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Age related interaction of dopamine and serotonin synthesis in striatal synaptosomes

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ABSTRACT: Tyrosine hydroxylase and tryptophan hydroxylase are key rate limiting enzymes in the biosynthesis of dopamine and serotonin, respectively. Since both enzymes are active in striatum, and affected by age, this study was undertaken to investigate interaction between dopamine and serotonin synthesis in brain striatal synaptosomes of aging rat. Male Wistar rats (3 and 30 month old) were killed by decapitation and brain striatal synaptosomes were prepared by discontinuous Ficoll/sucrose gradient technique. Synaptosomes were incubated in the presence of added pargiline (monoamineoxidase inhibitor), dopamine or serotonin synthesized during 25 min was measured by HPLC, employing electrochemical detection. Dopamine synthesis in synaptosomes prepared from young animals was markedly inhibited by addition of 5 μM serotonin concentrations (30%) and increasing serotonin concentrations up to 50 μM caused only a smaller additional inhibition. Dopamine synthesis in synaptosomes obtained from old rats was significantly lower than that of youg animals and addition of serotonin concentrations up to 50 μM had little effect on these preparations. In case of serotonin synthesis, exogenously added 5 μM dopamine inhibited serotonin synthesis in the synaptosomes of both ages by about 40%, whereas with higher concentration of dopamine (10-50 μM) the rate of inhibition was highly pronounced in old rats as compared to that of young animals. It is concluded that dopamine and serotonin interaction may be significant, and that these should be considered in long-term treatments of Parkinson's disease with L-DOPA.

Introduction

Tyrosine hydroxylase (TH, EC 1.14.16.2) and tryptophan hydroxylase (TPH, 1.14.16.4) are key rate limiting enzymes in the biosynthesis of dopamine and serotonin respectively (Nagatsu *et al.*, 1964; Lovenberg *et al.*, 1968). A number of investigations on partially purified cell free preparations of TH and TPH have indicated that these enzymes interact with several different molecules, such as non-heme iron (II), molecular oxygen and reduced pteridine cofactor BH4 to catalyze the conversion of tyrosine to dihydroxyphenylalanine and 5-hydroxytryptophan (Hamon *et al.*, 1981; Andersson *et al.*, 1992). They utilize molecular oxygen to oxidize the reduced pteridine cofactor BH4. Based on kinetic studies of rat TH-catalyzed reaction, a sequential reaction mechanism has been proposed with an ordered binding of BH4 as the first substrate, followed by dioxygen and tyrosine which is subjected to feedback inhibition by catecholamines (Flatmark *et al.*, 1999). This inhibition is suggested to be competitive with BH4 and not with tyrosine (Flatmark *et al.*, 1999). Similar studies on a possible serotonin TPH feedback loop have been complicated by difficulty of preparing adequate of purified enzyme of sufficient stability (Vitto and Mandell, 1982). However, a number of reports indicated

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that both enzymes are tightly regulated by inhibitory feedback via autoreceptors, a phenomenon which has been termed "receptor mediated feedback inhibition" (el Mestikawy and Hamon, 1986; Mestikawy *et al.*, 1986). Moreover, it is now well established that serotonin and catecholamines inhibit TH and TPH activities respectively (Devau *et al.*, 1987; Johnson *et al.*, 1993).

Synaptosomes retain the natural pteridine cofactor necessary for tyrosine and tryptophan hydroxylation and also the ability to respond to exogenously applied substances in a manner similar to that reported for *in vivo* experiments (Devau *et al.*, 1987; Johnson *et al.*, 1993; Makoto *et al.*, 1994; Lladó *et al.*, 1996). Since dopamine and serotonin share a common synthetic pathway and the synthesis of striatal neurons transmitter is influenced by age (Ponzio *et al.*, 1982; Hashiguti *et al.*, 1993) this study was undertaken to investigate the interaction between dopamine and serotonin synthesis in brain striatal synaptosomes in the aging rat.

Materials and Methods

Animals and chemicals

Male Wistar rats were housed in a temperature-controlled room at 20-24ºC and maintained in a 12-h light/dark cycle. Food and water were provided ad libitum. 'Young rats' were 3 months old and weighed 200-250 g, while 'old rats' were 30 months old and weighed 650-720 g. All procedures were approved by the Ethical Committee of the Isfahan University of Medical Sciences, and conducted in accordance with the 'Principles of Laboratory Animal Care' (National Institutes of Health publication no. 86-23, revised 1985).

All chemicals were of reagent grade and obtained from Sigma Chemical Company (Germany). Deionized water was used throughout this study.

Preparation of synaptosomes

Rats were killed by decapitation between 8 to 9 AM and the brain was dissected on ice by the method of Glowinski and Iversen (1966). Synaptosomes were prepared from the dissected striatum essentially as described by Booth and Clark (1978). Briefly, the dissected brain striatum from 6 young or 4 old rats were dropped into ice-cold isolation medium (0.32M-sucrose/L mM-potassium EDTA/l0 mM-Tris HCl, pH7.4) and chopped into small pieces with scissors. Blood and other debris were washed out and the chopped tissue was then homogenized in a glass homogenizer using a glass pestle with 0.1 mm clearance. This homogenate was diluted to 60 mL with isolation medium and centrifuged at 1200 *g* for 3 min at 4ºC. The supernatant was then centrifuged at 16000 *g* for 10 min, producing the crude mitochondrial/synaptosomal pellet. This pellet was resuspended in 30 mL of Ficoll/sucrose medium [12% (w/w) Ficoll, 0.32 M sucrose, 50 M potassium EDTA, pH 7.4] and homogenized. The suspention was transferred into a centrifuge tube and above this 5 mL of 7.5% Ficoll/sucrose medium [7.5% (w/w) Ficoll, 0.32M-sucrose, 50 pM-potassium EDTA, pH 7.4] was carefully layered. Finally, 5 mL of isolation medium was also carefully layered on top. Then the tubes were centrifuged at 70000 *g* for 40 min at 4ºC. Synaptosomes were gently sucked off from the the second interphase and resuspended in 10 mL of cooled incubation medium (125 mM NaCI, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 1mM ascorbic acid, and 15 mM sodium phosphate buffer) and centrifuged at 5500 *g* for 10 min. The pellet was resuspended in incubation medium to give a protein concentration of 8 to 10 mg/mL (as determined by the Lowry's method, after lysing synaptosomal membranes in a 2%, w/v, Na-deoxycholate solution). The optimal pHs for dopamine (Patrick and Barchas, 1974) and serotonin synthesis (Karobath, 1972) were adjusted by using different concentrations of Na_2HPO_4 or NaH_2PO_4 at 6.1 and 7.4 respectively. All procedures were performed at 4ºC.

Lactate dehydrogenase assay

The integrity of the synaptosomal membrane was assessed by lactate dehydrogenase (LDH) activity in the presence and absence of 0.1 % Triton X-100 (Booth and Clark, 1978). The assay was performed in the same incubation media with the optimal pHs used for determination of tyrosine hydroxylase (pH 6.1) and tryptophan hydroxylase (pH 7.4) plus NADH (0.2 mM) at 25ºC using a Pye Unican spectrophotometer (SP 8199). The reaction was started by addition of pyruvate (0.2 mM) and oxidation of NADH (0.2 mM) at 340 nm was monitored against a blank containing all compounds except pyruvate.

Measurement of dopamine and serotonin synthesis

The methods involved determination of the levels of dopamine or serotonin by HPLC before and after incubation of synaptosomes, in the presence of a monoamine oxidase inhibitor, pargyline (Messripour

and Clark, 1982; Rastegar *et al.*, 1993). Briefly, 200 µL (2 mg protein) aliquots of resuspended synaptosomes were incubated in the presence of either 40 µM tyrosine or tryptophan and 100 µM pargyline. The incubations were carried out at pHs of 6.1 or 7.4 for 15 min at 37ºC and the reactions were stopped by freezing the samples over solid CO_2. The concentration of the amines in non incubated samples (blanks) were subtracted from the amine content of the incubated samples to calculate the net rate of dopamine or serotonin formed in an appropriate incubation period. The results are expressed as the amount of the amine formed/min/mg protein.

High performance liquid chromatography

Dopamine and serotonin were extracted from samples as previously described (Messripour and Clark, 1982; Rastegar *et al.*, 1993) and HPLC assays of the amine content of the extracts were carried out on an Altex 15cm X 4.6mm I.D. Ultrasphere-IP column, using an Altex model 110A pump and LC-4 amperometric controller with a TL-4 detector compartment (Bioanalytical systems) and linked to a Hewlett-Packard integrator recorder (HP 3380A). The mobile phase for chromatography of dopamine was composed of 90% 0.1M potassium dihydrogen orthophosphate, 0.1 mM potassium EDTA, 0.3 mM sodium octyl sulphate and 10% HPLC grade methanol (final pH 3.0). The same mobile phase was used for estimation of serotonin except that a lower (0.3 mM) concentration of sodium octyl sulphate was used.

Statistical analysis

Results were expressed as mean±SD. The intergroup variations were evaluated by one way analysis of variance (ANOVA) followed by Tukey's test, using SPSS (Statistical Package for the Social Sciences. Statistical level of significance was set at $P < 0.05$.

Results

The LDH latency (the ratio between apparent LDH activity in the presence or absence of Triton X-l00) showed no significant differences at pH 6.1 (17.7 ± 0.5, n=6) and 7.4 (17.6 ± 0.6, n=6). However, the synthesis of dopamine and serotonin by striatal synaptosomes was affected by pH, being high for dopamine and low for serotonin at pH 6.1, and high for serotonin and low for dopamine at pH 7.4.

The rates of dopamine synthesis by striatal synaptosomal preparations from young and old rats were 14.55 ± 1.3 and 10.79 ± 0.66 pmol/min/mg protein, respectively. The differences were statistically significant. However, the rate of serotonin synthesis by synaptosomes was not significantly different between young and old rats (2.48± 0.33 and 2.63± 0.29 pmol/min/mg protein, respectively).

Figure 1 shows the effect of a range of serotonin concentrations on dopamine synthesis by striatal synaptosomes prepared from young and old rats. Dopamine synthesis by synaptosomes from young rats was markedly inhibited (~30 %) by the addition of 5-50 µM serotonin. However, these concentrations of serotonin had no effect on dopamine synthesis by synaptosomes from old rats.

Figure 2 shows the inhibitory effect of a range of dopamine concentrations on serotonin synthesis by

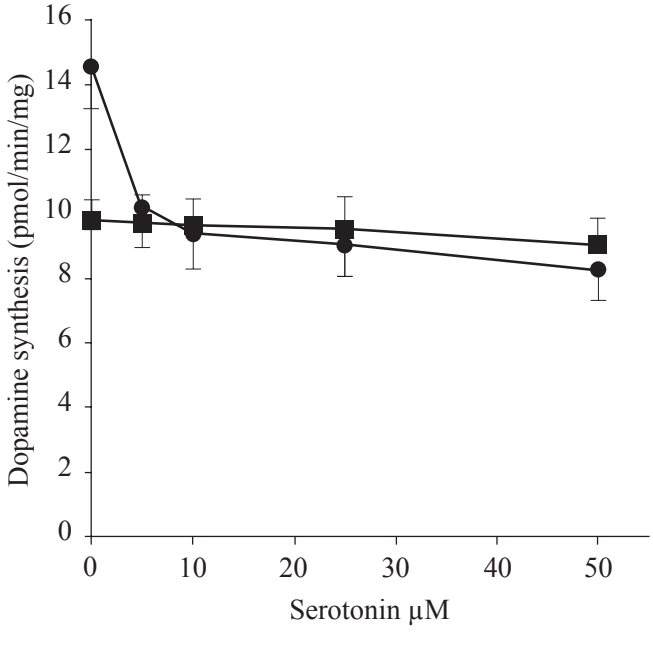

FIGURE 1. The effect of different concentration of serotonin on dopamine synthesis in young and old rats. The striatal synaptosomal preparations from 3 months (●-●) and 30 months (■-■) old were incubaed in different concentrations of serotonin (0 to 50 µM), and the rate of dopamine synthsis were measured in the presence of tyrosine (40 mM) and pargyline (100 mM) by HPLC method. Each point represents the mean ± SD of 6 separate experiments performed in triplicate. Differences between young and old rats in the absence of serotonin (0 µM) is statisticaly significant.

striatal synaptosomes from both young and old rats. Synthesis was inhibited by the synaptosomes at both ages by about 40% ($P<0.005$) whereas with higher concentration of dopamine (10-50 μM), the rate of inhibition was highly pronounced in the synaptosomes of old rats as compared to those of young animals.The dose response curve was a non linear fashion for both ages, but the pattern of inhibition was different to that seen for dopamine synthesis. The effect is more marked in synaptosomes from old than from young rats.

Discussion

Membrane integrity of synaptosomes is believed to be an important factor for the maintenance of the enzymes and the reduced pteridine cofactor at near optimal levels for dopamine and serotonin synthesis (Karobath, 1972; Patrick and Barchas, 1974). As has been indicated previously (Booth and Clark, 1978; Messripour and Clark, 1982) the measurement of dopamine and serotonin synthesis in purified synaptosomes in presence of a monoamine oxidase inhibitor gives rise to the optimal rate at which the neurotransmitters can be synthesized at the synapses from their precursors tyrosine and tryptophan respectively. The procedure for synaptosomal preparation may have important implications for the final metabolic and membrane integrity of the obtained synaptosomes and hence on its suitability as a model of the nerve ending for studying neurotransmitters interaction at the synaptic region of the neurons of the brain.

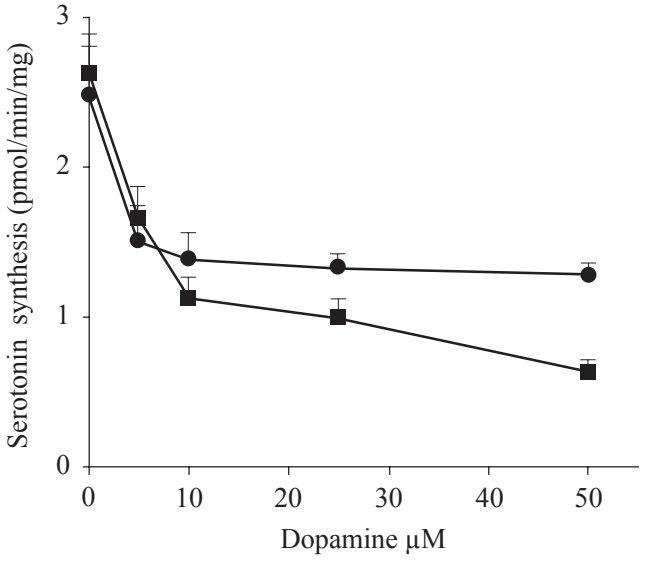

FIGURE 2. The effect of different concentration of dopamine on serotonin synthesis in young and old rats. The striatal synaptosomal preparations from 3 months (●-●) and 30 months (■-■) old were incubated in different concentrations of dopamine (0 to 50 μM), and the rate of serotonin synthesis was measured in the presence of tryptophan (40 mM) and pargyline (100 mM) by HPLC. Each point represents the mean± SD of 6 separate experiments performed in triplicate. Differences between young and old rats with dopamine concentrations of 25 and 50 μM are statistically significant.

The either strong or weak responses of synaptosomes to low (up to 5 μM) and high (5-50 μM) concentrations of the exogenously added neurotransmitters (Figs. 1 and 2) is supposed to be effected by at least 2 mechanisms; (a) by uptake of the serotonin or dopamine from the assay mixture thus increasing intra-membrane concentrations of the neurotransmitter and causing inhibition of the hydroxylase enzymes and (b) by presynaptic receptors, which may influence neurotransmitter release and hence intraterminal neurotransmitters via Ca^{2+} and cyclic AMP dependent mechanisms cause regulation of the enzyme protein itself. This study confirms the hypothesis of an inhibitory control by serotonin on TH activity in the dopaminergic neurons of the sriatum, indicating that serotonin could regulate the activity of the enzyme through specific serotoninergic receptor (De Deurwaerdère *et al.*, 2004) . However, the results of this study indicate (Fig. 1) that dopamine synthesis in the synaptosomal preparations of old rat striatum was not affected by addition of serotonin. It is suggested that dopaminergic modulatory mechanisms on the presynaptic regions of striatal neurons are not functioning in old rats as they are doing in the neurons of young animals. Conversely, the synthesis of serotonin in the synaptosomal preparation of old animals was more sensitive to the addition of dopamine as compared to that of young animals (Fig. 2). It appears that dopamine/serotonin "cross talk" regulates the neurotransmitter levels at synapses is significantly modulated during the aging process. Such interaction might be noteworthy, where long-term treatment with the dopamine precursor, L-DOPA of patients suffering from Parkinson's disease, renders the drug less effective and patients experience fluctuations in response and even psychiatric problems (Rascol *et al.*, 2000) which might be mediated by decreased serotonin levels.

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