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Ultrastructural study of synaptogenesis in the olfactory bulb NMRI mice

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Abstract

During the development of the olfactory bulb shows great cytological plasticity, being a widely used model to try to understand the mechanisms involved in neuronal growth and their functionalism, having consideration that it is a system establishing diversity of synaptic interactions. The purpose of this study was to know the process of formation of axodendritic and dendrodendritic synaptic contacts during the development of the olfactory bulb. Were used NMRI mice from NMRI mice were used from 17 days embryonic (E) until to 9 days postnatal (P). The tissue was processed for ultrastructural study, and variance analysis (ANOVA) was performed using the statistical program SPSS version 15. At E17 internal thickening were observed in axonal and dendritic membranes, and synaptic vesicles attached in proximity to the membrane thickenings, which increased in number and thickness older ages. Axodendritic synapses immature increase in size, thickness and number from E17 until P7, and the following quantitative differences are identified: between E17 and E19 there was an increase of 39.8%, from E19 to E21 of 4.95%, between E21 and P0 there was of 5.84%, P1 day there was a difference of 16.58% over the previous day, and from this day until P7 there was a continuous increase in the number of synapses. Moreover, the formation of dendrodendritic synapses is an exclusively postnatal phenomenon that starts the day P3, increasing their number up to P7, as evidenced by the following data found: between P3 and P5 there was a difference of 41.03% between P5 and P7 of 54.02%, and between P7 and P9 the difference there was 61.5%. In conclusion, the amount of axodendritic and dendrodendritic synapses increased until P7 day; and was at P9 when both synapses reached morphological maturity.

Keywords

Olfactory Bulb, Synaptogenesis, Neural Development, Venezuela

1. Introduction

The olfactory system during late embryonic and early postnatal development has a marked cytological plasticity and diversity of physiological responses (1), constituting one of the most studied models to understand the mechanisms involved in the growth and dynamism neuronal during the nervous system formation and consolidation processes, because it is a region where many different synaptic interactions are established (2-3).

Although it has been reported the existence of alterations of the olfactory system in pathologies such as Parkinson's disease, Alzheimer's disease, Korsakoff syndrome, Down syndrome, HIV infections, hepatic encephalopathy and Kallmann syndrome (4-14), there are few histological and ultrastructural studies on the subject and which can provide information on the cytological changes occurring in these pathologies olfactory level. This motivated us to deepen, experimentally, in the study of olfactory bulb during neurogenesis, emphasizing the mechanisms of formation of interneuron connections as well as in neuronal plasticity processes that occur at different stages of olfactory bulb development whose results can be compared to a normal pattern in studies of diseases with neuropathic olfactory component as mentioned above (15-20).

The olfactory system has important physiological and ultrastructural characteristics that they are shaping and

defining during embryonic and early postnatal development being a widely used model to understand the growth mechanisms of neuronal cells by morphological simplicity, although it is also a model synaptic complex by the particular synaptic interactions required to connect the olfactory epithelium, olfactory bulb and olfactory cortex, which allows to detect volatile, organic, and low molecular weight chemical compounds, in order to perform its role in olfactory entirety. In recent years we have made great progress in better understanding of the olfactory pathways, which are responsible for making contact an odorant molecule with a receptor at the olfactory epithelium, decode and process the information generated, storing this information on the olfactory memory and produce the sensory response (21).

Studying in a lower vertebrate the cell maturation and formation of synapses that occur in the olfactory bulb during the period between the late prenatal and early postnatal development, can get a better understanding of the functionality of this organ. The results presented in this paper, when extrapolated to human, can allow to understand the relationship of olfactory disorders with some diseases, as is the case of known cytoarchitecture cortical dysplasia that correlates with epileptic disease (22).

2. Materials and Methods

2.1. Animals

The study was carried out on mice of the strain Naval Medical Research Institute (NMRI), provided by the Central Animal Laboratory of the University of Los Andes, Mérida state, Venezuela. Animals selected for the study were treated according to the regulations of the Bioethics Committee of that Laboratory, to the Standards for the Use of Animals in Research contained in the Code of Bioethics and Safety of National Found for Science, Technology and Innovation (FONACIT, Caracas, Venezuela) and the requirements of National Institute of Health-USA (NIH) (23, 24). Embryos (E) of mice 13, 15, 17, 19 and 21 days of development were used, which were obtained from two females gestated for each age. Five mice of each of the following postnatal age (P): 0, 1, 3, 5, 7 and 9 days, were also used. Mice were anesthetized with Ketamina^{R} at a dose of 240 mg / kg-weigh, and female mice with the programmed time of gestation, we performed an abdominal incision to expose the uterus and remove the sedated embryos, which were immediately placed in a fixative solution. Were to remove the olfactory bulbs both embryos and postnatal mice, and then they were fixed in mixture 3:3 for ultrastructural study.

2.2. Ultrastructural Analysis

For ultrastructural analysis olfactory bulbs after removal, were immersed in fixative mixture 3:3 (3% glutaraldehyde plus 3% formaldehyde in 0.1 M cacodylate buffer pH 6.3) (25), at 4°C conditions in which they were kept for 12h, after having been sectioned in blocks of approximately 3 mm^3. The tissue blocks were washed with 0.1 M cacodylate buffer pH 6.3 and, then be post-fixed, for 18h osmium tetroxide in 1% prepared in the same buffer. Then washed again in buffer, dehydrated with ethyl alcohol, and infiltrated with epoxy resin. Sections were made using a Porter-Blum Sorvall MT2-B ultramicrotome, and observed through a transmission electron microscope Hitachi H-7000.

2.2.1. Statistical Analysis

Quantification of interneuronal contact in different prenatal and postnatal ages was studied, since the appearance of the first intercellular contacts to the formation of morphologically mature axodendritic and dendrodendritic synapses. Statistical analysis of variance (ANOVA) with the application of SPSS version 15. The results were expressed in average mean ± standard deviation (Mean ± SD), taking the 95% as statistical confidence index ($p < 0.05$). The results allowed to compare the increase in the number of axodendritic and dendrodendritic synapses and the relationship of these with the development and maturation of the olfactory bulb.

3. Results

3.1. Ultrastructural Analysis

At day E17 between cell bodies, immature neuronal processes are observed which present thin and short thickenings on the inner of the neuronal membrane, and vesicular-like formations of different sizes, some of them similar to synaptic vesicles. Furthermore, the presence of small clusters of electron dense attached to the inner side of the membrane cell processes, which can be interpreted as the first intermembrane contact and therefore the beginning of the process of synaptic formation in the species studied.

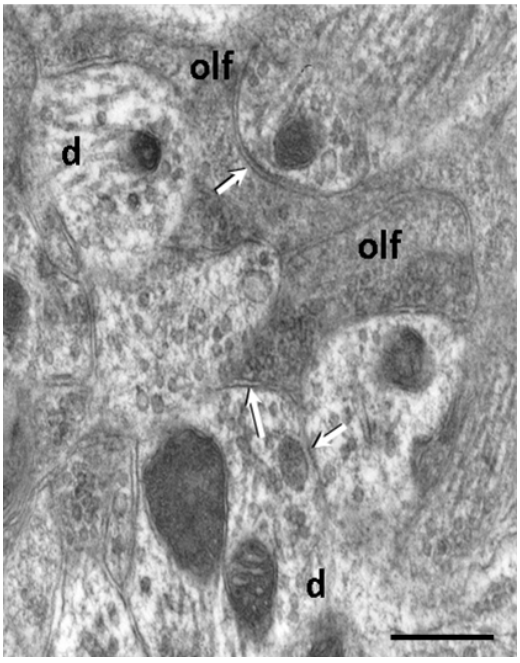

Figure 1. *Neuronal processes with synaptic vesicles in mouse olfactory bulb P3. Arrows indicate the onset of thickening of the neuronal membrane. More electron dense processes correspond to branches of the olfactory nerve (olf); d, dendrites of mitral and tufted cells. Bar=0.54µm.*

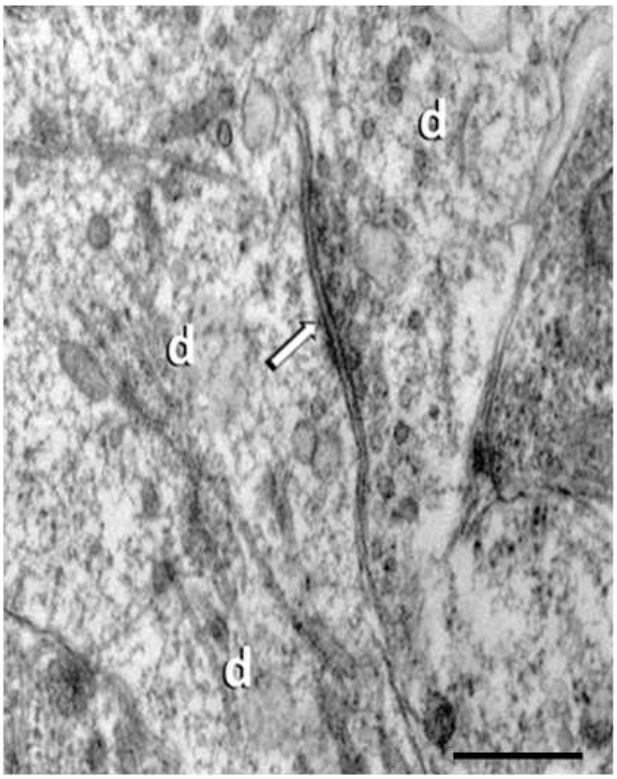

Figure 2. *Initial stage of the formation of a dendrodendritic synapses (arrow) between dendritic processes (d) in mouse olfactory bulb P3. Bar=0.54 µm.*

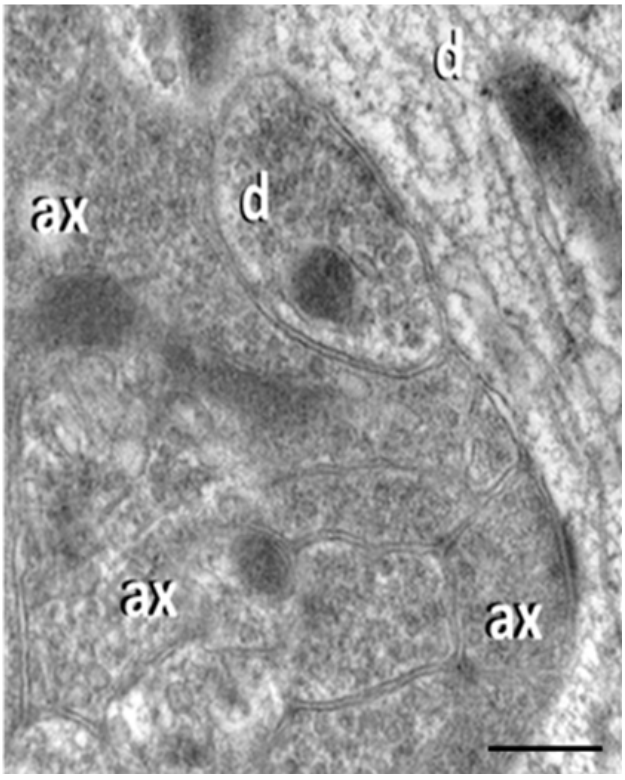

Figure 3. *Synaptic contacts observed in mouse olfactory bulb P7. ax, axon; d, dendrite. Bar=0.68 µm.*

At older ages analyzed is determined that the increase in the number of interneuronal contacts is very slow although the increase is continuous; further a slight and progressive increase in the densification of the inner sheet of the membrane at some sites in the dendritic structures is observed.

It is from P0 when a more pronounced increase in the number of synaptic formations is observed, P1 day begin to differentiate the axodendritic synapses, and to the day P3 can be clearly identified these synapses contacts between olfactory nerve processes and cellular dendritic of the olfactory bulb (Fig. 1). Moreover, the dendrodendritic synapses begin to display a day P3, mainly in the neuropil of the deepest parts of the plexiform layer, as illustrated in Figure 2, where the interrelationship between two dendrites is observed, being noted the presence of electron dense material internally attached to the dendritic membranes and the existence of few synaptic vesicles in proximity to this area. Both axodendritic as dendrodendritic synaptic contacts increase quantitatively to the days P7 and P9, ages at which the greatest number of synapses is recorded, which coincides with the observation that P7 is on the day when synaptic contacts in the mouse olfactory bulb showed their definitive characteristics, which are consolidated at day P9.

That is, in the P7 (Fig. 3) and P9 days (Fig. 4) axodendritic and dendrodendritic synaptic contacts have all the features of morphological maturity and number of synapses is higher in relation to the previous ages (Figs. 5 and 6).

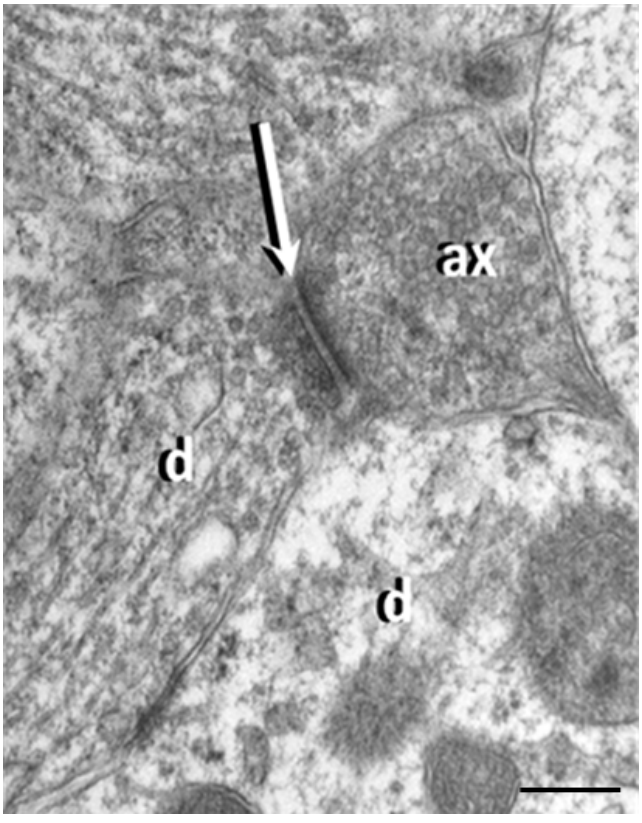

Figure 4. *Synapse axodendritic (straight arrow) in P9 mouse olfactory bulb. ax, axon; d, dendrite. Bar = 0.68 µm*

3.2. Statistical Analysis

The results obtained after ANOVA statistical analysis of the number of axodendritic synapses in the mouse olfactory bulb

from E17 to P9 (Table 1), show a value of p = 0.000, indicating that there are very significant differences between the number of axodendritic synapses in ages studied.

Table 1. *Variability in the number of axodendritic synapses in the mouse olfactory bulb during late embryonic and early postnatal development (n = 50).*

Ages	Number of synapses/µm^2 Averages ± SD	p
E17	0.97±0.29	
E19	1.61±0.21	
E21	1.69±0.50	
P0	1.80±0.47	
P1	2.16±0.57	0.000
P3	3.34±0.42	
P5	4.38±0.58	
P7	5.81±0.78	
P9	6.42±0.89	

The values obtained in E17, and from P1 to P9 show a statistically significant difference, whereas between E19 to P0 no statistically significant difference is observed.

During the prenatal stage (E17 to E21) growth in the number of axodendritic synapses is very slow and tends to be constant.

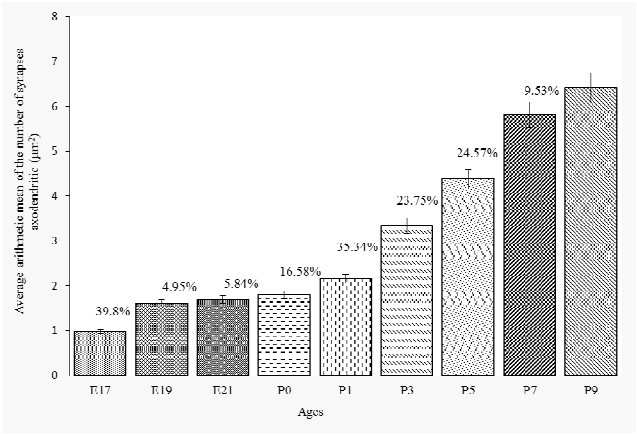

Figure 5. *Comparison of the percentage increase in the number of axodendritic synapses in embryonic and postnatal ages of mouse olfactory bulb.*

Table 2. *Variability in the number of dendrodendritic synapses in the mouse olfactory bulb during early postnatal development (n = 50)*

Ages	Number of synapses/µm^2 Averages ± SD	p
P3	0.80±0.47	
P5	1.95±0.56	0.000
P7	3.61±0.74	
P9	5.87±0.85	

It is from P0 when a slight increase in the number of these synapses is observed, although the true increase is from P1, as shown in Figure 5; this increase is progressive until P9, age in which synapses are already fully developed.

The statistical analysis of the number of dendrodendritic synapses in the mouse olfactory bulb during postnatal development (P3 to P9), gave a significance level of p = 0.000, which means there is a statistically significant difference between the ages studied, since p <0.05 (Table 2).

Averages in the number of dendrodendritic synapses are different for each age, which indicates that there are statistically significant differences in the number of these contacts in the ages studied.

Figure 6 shows, graphically, the steady and sustained increase in the number of dendrodendritic synapses in the mouse olfactory bulb during the period from P3 to P9.

The results obtained of the average arithmetic mean analysis in the number of axodendritic and dendrodendritic synapses indicate that the final assembly of both synapses is a postnatal event, from P1 in the case of axodendritic synapses and from P3 for dendrodendritic synapses, although in E17 the first signs of neuronal interrelationship are observed.

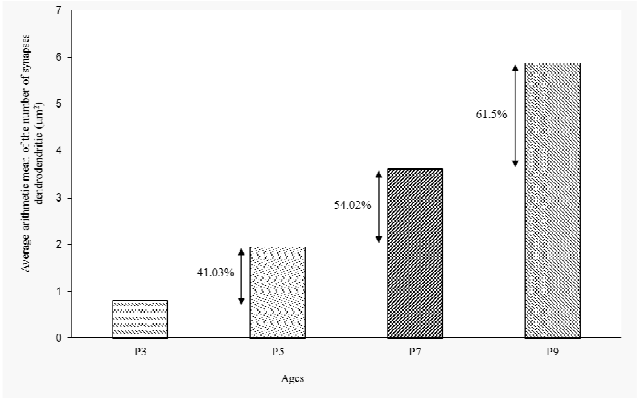

Figure 6. *Comparison of the percentage increase in the number of dendrodendritic synapses in postnatal ages of mouse olfactory bulb.*

4. Discussion

During the stage of cytological neurodifferentiation, the olfactory bulb undergoes a series of dynamic changes both cytomorphologicals as cytofuntionals that allow it to reach functional maturation by which it can exert the diverse and complex functions that fulfills despite being a relatively simple neural network (2, 26- 35) morphologically consolidates during early postnatal stage, when most neural interrelationships established. At P9 day reaches the synaptic maturity evidenced by the existence of axodendritic and reciprocal dendrodendritic synapses, the latter formed from a simple neural circuit controlling the modulation of the inhibitory activity of the mitral cells (3, 36-39) and consists of the mitral and anaxonic granule cells, making the olfactory bulb region complex synaptically.

According Blanchart et al. (40) in the mouse, electron-dense joints are observed from E13, and they were able to visualize synaptic vesicles in E14. However, our findings indicate that the onset of synaptogenesis in NMRI mouse olfactory bulb can be established on day E17 when thickenings are observed in the inside of some detached neuronal membranes. At older ages the amount of thickening increases mainly dendritic structures, becoming more pronounced and more electron density. Moreover, Marchand and Bélanger (41) have reported in rat that at day E16 is when axons begin to make contact in the olfactory bulb.

Relating axodendritic synapses quantitatively and age, we determine in E19 an increase of 39.8%, of synapses in relation to E17, then to P0 quantitative increase is slow with a percentage of synapse formation not exceeding 6% (E19 to E21 = 4.95%, and E21 to P0 = 5.84%). It is from P1, when a difference of 16.58% over the previous day, and begins a significant increase in the number of synapses is kept rising until P7, decreasing again synapse formation between P7 and P9 with a difference between them of 9.53%.

Through statistical analysis it can be conclude that the formation of dendrodendritic synapse is an exclusively postnatal event that starts the day P3, as a dynamic process with a high formation level; result that between P3 and P5 there is a difference of 41.03%, between P5 and P7 of 54.02%, and between P7 and P9 the difference is 61.5%.

Although in E17 axodendritic synapses are identified, their real differentiation is in P0; while the dendrodendritic synapses differ from P3, and both synapses increase quantitatively to the days P7 and P9. This allows us to affirm that the development and synaptic maturation in the mouse olfactory bulb is an event that occurs mainly in the early postnatal period, which coincides with the indicated Walton (42) and Kopel et al. (43). In addition, there are studies that suggest that the olfactory bulb of animals 3 days old is already capable of discerning olfactory stimuli it receives, which contributes significantly to the consolidation and final maturation of this sensory organ (15, 16, 44-49).

The results obtained in this study can determine the chronological moment in which the whole population neuronal olfactory bulb reaches its full integration to become an organ that can express all their functional capabilities. That moment, in the mouse corresponds to the age P7 ± 24h, we have defined as Critical Period, due to its physiological significance for animal biomodels use, because it allows to design more accurately experimental trials based on the objective to be pursued; ie, conditions studies involving neuronal connectivity processes will provide best results with experiments using animals with lower ages at Critical Period; while for the study of pathologies that develop when the nervous system is morphologically mature, model to choose is after the Critical Period age. The contributions of this work will allow designing experimental studies on diseases in which the olfactory system is involved.

5. Conclusions

This study allowed us to determine the way the neural structures that form the olfactory bulb are interconnecting and interacting to constitute an important and fundamental synaptic circuit for olfactory tract functionality.

Ultrastructural and statistically it was found that the dendrodendritic synapses are postnatal morpho-funtional expression, starting from the day P3; while axodendritic synapses are observed from the old E17, clearly expressing the P0 age. Both contacts increase their number and morphological maturity of continuous and consistent way to P7, age when the olfactory bulb expresses its functional potential.

The study and understanding of the development of the olfactory bulb, as well as knowledge of different morphological and synaptic events that occur during the embryonic and postnatal maturation, they are essential to understand the relationship of this region of the CNS with different neuronal pathologies and behavioral expressions, so much in biomodels both mammals and birds as in the humans. Analysis of the processes of morphogenesis that occur in this region of the nervous system is important to the understanding and treatment of diseases in which the olfactory bulb is involved.

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