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Dopamine D1 and D2 Receptor Subtypes Functional Regulation in Unilateral Rotenone Lesioned Parkinson's Rat Model: Effect of Serotonin, Dopamine and Norepinephrine

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta leading to marked reduction of dopamine levels in the cerebral cortex. The present study analysed the effect of serotonin, dopamine and norepinephrine as treatment on rotenone induced hemi-Parkinson's disease in rats and its role in the regulation of Dopamine receptor subtypes in the cerebral cortex of the experimental rats. Unilateral stereotaxic single dose infusions of rotenone were administered to the substantia nigra of adult male Wistar rats. Neurotransmitters –serotonin, dopamine and norepinephrine treatments were given to rotenone induced hemi-Parkinson's rats. Scatchard analysis of Dopamine D1 and D2 receptor showed a significant increase ($p<0.001$) in the cerebral cortex of the Parkinson's rats compared to control. These altered parameters were reversed to near control in the serotonin and norepinephrine treated Parkinson's disease rats and no change was observed in Dopamine treated Parkinson's disease rats. Real-time PCR results confirmed the receptor data. Cognitive and sensorimotor activities were reduced in Parkinson's disease rats which were reversed by serotonin and norepinephrine. Our results showed serotonin and norepinephrine functionally reversed the Dopamine receptors significantly in rotenone induced hemi-Parkinson's rat. This has clinical significance in the therapeutic management of Parkinson's disease.

Keywords

Parkinson's disease; Rotenone; Substantia nigra; Cerebral cortex; Dopamine receptors.

Introduction

Parkinson's disease (PD), originally described in 1817 by James Parkinson, is currently regarded as the most common degenerative disorder of the ageing brain after the Alzheimer's dementia. It is characterized by muscle rigidity, tetrad of tremor at rest, postural instability, bradykinesia and in extreme cases akinesia [1]. The symptoms are the results of decreased stimulation of the motor cortex by the basal ganglia, normally caused by the insufficient formation and action of dopamine, which is produced in the dopaminergic neurons of the substantia nigra pars compacta (SNpc) of brain.

PD patients are characterized by systemic mitochondrial dysfunction, marked by inhibition of complex I of the mitochondrial electron transport chain. To model the systemic defect in complex I reported in PD, researchers have used rotenone exposure. Rotenone is a commonly used pesticide and potent, specific inhibitor of mitochondrial complex I. Rotenone because of its lipophilic nature, crosses biological membranes easily and independent of transporters. As a result, systemic rotenone exposure inhibits complex I uniformly throughout brain [2]. Unilateral infusion of rotenone reproduces neurochemical and neuropathological features of hemi-Parkinsonism in rats and indicates an active involvement of oxidative stress in rotenone-induced nigrostriatal neurodegeneration [3].

Dopamine (DA), one of the major neurotransmitter in central

nervous system is involved in the control of motor and cognitive functions [4]. Dopamine supplied as medication cannot cross the blood-brain barrier [5]. Targeting neurotransmitter systems beyond the dopamine system is an interesting approach, both for the motor and nonmotor problems of PD [6]. Non-dopaminergic neurotransmission is also affected in PD. The dysfunction of non-dopaminergic systems explains the principal non-dopaminergic symptoms, such as 'axial' signs and cognitive impairment. The non-dopaminergic neurotransmitters affected in PD are noradrenaline (norepinephrine), serotonin (5-hydroxytryptamine; 5-HT), glutamate, gamma-aminobutyric acid (GABA), acetylcholine and neuropeptides [7]. Dysfunction of these systems can lead to some of the motor symptoms of the disease and provide targets for pharmacological interventions to treat these symptoms. Accordingly, alterations in serotonergic function accounts for behavioural disturbances commonly observed during PD. Recent advances in understanding the role of 5-HT in Parkinsonism and the generation of side-effects of dopamine replacement therapy (e.g. wearing-off and levodopa-induced dyskinesia) have identified 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors as potential therapeutic targets in Parkinson's disease [8].

Although Parkinson's disease is characterized primarily by loss of nigrostriatal dopaminergic neurons, there is a concomitant loss of norepinephrine neurons in the locus coeruleus (LC). Norepinephrine is important for regulating the activity of dopamine neurons. The dopamine neurons and the norepinephrine neurons function in concert. As the dopamine neurons start dying, the norepinephrine neurons compensate by signalling the surviving dopamine cells to dramatically increase their activity and the output of dopamine. Results of Rommelfanger et al. [9] indicate that having a normal complement of dopamine neurons is not enough for normal motor function but that norepinephrine also needs to be present to ensure proper dopamine release.Rommelfanger and Weinshenker [10] examined the evidence that NE is neuroprotective and that LC degeneration sensitizes DA neurons to damage. In patients with Parkinson's disease, the cerebellar cortical norepinephrine levels were significantly below normal [11]. Altered norepinephrine metabolism contributes to some aspects of intellectual dysfunction in PD [12-14]. Restoring NE can have important therapeutic potential in PD.

In the present work, the effects of 5-HT, DA and NE supplementation intranigrally to substantia nigra as treatment individually on rotenone induced Hemi-Parkinson's disease in rats were analyzed. Dopaminergic D1 and D2 binding parameters investigated its role in the regulation of dopamine receptor subtypes in the cerebral cortex of the experimental rats. Real-Time PCR work was done to confirm the binding parameters.

Experimental and Methods

Animals

Experiments were carried out on adult male Wistar rats of 250-300g body weight purchased from Kerala Agricultural University, Mannuthy. They were housed in separate cages under 12 hrs light and 12 hrs dark periods and were maintained on standard food pellets and water ad libitum. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health guidelines and CPCSEA guidelines.

Chemicals

Biochemicals

Rotenone, dopamine, 5-hydroxy tryptamine, norepinephrine, ascorbic acid, pargyline, calcium chloride, sulpiride, SCH 23390, amphetamine and apomorphine were purchased from Sigma Chemical Co., St. Louis, MI, USA. All other reagents were of analytical grade purchased locally.

Radiochemicals

[^{3}H]SCH 23390 (Sp. activity 83Ci/mmol), [^{3}H]YM-09151-2((Sp. activity 85Ci/mmol) and [^{3}H] Dopamine (Sp. activity 45Ci/mmol) were purchased from NEN Life Sciences Products, Inc. Boston, USA.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, MI, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR - Dopamine D_1 (Rn_02043440), Dopamine D_2 (Rn_00561126) endogenous control (β-actin) were purchased from Applied Biosystems, Foster City, CA, USA.

Experimental Design

The experimental rats were divided into the following groups i) Control ii) Rotenone infused (Rotenone) iii) Rotenone infused supplemented with DA (Rotenone + DA) iv) Rotenone infused supplemented with 5-HT (Rotenone + 5-HT) v) Rotenone infused supplemented with NE (Rotenone + NE). Each group consisted of 6-8 animals.

Unilateral intranigral infusion of Rotenone

Rats were anesthetized with urethane (100 mg/100g body weight. i.p.). The animal was placed in the flat skull position on a cotton bed on a stereotaxic frame (BenchmarkTM, USA) with incisor bar fixed at 3.5 mm below the interaural line. Rotenone dissolved in DMSO: PEG (1:1) was infused 1µl into the right SNpc at a flow rate of 0.2µl/min. After stopping the infusion of the toxin, the probe was kept in the same position for a further 5 min for complete diffusion of the drug and then slowly retracted. The stereotaxic coordinates for SNpc were: lateral (L) =+0.20 cm, antero-posterior (AP, from the bregma point) =−0.53 cm and dorsi-ventral (DV) = +0.75 cm. The stereotaxic co-ordinates were calculated for the dopaminergic neuronal cell body region, SNpc following the "Rat Brain Atlas" [15]. All the groups except Control group were infused with Rotenone and in control animals, 1 µl of the vehicle (DMSO: PEG (1:1)) was infused into the right SNpc. Proper postoperative care was provided till the animals recovered completely.

Rotational Behavior

Amphetamine-induced rotational behavior was assessed as described earlier [16,17]. Rats were tested on the 14th day after

intranigral injection of Rotenone. After acclimatization for at least 10 min, amphetamine (5 mg/kg, i.p.) was administered. Animals that had completed a 360◦ circle towards the intact (contralateral) side and the lesioned (ipsilateral) side were counted for 240 min continuously and recorded separately. Rats were tested on the 16th day following intranigral injection of Rotenone. After acclimatization in the experimental cage for at least 10 min, apomorphine (1 mg/kg, s.c.) was administered. Animals that had completed a 360◦ circle towards the intact (contralateral) and the lesioned (ipsilateral) sides were counted for 60 min continuously and recorded separately. Animals showing more than 350 contralateral rotations during the initial 1 hr were separated on the 16th day. (Animals that showed no significant contralateral rotations were excluded from the study).

Treatment

Animals in group iii, iv, v were anaesthetized with urethane (100 mg/100g body weight. i.p.) on the 18th day, and Stereotaxic single dose of 1µl of DA (10µg/µl), 5-HT (10µg/µl) and NE (10µg/µl) was infused into the right SNpc at a flow rate of 0.2 µl/min into the respective groups. The stereotaxic co-ordinates are: lateral (L) =+0.20 cm, antero-posterior (AP) =−0.53 cm and dorsi-ventral (DV) = +0.75 cm from the bregma point. After stopping the infusion, the probe was kept in the same position for a further 5 min for complete diffusion of the drug and then slowly retracted.

Tissue Preparation

All the control and experimental rats were sacrificed on the 25th day by decapitation. The brain regions and body parts were dissected out quickly over ice [18] and the tissues were stored at -700C for various experiments. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

Dopamine Receptor Binding Studies Using [^{3}H]Radioligands In the Brain Regions of Control and Experimental Rats

Dopamine receptor binding studies using [^{3}H] Dopamine

DA receptor assay was done using [^{3}H]DA according to Madras et al. Cerebral cortex were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01%ascorbic acid, 4mM $MgCl_2$, 1.5mM $CaCl_2$, pH. 7.4 and centrifuged at 38,000 x g for 30min at 4°C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000 x g for 30min at 4°C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-1.5nM of [^{3}H]DA in 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 1mM $MgCl_2$, 2mM $CaCl_2$, 120mM NaCl, 5mM KCl, pH.7.4 in a total incubation volume of 250µl containing 200-300µg of proteins. Specific binding was determined using 100µM unlabelled dopamine. Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D1 Receptor Binding Studies Using [^{3}H]SCH 23390

Dopamine D1 receptor binding assay using [^{3}H]SCH 23390 in the cerebral cortex were done [19]. The tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM $MgCl_2$, 1.5mM $CaCl_2$, 5mM KCl, pH 7.4. The homogenate was centrifuged at 40,000 x g for 30min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000 x g for 30min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.5 - 5.0nM of [^{3}H]SCH 23390 in 50mM Tris-HCl buffer (pH 7.4), along with 1mM EDTA, 4mM $MgCl_2$, 1.5 mM $CaCl_2$, 5mM KCl with 12µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250µl containing 150-200µg protein. Specific binding was determined using 50µM unlabelled SCH 23390. Competition studies were carried out with 1.0nM [^{3}H]SCH 23390 in each tube with unlabelled ligand concentrations varying from 10-9-10-4 M of SCH 23390.

Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters. The filters were washed quickly by three successive washing with 5.0ml of ice cold 50mM Tris buffer, (pH 7.4). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D2 receptor binding studies using [^{3}H]YM-09151-2

Dopamine D2 receptor binding assay was done according to the modified procedure of Madras et al., Unis et al. [20,21]. The dissected Cerebral Cortex was weighed and homogenised in 10 volumes of ice cold 50mM Tris-HCl buffer, pH.7.4 along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl and 5mM KCl. The homogenate was centrifuged at 40,000xg for 30 min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000xg for 30 min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.1 - 2.0nM of [^{3}H]YM-09151-2 in 50mM Tris-HCl buffer, pH 7.4, along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl, 10µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250µl containing 150-200µg of protein. Specific binding was determined using 5.0 µM unlabelled sulpiride.

Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Protein Determination

Protein was measured according to Lowry et al. [22] using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read

in Spectrophotometer at 660nm.

Analysis of the Receptor Binding Data

Linear Regression Analysis for Scatchard Plots

The data was analysed by Scatchard et al. [23]. The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

Gene Expression Studies of Dopamine Receptor Subtypes in Cerebral Cortex of Control and Experimental Rats

Preparation of RNA

RNA was isolated from the different brain regions of control and experimental rats using the Tri reagent from Sigma Chemical Co., St. Louis, MI, USA.

Isolation of RNA

Tissue (25-50mg) homogenates were made in 0.5ml Tri Reagent and was centrifuged at 12,000 x g for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5min. 100µl of chloroform was added to it, mixed vigorously for 15sec and allowed to stand at room temperature for 15min. The tubes were then centrifuged at 12,000 x g for 15min at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10min. The tubes were centrifuged at 12,000 x g for 10min at 4°C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000 x g for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was 1.7. The concentration of RNA was calculated as one absorbance 260 = 42µg.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2ml microfuge tubes. The reaction mixture of 20µl contained 0.2µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25°C for 10min and 37°C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Fosterity, CA, USA designed using Primer Express Software Version (3.0).

Real-Time PCR Assay

Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe (designed by Applied Biosystems). Endogenous control, β-actin, was labelled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20µl contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probe for Dopamine DA D_1 (Rn_02043440_s1), Dopamine DA D_2 (Rn_00561126_m1), endogenous control (β-actin) and 12.5µl of TaqMan 2X Universal PCR MasterMIX (Applied Biosystems). The volume was made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50ºC -- 2 minutes --- Activation
95ºC -- 10 minutes --- Initial Denaturation
95ºC -- 15 seconds --- Denaturation 40 cycles
50ºC -- 30 seconds --- Annealing
60ºC -- 1 minute --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

Statistics

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve fitting procedure (GraphPad PRISMTM, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

Scatchard analysis using [^{3}H] Dopamine against Dopamine

Binding studies of [^{3}H] Dopamine against Dopamine for DA general receptors showed a significant increase in B_{max} ($p<0.001$) in the Rot group compared to control. B_{max} of 5-HT and NE treated groups - Rot+5-HT, Rot+NE reversed towards control values. Rot+DA did not reverse the changes compared to other two groups (Table 1).

Animal status	B_{max}(fmoles/mg protein)	K_d (nM)
Control	146.00 ± 3.16	3.24 ± 0.19
Rotonone	274.16 ± 3.17 ***	3.40 ± 0.14
Rot +DA (Dopamine)	264.00 ± 3.15 ***	3.26 ± 0.16
Rot+5-HT(Serotonin)	180.00 ± 3.16 ψψψ	3.28 ± 0.20
Rot +NE(Norepinephrine)	182.00 ± 3.13 ψψψ	3.27 ± 0.19

Table 1: Scatchard Analysis of [^{3}H] Dopamine Binding against Dopamine in the Cerebral. Cortex of Control, Rotonone, Rot+DA, Rot+5HT and Rot+NE.
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats.
B_{max} – Maximal binding; K_d – Dissociation constant, *** $p<0.001$ when compared to Control, ψψψ $p<0.001$ when compared to Rot, C – Control, Rot – Rotenone infused, Rot+DA – Rotenone infused treated with Dopamine, Rot+5-HT – Rotenone infused treated with Serotonin, Rot+NE – Rotenone infused treated with Norepinephrine.

Scatchard analysis using [^{3}H] SCH 23390 against SCH 23390

Binding studies of [3H] SCH 23390 against SCH 23390 for DA D1 receptors showed a significant increase in Bmax ($p<0.001$) in the Rot group compared to control. B_{max} of 5-HT and NE treated groups - Rot+5-HT, Rot+NE reversed towards control values. Rot+DA did not reverse the changes compared to other two groups (Table 2).

Animal status	B_{max}(fmoles/mg protein)	K_d (nM)
Control	43.00 ± 2.05	3.08 ± 0.15
Rotonone	157.00 ± 2.77 ***	3.37 ± 0.12
Rot +DA (Dopamine)	101.00 ± 2.05 ***	2.98 ± 0.15
Rot+5-HT(Serotonin)	52.00 ± 2.16 ψψψ	2.72 ± 0.20
Rot +NE(Norepinephrine)	59.00 ± 2.33 ψψψ	2.78 ± 0.18

Table 2: Scatchard Analysis of [^{3}H] SCH 23390 Binding against SCH 23390 in the Cerebral Cortex of Control, Rot, Rot+DA treated, Rot+5-HT treated and Rot+NE treated group rats.
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats.
B_{max} – Maximal binding; K_d – Dissociation constant. *** $p<0.001$ when compared to Control. ψψψ $p<0.001$ when compared to Rot. C – Control, Rot – Rotenone infused, Rot+DA – Rotenone infused treated with Dopamine, Rot+5-HT – Rotenone infused treated with Serotonin, Rot+NE – Rotenone infused treated with Norepinephrine.

Scatchard analysis using [^{3}H]YM-09151-2 against Sulpiride

Binding studies of [^{3}H]YM-09151-2 against Sulpiride for DA D2 receptors showed a significant increase in Bmax ($p<0.001$) in the Rot group compared to control. Bmax of 5-HT and NE treated groups - Rot+5-HT, Rot+NE reversed towards control values. Rot+DA did not reverse the changes compared to other two groups (Table 3).

Animal status	B_{max}(fmoles/mg protein)	K_d (nM)
Control	39.00 ± 2.05	3.11 ± 0.19
Rotonone	160.00 ± 3.77 ***	3.40 ± 0.14
Rot +DA (Dopamine)	104.00 ± 3.05 ***	3.04 ± 0.16
Rot+5-HT(Serotonin)	60.00 ± 4.16 ψψψ	2.72 ± 0.20
Rot +NE(Norepinephrine)	62.00 ± 3.33 ψψψ	2.82 ± 0.19

Table 3: Scatchard Analysis of [3H] YM-09151 Binding against Sulpiride in the Cerebral. Cortex of Control, Rotonone, Rot+DA, Rot+5HT and Rot+NE.
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consist 6-8 rats.
Bmax – Maximal binding; Kd – Dissociation constant, *** $p<0.001$ when compared to Control, ψψψ $p<0.001$ when compared to Rot, C – Control, Rot – Rotenone infused, Rot+DA – Rotenone infused treated with Dopamine, Rot+5-HT – Rotenone infused treated with Serotonin, Rot+NE – Rotenone infused treated with Norepinephrine.

Real-Time PCR analysis of DA D1 receptors

The gene expression studies by real-time PCR analysis showed that DA D1 receptor mRNA was significantly up regulated in Rot ($p<0.001$) groups. 5-HT treatment and NE treatment to Rot rats – Rot+5-HT and Rot+NE significantly ($p<0.001$) reversed the up regulation compared to PD groups. Rot+DA group did not reverse the changes compared to other two groups (Figure 1).

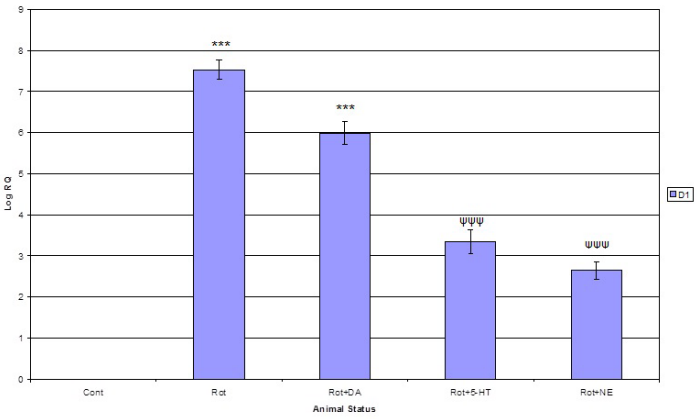

Figure 1: Real Time PCR amplification of Dopamine D1 receptor mRNA from the Cerebral Cortex of Control, Rot infused, Rot+DA, Rot+5-HT, Rot+NE treated rats.
Representative graph showing Real-Time amplification of Dopamine D1 receptor subunit mRNA from the cerebral cortex of control and experimental rats. The 2-ΔΔCT method of relative quantification was used to determine the fold change in expression. Values are mean ± S.E.M of 4-6 separate experiments. C – Control, Rotenone – Rotenone infused, Rotenone + DA – Rotenone treated with Dopamine, Rotenone + 5-HT – Rotenone infused treated with Serotonin, Rotenone + NE– Rotenone infused treated with Norepinephrine. ***$p<0.001$ when compared to control, ψψψ$p<0.001$ when compared to Rotenone group.

Real-Time PCR analysis of DA D2 receptors

The gene expression studies by real-time PCR analysis showed that DA D2 receptor mRNA was significantly up regulated in Rot and Rot+DA ($p<0.001$) groups. 5-HT treatment and NE treatment to Rot rats – Rot+5-HT and Rot+NE significantly ($p<0.001$) reversed the up regulation compared to PD groups. Rot+DA group did not

reverse the changes compared to other two groups (Figure 2).

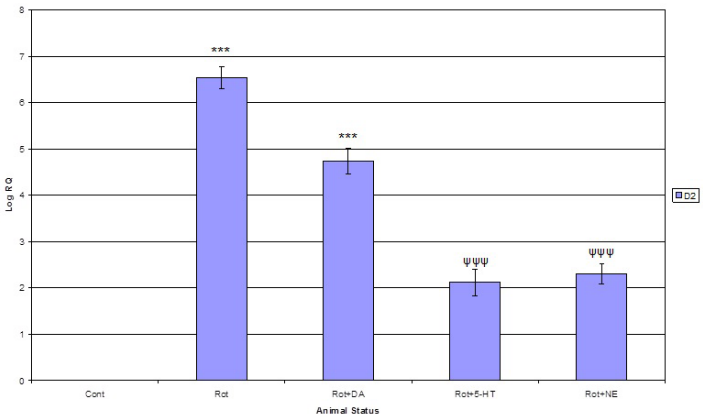

Figure 2: Real Time PCR amplification of Dopamine D2 receptor mRNA from the Cerebral Cortex of Control, Rot infused, Rot+DA, Rot+5-HT, Rot+NE treated rats.

Representative graph showing Real-Time amplification of Dopamine D2 receptor subunit mRNA from the cerebral cortex of control and experimental rats. The 2-ΔΔCT method of relative quantification was used to determine the fold change in expression. Values are mean ± S.E.M of 4-6 separate experiments. C – Control, Rotenone – Rotenone infused Rotenone + DA – Rotenone treated with Dopamine, Rotenone + 5-HT – Rotenone infused treated with Serotonin, Rotenone + NE– Rotenone infused treated with Norepineherine. ***p<0.001 when compared to control, ψψψp <0.001 when compared to Rotenone group.

Discussion

PD is a progressive motor system disorder characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc) leading to marked reduction of dopamine levels in the striatum [24-26]. Administration of rotenone, a well-characterized, high-affinity, specific inhibitor of complex-I of the inner mitochondrial membrane involved in oxidative phosphorylation [27] has been demonstrated convincingly to produce nigrostriatal dopaminergic neurodegeneration, as well as behavioral and neuropathological hallmarks of PD [28].

Serotonin and L-DOPA have been reported to exhibit protective effects on oxidative tissue damages [29]. Serotonin depresses lipid peroxidation of microsomes by Fe3+, ADP and NADPH system [30]. N-Acetylserotonin decreases the peroxidation of linoleic acid induced by 2, 2' -azobis (2-amidinopropane) [31]. Serotonin is reported to scavenge superoxide anion and hypochlorous acid (HOCl). Serotonin metabolises to the potential neuroprotective antioxidants, normelatonin and melatonin, which also helps to prevent oxidative damage caused as a result of rotenone administration. N-acetyl-serotonin (normelatonin) and melatonin protect neurons against oxidative challenges and suppress the activity of the transcription factor NF-kappaB [32]. Norepinephrine is a potent comitogen through binding to the ∞1 - adrenergic receptor [33]. The comitogen, NE, increased the proliferative response to EGF [34].

The cerebral cortex is critical to speech, emotion, reasoning, memory, movement and integration of information. In the cerebral cortex, DA receptor sub types showed significantly increased activity in PD rats and on supplementation of dopamine compared to control rats whereas its activity reversed to near control level in the 5-HT and NE supplemented groups. Real time PCR studies were conducted to evaluate the DA functional regulation at the mRNA level during PD and supplementation with DA, 5-HT and NE. We obtained an up regulation in the DA receptor subtypes mRNA during PD and those treated with DA. 5-HT and NE supplemented groups reversed the receptor gene expression to near control level.

The increased expression of DA receptor subtypes in the cerebral cortex of PD and those treated with DA is accounted for the supersensitivity of the DA receptor subtypes to DA owing to loss of dopaminergic neurons in the substantia nigra as a result of administration of rotenone. The antioxidant and comitogen 5-HT and NE triggered cell division of the dopaminergic neurons in the substantia nigra region, causing the DA neurons to secrete DA although not efficient as in the control group, thus leading to a reversal of the receptor gene expression to control level owing to decreased supersensitivity of the DA receptor subtypes in the cerebral cortex. The dopamine treated group does not show much significant change as compared to the other two groups as the deficiency of the neurotransmitter in the above two regions results in an increased utilization of the same.

Our experimental results show that 5-HT and NE play important role in the management of Parkinson's disease and no change was observed in DA treated PD rats. All our studies including behavioral and Real time PCR supports the above statement. We conclude from our studies that 5-HT and NE treatment potentiates a therapeutic effect by reversing the alterations in DA receptor subtypes binding and gene expression that occur during Parkinson's disease. Thus, it is evident that 5-HT and NE treatment to PD rats renders protection against oxidative and related motor and cognitive deficits which will have therapeutic significance in the management of Parkinson's disease.

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