32.13 ±.26 | 4.03 ±.26  | 4.03 ±.26  | 4.03 ±.26  | 4.03 ±.26 | 4.03 ±.26  |
| Layer D  | 33.87± 18  | 3.87± 28   | 3.87± 28   | 3.87± 28   | 3.87± 28  | 3.87± 28   |
| Layer E  | 29.30 ±.16 | 44.03 ±.26 | 43.53 ±.26 | 43.03 ±.43 | 32.3 ±.26 | 34.3 ±.26  |
| Layer F  | 43.87± 44  | 33.87± 28  | 53.56± 23  | 53.87± 28  | 23.87± 28 | 46.87± 28  |



## Discussion

The present study quantified synaptic number changes within the adolescent cerebrum from the adulthood pregnancy, using synaptophysin as an immunoreactive marker for synapses. We found decreases in overall synapse numbers within the ARwOP cerebrum at adulthood stage, and synaptic losses associated with female pregnancy. Layer-specific analysis revealed similar patterns of synaptic pruning between cortical layers, but age was significant in only some of the layers. All layers of the female ARWP show decreases in synapses at adulthood, though it was only significant in layer I. In the female ARWoP, again there appeared to be an increases in all layers at adulthood, but only layer V/VI was significant. Changes in synaptic numbers across the adulthood period may partly explain the deficits adolescents show on cognitive tasks that involve the cerebrum. Furthermore, they found that socially isolated rats showed deficits in synaptic plasticity proteins, including decreases in frontal synaptophysin levels, suggesting a link between adolescent stressors and increased synaptic pruning [31-39].

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