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Age-Dependent Dopamine-2 Receptor (D_2R) Plasticity in Mouse Motor Cortex and Hippocampus

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ABSTRACT

Dopamine-2 receptor (D_2R) is shown to be important in motor and memory consolidation. Some neuro-psychiatry disorders are said to arise as developmental problems hence age-dependent D_2R was studied in mice. The study was designed to show if there is the presence of D_2R in mice motor cortex and hippocampus and if it changes as the animals grow with age. Four adult male and female mice were used for breeding pups used for the experiments. The animals were allowed to mate freely. At birth (P0) three pups were sacrificed by decapitation, brain was excised rinsed in normal saline and transferred to specimen bottle containing formal saline solution. Three pups sacrificed by cervical dislocation at postnatal day 14, 28 and 42 respectively were fixed transcardially using formal saline. The brain was excised immersion-fixed in formal saline. The brains were processed immunohistochemically to identify D_2R in the motor cortex and hippocampus. Number of D_2R expressing cells were counted using ImageJ software, and data were represented on a line graph to show age-related changes using GraphPad software V.5.0. D_2R was expressed in the motor cortex from birth, hippocampus from P14 till P42. Expression of D_2R peaks at P28 in the motor cortex and hippocampus and decline at P42.

Key words: *Dopamine-2 receptor, plasticity, hippocampus, motor cortex*

INTRODUCTION

Postnatal brain development is important in attaining the functional circuitry of the brain (Rakic et al. 1986), and involves synaptogenesis, synaptic pruning, receptors rearrangement and controlled cell death (Zangen et al. 2001). Postnatal development occurs at different stages in different species and different regions undergo a different process in attaining adult morphology (Andersen et al. 1997).

Dopamine a neurotransmitter produced mainly in the substantia nigra is shown to be responsible for motor coordination (Barnes and Sharp 1999). It has been shown anatomically that the basal ganglia receive inputs and send efferents to the cortex to mediate its functions (MacLean et al. 1985). The action of dopamine on the brain is mediated by dopamine receptors which have been classified as dopamine-1 (D_1R) and dopamine-2 receptors (D_2R) (Pearson et al. 1990). Another subtype has been identified ranging from D_3, D_4 and D_5 (Bouthenet et al. 1991). Dopamine projections to the motor cortex is responsible for the basal ganglia inputs for cortex coordination of movement (Garraux et al. 2007) and to the hippocampus for emotional learning (Torres et al. 2003). The dopaminergic neurons projected will

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release dopamine at the synapse that act on the postsynaptic neurons which carries the receptors.
Using the mRNA expression to study dopamine receptors distribution in rats, it was observed that D_2R was mainly expressed in the entorhinal cortex at high level, anterior cingulate, orbital and insular cortex has moderate expression, while scattered cells in layers IV-VI of frontal, parietal and temporal and occipital cortex also express D_2R mRNA (Mansour and Watson 2000). Dopaminergic stimulation plays a role in hippocampal functions (Saab et al. 2009). Studies from rats indicate that D_2R is not directly present in the hippocampus but in regions close to the hippocampus (Khan et al. 1999).
No studies have indicated the presence of D_2R in mice motor cortex and hippocampus and or its plasticity with aging as most neuropsychiatry disorders are said to arise as a developmental problem in the brain. Hence, this study is designed to investigate the age-dependent plasticity of dopamine-2 receptor (D_2R) in the motor cortex and hippocampus.

MATERIALS AND METHODS

Animal care
Eight (8) adult mice comprising of four males and four females were used for the experiment. The animals were gotten from Animal Holdings Unit, Afe Babalola University Ado-Ekiti. They were housed in standard housing cages, two females and two males' mice per cage in the animal house. Food and water were provided ad libitum and 12-hour day and night cycle was maintained in the animal house.
All experimental protocol and handling was done in accordance with Nigeria Ethical code of animal research as approved by Afe Babalola University animal research ethical committee.

Mating
The animals were allowed to mate freely. The animals were checked upon twice a day (morning and evening) when pregnancy is suspected for knowing the date of delivery.

Animal Sacrifice
After the female mice had littered, three pups were sacrificed from the day of birth (P0), and every two weeks till sixth week after delivery (i.e., P0, 14, 28, and 42).
Day 0 pups were sacrificed by decapitation, the brain was excised, rinsed in normal saline and fixed by immersing in 10% formal saline for 24 hours.
Day 14-42 pups were sacrificed by cervical dislocation. Transcardial perfusion fixation was done by first flushing out the blood with 0.9% normal saline through the left ventricle and drained out through the right atrium. After the blood was flushed out, 10% formal saline was injected through the ventricle to perfuse the whole animal through blood vessels. Perfusion was complete when the animal starts to twitch and remained turgid. After these, the animals were decapitated and the brain excised out and put in specimen bottle containing 10% formal saline for 24 hours.
The brain was then transferred into 20% formal sucrose solution (cryopreservation) and kept inside fridge (at $4^{o}C$). The brains were then thawed to room temperature, dehydrated in grades of alcohol and embedded in paraffin. Serial section was done at 5 µm with rotatory microtome using systemic random sampling (SRS) to pick sections on slide. Slides were later processed for immunohistochemistry (IHC) stain for D_2R.

Purchase of Antibodies
Mouse/Rat anti-D_2R primary antibody was a gift from Dr. Ogundele's Laboratory. Goat anti-rabbit/mouse (polyvalent) secondary antibody (ab64238) and DAB substrate kit (ab93705) was also purchased from Abcam USA.

Immunohistochemistry (IHC) Protocol
IHC was done to the brain slices using heat method of antigen retrieval for paraffin embedded tissue, this was done by heating the slides in antigen retrieval solution (citric acid pH 7.0) at 700C for 50 minutes. After which the slides were rinsed in 1X phosphate buffered saline (PBS) solution for 5 minutes twice, endogenous peroxidase was blocked by incubating in peroxide block solution for 30 minutes at room temperature followed by rinsing in 1X PBS thrice for 3 minutes each. Protein block was done by incubating the slides in protein block solution for 10 minutes at room temperature followed by rinsing in 1X PBS thrice for 3 minutes each. The peroxidase and protein block solutions came with the diaminobenzedine (DAB) detection kit. The slides are incubated with primary antibody (anti D_2R 1:1000) overnight at 100C. Next day the slides were rinsed thrice in 1X PBS for 5 minutes each and then incubated with secondary antibody (goat anti-rabbit/mouse) at room temperature for 30 minutes. The slides are then rinsed in 1X PBS thrice for 5 minutes each, incubated in streptavidin peroxidase solution, 3-amino-9-ethyl carbazole single solution and DAB solution all for 10 minutes at room temperature, rinsing in 1X PBS was done in between each incubation. After which counterstaining was done in haematoxylin solution for 10 minutes, dehydrated in alcohol, xylene and cover slipped.

Stereology Protocol
A number of immunopositive cells were counted using ImageJ software following the method of Reinking (2007). Photomicrograph was acquired using Olympus J50 light microscope coupled with

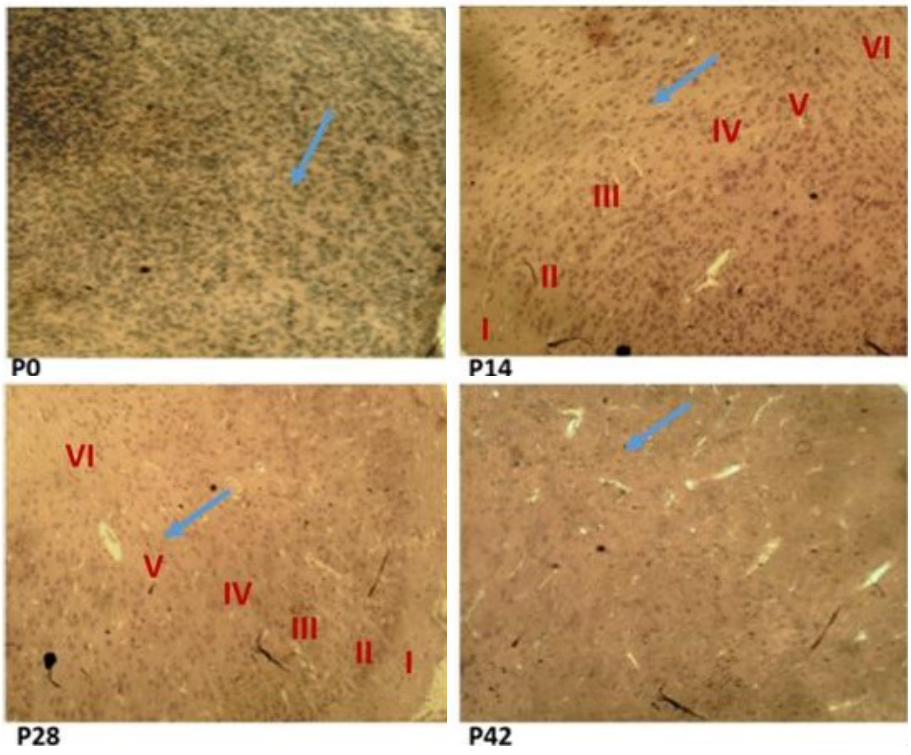

Figure 1: Representation of immunohistochemical image of D2R expression in the motor cortex of mice from postnatal day 0 (P0) to P42 at x10 objective lens. Blue arrow showing the immunopositive cells. Roman numerals represent the cortical

WinJoe cameroscope. The micrographs were scaled on imageJ software, superimposed with Cartesian grid to estimate the area. Region of interest was examined at x40 obj lens and brow deposit associated with a nucleus is counted as positive cells. Data were represented on a line graph using Graphpad software V.5.0.

RESULTS

D_2R Expression in Motor Cortex and Hippocampus

Immunohistochemical results reveal immuno-positive cells expressing D_2R present in the motor cortex area of pups. The positive cells were observed from P0 till P42 with different expressing patterns (Figure 1).

At birth, the hippocampus has not yet differentiated from the limbic system so no data was obtained at this age. D_2R was only present in the hippocampus in the region of the dentate gyrus (DG) and cornu ammonis regions (CA1, CA2, CA3) from postnatal day fourteen to forty-two (P14-42), a period when the hippocampus has fully developed and neurons expressing D_2R are abundant in both the DG and CA regions (Figure 2).

Age-Dependent Plasticity of D_2R in Motor Cortex and Hippocampus

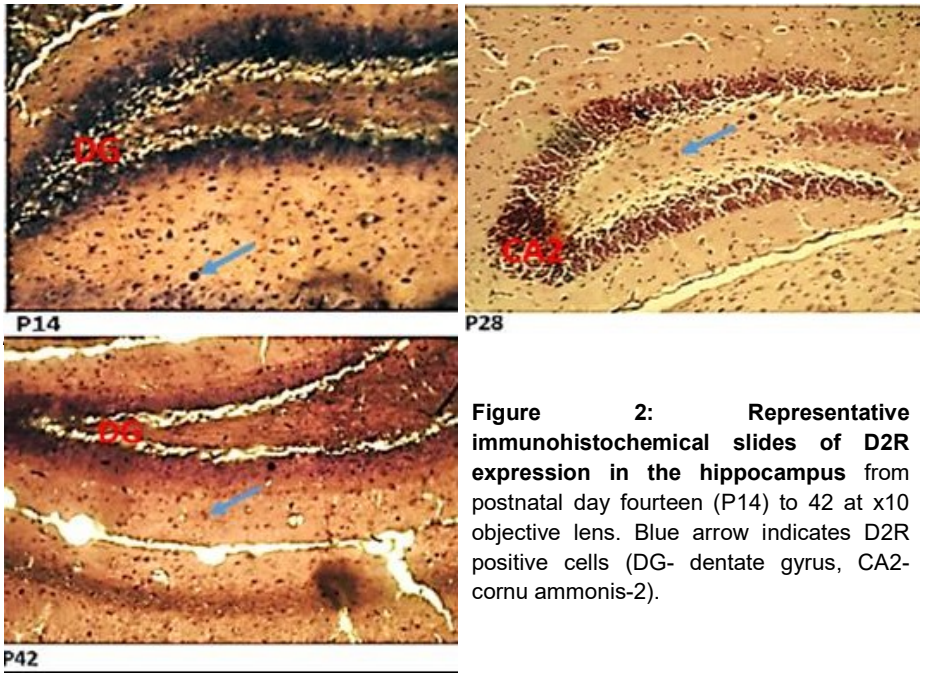

Figure 2: Representative immunohistochemical slides of D2R expression in the hippocampus from postnatal day fourteen (P14) to 42 at x10 objective lens. Blue arrow indicates D2R positive cells (DG- dentate gyrus, CA2- cornu ammonis-2).

D_2R immunopositive cells were counted using the ImageJ software. Data collected are represented as line graph to show the age-related changes of D_2R in motor cortex. Expression of D_2R had a sharp rise at an early stage of postnatal development (P14-P28) and peaked at P28. This later decreased as the adult reaches adulthood (P42; see Figure 3).

There was a steady increase in the number of D_2R expressing cells from P14 peaking at P28 (adolescent age in rodents) and then a decline back till P42 which marks the beginning of the adult stage in rodents (Figure 4).

DISCUSSION

D_2R Expression in Motor Cortex

Dopaminergic projection to motor cortex is said to play a role in the formation of motor memories, i.e. learning of novel movements (Garraux et al. 2007), control of voluntary movements and important in postnatal development of the brain (Gregg et al. 2001) Most studies on dopamine receptors densities in the brain focused on rats, monkeys, cats and humans.

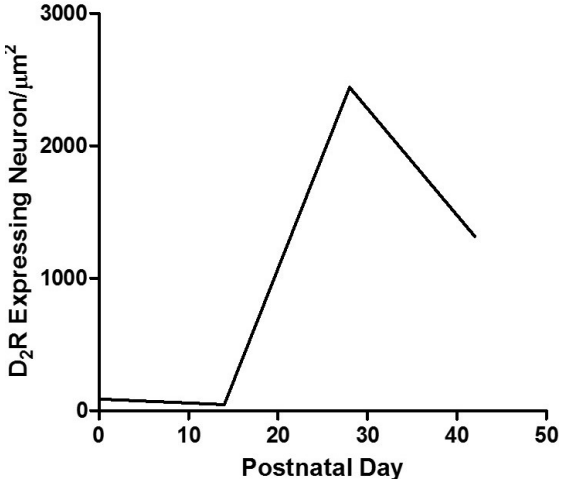

Figure 3: Line graph showing the number of neurons expressing D_2R in the motor cortex from postnatal day 0 (P0) to 42. The number of neurons expressing D_2R maintains a level before off shooting at P14 which then peaks at P28 and falls back as the animals' approach adulthood (P42).

D_2R was shown to be present in the mice motor cortex in this study. Similar D_2R presence was reported from other species like rats (Bouthenet et al. 1985; Mattes et al. 1986), cat (Richfield et al. 1987), monkey (Christian et al. 2009), and humans (Camus et al. 1986). In other species, D_2R is said to be predominantly located on the small interneurons in the cortex in all layers (Conde´ et al. 1994) while D_1R is said to be located on pyramidal neurons (Graybiel et al. 1981). In this study, D_2R was found in the small interneurons similar to what is obtained in rats (Mattes et al. 1986) and other species.

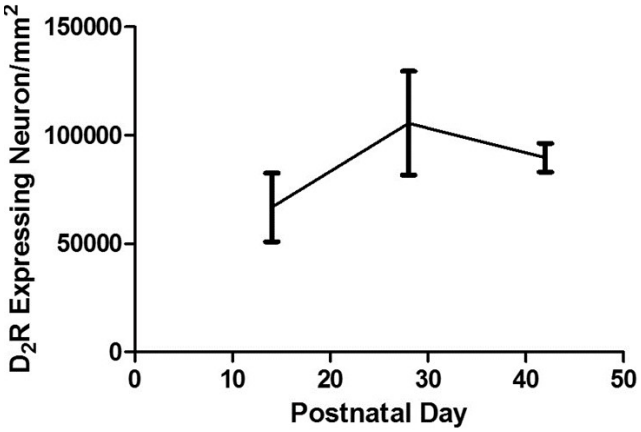

Figure 4: Line graph showing the number of neurons expressing D_2R in the hippocampus (DG and CA1-CA3) from postnatal day 14 (P14) to 42. The number of neurons expressing D_2R increased at an early stage of development and peaks at P28 which then decreased as the animal is growing in age till adult.

Dopamine system controls the cortex through 2 direct and indirect pathways (Goldman-Rakic 1998). The indirect pathway is said to synapse on the interneurons using GABA to control the pyramidal neurons which are cortex outflow (Horn 1990). This explains why 4th generation antipsychotics (e.g. haloperidol) can cause movement disorder since it acts mainly on D_2R (Joyce and Meador-Woodruff 1997). Ogundele et al (2015) and Ishola et al (2015) have shown that administration of haloperidol (which is a D_2R blocker) to mice leads to motor deficit seen in Parkinsonism and has been used as a model for studying Parkinsonism.

Mansour and Watson (2000) reported lamina variations in the expression of dopamine receptors in cats. In this study, D_2R was present in all layers of the motor cortex indicating that dopamine plays a vital role in motor coordination of mice. In a study that uses mRNA analysis to study the distribution of D_2R in the brain of rats (Stool and Kebabian 1984) it was reported that scattered cells in layers IV-VI of the cortex expressed D_2R (Mansour and Watson 2000). Although due to the complex mechanism of gene expression regulation in eukaryotes (Bouthenet et al. 1991), the presence of mRNA may not translate to the production of functional receptors. The wider distribution of D_2R in mice indicates that mice are sensitive to antipsychotics (e.g. haloperidol) than rats. This was evidenced from administering haloperidol to mice for 14 days to mimic Parkinsonism (Joyce and Meador-Woodruff 1997) while treatment for 28 days led to dyskinesia (Bankole et al. 2015).

D_2R Expression in the Hippocampus

The hippocampus was not yet developed in mice at birth as reported earlier in rats (Ishola and Adeniyi 2014). At P14 the hippocampus had developed and D_2R was expressed in granule cells of DG and CA1-CA3. This is different from other studies that reported a lack of D_2R in the hippocampus (Levey et al. 1993; Ciliax et al. 2000). There is evidence that dopaminergic inputs enter the hippocampus (Swanson 1982) and that they play important role in hippocampal function (Torres et al. 2003).

This study showed that mice hippocampus expressed D_2R in DG and CA1-CA3 regions. In rats, D_2 receptors were shown to be expressed in the retro hippocampus than the hippocampus proper (Kohler et al. 1985). The layers 1 and 3 of the entorhinal cortex and the layer 2 of the presubiculum were reported to be rich in specific binding sites for D_2R (Halldin et al.

1993). Studies from monkeys indicate that highest densities of D_2R were detected in the deep layers of the entorhinal cortex and in the layer 2 of the presubiculum. High expression of D_2R was also found in the granule cell layer of the area dentate (Khan et al. 1999).

In the hippocampus of a cat the highest expression existed in the inner one-third of the molecular layer of the dentate gyrus (DG) (Goldsmith and Joyce 1994). There were also significant numbers of D_2 receptors in strata radiatum and oriens of the CA subfields, with almost undetectable levels in lacunosum moleculare and subiculum (Goldsmith and Joyce 1994).

Post-mortem studies from human brain showed less specific binding compared to the monkey and rat. The highest expression is in the outer layers of the presubiculum and in the hilus of the area dentata (Kohler et al. 1985). There are the only minute amounts of D_2R in the entorhinal cortex (Lidow et al. 1989).

The distribution of dopamine D2-like receptors in the rat, monkey, and the human brain has been analyzed by autoradiography (Boyson et al. 1986; Bouthenet et al. 1991). These findings show that D_2 receptors are present in the hippocampal region and the retrohippocampus is enriched in dopamine D_2 receptors including the entorhinal cortex (Hogberg et al. 1991).

Age-Dependent Plasticity of D_2R

The brain undergoes plasticity with age and exposure to different factors (Gregg et al. 2001) ranging from environmental, biological and social factors (Blumberg et al. 2010). Some neurodegenerative diseases seen at an adult are associated with developmental problems during postnatal brain development (Dorn and Chrousos 1997). Symptoms of some diseases like attention deficit hyperactivity disorder (ADHD), Tourette syndrome and autism vary depending on the stage of the patient either during pre-pubertal or post-pubertal periods (Dorn and Chrousos 1997).

Receptors play an important role in postnatal development of the brain (Kohler et al. 1985), and receptor distribution vary in animals like rats (Kohler et al. 1985), monkeys (Pani et al. 2001) and man (Kohler et al. 1991). Receptors dynamics in postnatal brain development is high in number with increase in synaptic number which is later reduced starting from adolescence age to reach adult conformity (Adolfsson et al. 1979).

The result from this study shows that D_2R expression in the motor cortex was low at birth until postnatal day 14, and peak at postnatal day 28 (adolescent stage) and started to decline to reach the adult stage at P42 (Figure 3). This is similar to what is achieved in another mammalian brain where there was an overshoot of receptors and synapse at periadolescence followed by pruning (Andersen 2003). Most regions of the brain developed at a different stage in the rats where in the cortex D_2R increase with development and reach adult level by P21 (Murrin and Zeng 1986) while D_2R in globus pallidum reach an adult state at P30 (Rao et al. 1991). D_2R over expression and pruning back was not observed in rats except in the substantia nigra (Teicher et al. 1995).

Results from this work are similar to what is reported from monkey that D_2R decreases within one month after birth and increase to peak at 2-3 years and decline back to reach adult status (Rosenberg and Lewis 1995).

Movement study from mice corroborates the D_2R variation in the age as the animal at birth lacks coordinated movement. At P14, the animals move with more of jerky movement but start having coordinated movement as they approach adolescent stage (personal observation)

Midbrain structure (substantia nigra) project dopaminergic inputs into the hippocampus (Ahmed et al. 2010), and so it is hypothesized that dopamine plays role in memory formation (Torres et al. 2003). Results showed that at birth the hippocampus is not fully developed so there is no confirmation of D_2R expression in the hippocampus. From postnatal day fourteen to forty-two (P14-42), the hippocampus is fully developed and the neurons expressing the D_2 receptors are abundant in the DG and CA regions. From P14 to P28 there was a steady rise in D_2 which peaked at P28 which was the adolescent age in rodents and to decrease till the beginning of the adult stage in rodents which were P42. The pattern of distribution seen from this study is similar to the monkey (Kohler et al. 1991) as they too have overshoot in receptors at adolescent in their cortex. A similar distribution was observed in rat's striatum (Kohler et al. 1985) and post-mortem studies in man (Lidow et al. 1989). Insults to the brain during postnatal development have been shown to have long-term effects on the brain at adult (Carroll et al. 1991). Results from this study showed that D_2R density was high in the hippocampus at adolescent (Figure 4), this shows the window of high susceptibility of the hippocampus to insults. This is similar to other brain regions like substantia nigra, prefrontal cortex (Santos et al. 2008), striatum (Christian et al. 2009), and monkey cortex (Pani et al. 2001).

CONCLUSION

D_2R is present in mice motor cortex at birth, present in all the layers and mostly present in the small interneurons. Age-dependent distribution showed that D_2R expression was low after birth but peaks at adolescent and decline back to reach to adult status.

The hippocampus was not fully developed at birth so the presence of D_2R was not confirmed. But from P14 to P28 the hippocampus was fully developed showing the expression of D_2R. Age-dependent distribution showed the D_2R expression was

increased from P14 but peaked at adolescent P28 and later declined during adult years.

Conflict of Interest

The authors declare no conflict of interest in the conduct of this research work

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