Age-related Changes in 5-HT$_2$ Serotonin Receptor on Human Platelets

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Numerous studies suggest that serotonergic nervous system may be involved in the neurophysiological processes in age-related learning and memory. A reduction in biochemical markers of serotonergic synapses also has been reported. Besides the central nervous system, 5-hydroxytryptamine (5-HT) is also found in blood platelets. Platelets and serotonergic nerve endings share many morphological, biochemical and pharmacological characteristics. The present experiment is performed by using platelet as a model for 5-HT neuron activity. By using radioligand binding technique, 5-HT$_2$ serotonin receptor subtype has been identified and characterized in human platelet membrane. [^{3}H]-spiperone in various concentrations (0.2-10 nM) used as radioligand whereas ketanserin, a selective 5-HT$_2$ serotonin antagonist was used to determine the non-specific binding. The association binding was reached to equilibrium within 30 min and remained constant for at least two hr. The saturation experiment revealed a single binding site with a dissociation equilibrium constant (Kd) of 3.41 ± 0.95 nM and a receptor density (Bmax) of 86.59 ± 9.09 f mol/mg protein. Several drugs were used to compete with [^{3}H]-spiperone binding having the descending order of potency : ritanserin》prazosin 〉pipamperone〉pirenperone〉ketanserin〉spiperone〉methysergide〉chlorpromazine〉haloperidol〉sulpiride〉serotonin〉imipramine. The results indicate that human platelets contain 5-HT$_2$ serotonin receptor binding. The Bmax value of the elderly is significantly reduced when compared with young adult subjects (39.96 ± 5.42 f mol/mg protein, 86.59 ± 9.09 f mol/mg protein, respectively), whereas the Kd value is non-significant different between these two groups, elderly healthy and young adult subjects, (2.84 ± 0.48 and 3.41 ± 0.95 nM respectively).

5-Hydroxytryptamine (5-HT) is present in many neurons , including cells located in brain stem, and project to most parts of the mammalian forebrain and spinal cord (1). The existence of such neuronal network is reflected by the numerous studies which implicate involvement of 5-HT in diverse physiological and behavioral processes such as nociception (2) ingestion (3) sleep (4), cardiovascular regulation (5), and sexual behavior (2). Abnormal serotonergic function has been linked to certain disease states including depression (6), schizophrenia (7), and migraine (8-9). In addition, numerous studies also suggest that serotonergic nervous system may be involved in the neurophysiological processes in age-related learning and memory (10). The existence of multiple 5-HT binding sites, with some types located in brain regions related in learning and memory have been implicated (10). A reduction in biochemical markers of the serotonergic synapse, including 5-HT uptake (11), the maximal number of 5-HT binding sites and the increase in 5-HT turnover also have been reported to be associated with the age-related processes (12). However, the reported changes in the serotonergic nervous system in terms of the cause or treatment of the cognitive deterioration associated with certain age-related neurodegenerative disorders is not known. The major reason for this, is due to the past inconsistences observed at the animal level and the biochemical studies in human nervous system have certain limitation.

Besides the central nervous system, 5-HT is also found in many other tissues, eg. blood platelets, mast cells and the enterochromaffin cell of the gut. Almost all circulating 5-HT is contained within platelets (13), mostly in specialized storage granules. It is believed that the main sources of the amine are the enterchromaffin cells of the gut (14).The platelet and 5-HT can interact in several ways : 1. Storage of the amine, within organelles as well as the cytoplasm (15). 2. Uptake, by passive diffusion and more importantly by a specific active uptake process effective considerable concentration gradients(16). 3. Release, probably mainly by exocytosis in vivo . 4. 5-HT-induced platelet shape change

and aggregation , the latter usually accompanied by the release of 5-HT , probably by exocytosis, and also of other cell constituents such as ATP, beta-thromboglobulin, and preceded by a change in cell shape from flattened discoid form a spherical one , with pseudopodia formation (17).

Platelets and serotonergic nerve endings share many morphological, biochemical and pharmacological characteristic (9,18-22).Similarities of importance functions such as the accumulation and storage of serotonin and the stimilus response mediated release of biologically active substance like serotonin, catecholamines and prostaglandins.Their respective cell membranes contain similar supplies of receptors (alpha2 ,beta2, 5-HT_2) and specific imipramine binding sites (22). Both cell types also have lipid metabolizing systems and the intracellular mediates of secretion involve Ca^{2+} and metabolistes of the phosphatidylinositol, phosphate cycle and the prostanoid pathways, enzymes which are common in both cells, such as monoamine oxidase (MAO), gamma aminobutyric acid (GABA) aminotransferase and neuron-specific enolase (NSE) . Moreover a common origin of platelets and serotonergic neuron from the embryonic has been suggested (18). From the accumulated knowledge in the field has led to suggest that biogenic amine functions of platelet closely resemble those of serotonergic neurons (9). The present experiment is performed by using platelet as a model for 5-HT neuron activity. The [^{3}H]-spiperone binding to platelet membrane has been identified and characterized and the receptor sites in young and elderly healthy subjects are compared.

MATERIALS AND METHODS

Radioligands and Chemicals

[Phenyl-4 ^{3}H]- spiperone (specific activity 27.5 Ci/mmol) was purchased from Amersham International plc. (Amercham,U.K).Ketanserin, pipamperone, and ritanserin were gift from Janssen Research Foundation (Bierse, Belgium). Sulpiride was

purchased from Delagrange (Paris).Pirenperone was a gift from Sandoz (Bassel Switzerland). Serotonin, chlorpromazine, imipramine were purchased from Sigma (St. Louis, MO) .Prazosin was purchased from Pfizer, Inc. Groton, Conn.All other chemicals and reagents were the purest commercially available grade, purchased mainly from Sigma (St. Louis, U.S.A.) , E. Merck (Darmstadt,Germany) and May & Baker Ltd. (Dagenham, U.K.). All solutions containing the drugs were freshly prepared for each experiment.

Subjects

The young adult subject age was 27.67±2.34 years and the elderly subjects was 72.86±1.82 years. They all were drug-free and had no history of medical or neurological illness.

Platelet membrane preparation

Blood (10-20 ml) was taken from the antecubital vein with a 19-gauze needle into plastic syringes and transferred into plastic tubes containing with 0.38% (final concentration) sodium citrate as anticoagulant. Whole blood was centrifuged at 200xg for 10 min, the resultant platelet-rich plasma (PRP) was obtained. A 100 µl of PRP sample was taken for a platelet count and the number of aggregation by using phase-contrast light microscope. PRP preparations were then mixed with half of their original volume of a hypotonic medium (5 mM Tris HCl, pH 7.5) and homogenized for 15 sec with a tissue homogenizer (Ultra Turrax T25), setting at 13,500 /min. This suspension was centrifuged at 12,000 x g for 10 min in a refrigerated centrifuge (HITACHI, SCR 20B). The supernatant was decanted and the membrane pellet was resuspended in 20 volumes of ice cold, 50 mM Tris HCl, pH 7.5 and homogenized. The process was repeated twice with 50 mM Tris HCl (pH 7.5) for washing the membrane pellet. The membrane pellet was then resuspended into the incubation buffer (containing NaCl 120 mM; $MgCl_2$ 1mM,and $CaCl_2$ 2mM in 50 mM Tris HCl buffer pH 7.5) to form the final membrane suspension for binding studies.

Radioligand binding assay for serotonin receptor

The freshly prepared platelet membranes were used for the 5-HT serotonin receptor binding according to the method of Govitrapong (23); Macbride (24); and Biegon (25) with some modification. Membranes were resuspended in 20 volumes of ice cold 50 mM Tris HCl salt buffer (pH 7.5) containing; NaCl 120 mM; KCl 5 mM; CaCl2 2mM; MgCl2 1mM and homogenized for 15 sec with a tissue homogenizer (Ultra Turrax T25) setting at 13,500 / min. Since the specific [^{3}H]-spiperone binding increases linearly as a function of tissue protein concentration between 0.02-1.0 mg/ml, all binding assays were carried out by using sufficient membrane preparation to provide concentration between 0.1-0.35 mg/ml. Assays were performed by the addition of 400 µl platelet membrane suspension into glass tubes which containing 50 µl incubation buffer with or without appropriate drug in buffer. The reactions were started by adding 50 µl of [^{3}H]-spiperone to give a final concentration of 0.5 nM in final incubation volume of 0.5 ml. Ketanserin (10 µM) was chosen for the displacing agent to define non-specific binding. The assay mixtures were incubated at 37^{o}C for 30 min; during which time binding equilibrium was reached. The reaction was terminated by rapid filtration through Whatman GF/C filters were washed twice with 3 ml of ice cold 50 mM Tris HCl salt buffer (pH 7.5). Receptor-bound radioactive which was trapped on the filters was counted in 5 ml scintillation fluid containing of Triton X 100/ toluene base fluor (1:3) by scintillation counter. The counting efficiency for tritium was approximately 45%. Specific [^{3}H]-spiperone binding was accounted for 40-60 % of the total binding.

Determination of protein

The concentration of protein was determined according to the technique of Lowry (26) using bovine serum albumin as a standard.

Analysis of data

Saturation curve were initially analyzed by the method of Scatchard,plotting bound/free (B/F) versus bound (B) and finally analyzed by the nonlinear least-squared regression analysis computer programs, Enzfitter (27) and LIGAND (28) equilibrium constant (Kd) and the maximum number of binding sites (Bmax) were obtained.

The data are expressed as mean±SEM derived from experiments indicated. Statistical evaluation of the results was performed using the student's t-test.

RESULTS

Time course of [^{3}H]-spiperone binding to human platelet membrane

The time course of [^{3}H]-spiperone association to the human platelet membranes is presented in Fig. 1.. The binding was performed at 37° C using 2 nM [^{3}H]-spiperone and 10 μM ketanserin was used to determine the nonspecific binding. Under this condition, the specific binding reached equilibrium within 30 min and remained stable for at least 2 hrs. For the routine studies, the incubation time was maintained at 30 min.

Pharmacological characteristics of [^{3}H]-spiperone binding to human platelet membrane

To obtain information as to the subtype of serotonin binding site in this study, the ability of a number of compounds to compete for [^{3}H]-spiperone binding on human platelet membrane was studied. The ability of compounds that displaced the specific binding was examined and their potencies expressed as percent values. The concentration of inhibitors which inhibit the specific binding of [^{3}H]-spiperone was shown in Table 1. The serotonin antagonists, known to have a high affinity for 5-HT serotonin receptor sites such as ritanserin, pirenperone and pipamperone were shown to be the

most potent inhibitors which serotonin was the least potent inhibitor of [3H]-spiperone binding on human platelet membrane. The sequence of potency order is ritanserin > prazosin > pipamperone > pirenperone > ketanserin > spiperone > methysergide > chlorpromazine > haloperidol > sulpiride > serotonin > imipramine.

Saturation studies on human platelet membrane

The saturation of [^{3}H]-spiperone binding on human platelet membrane was examined at 37^{0}C by incubating the membrane suspension with [^{3}H]-spiperone at concentrations ranging from 0.2-

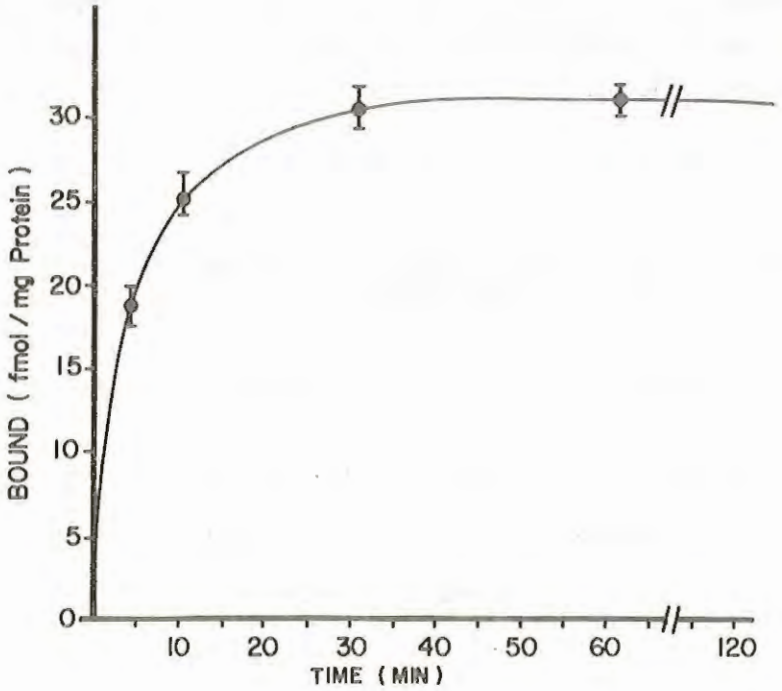

Fig.1. Time course of the specific [^{3}H]-spiperone binding to human platelet membranes. [^{3}H]-spiperone (2nM) binding was studied as described in the section of " Materials and Methods " for varying peroids of time from 0-120 min at 37^{0}C. The data were obtained from 4 sets of experiments, each of which was performed in triplicate.

10 nM, along with 10 μM of ketanserin to account for non-specific binding. Analysis of the data by Scatchard plot and fitted the data by LIGAND (28) and Enzfitter (27) the non-linear least squares regression analysis programs, it indicates the presence of single binding site.

The saturation curve and Scatchard analysis of [^{3}H]-spiperone binding on human platelet membranes. The comparison of the receptor density (Bmax) and dissociation equilibrium constant (Kd) on platelet membrane in young healthy adult and healthy elderly subjects were shown in Fig. 2. The average Kd values of the young adult and the elderly groups were 3.41±0.95 and 2.84±0.48 nM respectively and the Bmax values of the young adult and elderly groups were 86.59±9.09, and 39.96±5.42 f mol/mg protein respectively (Fig. 3.).

Table 1. Inhibition of [^{3}H]-spiperone binding to human platelet membrane by several serotonin related compounds.

Compounds	% of displacement		
Ketanserin	84.29	Chlorpromazine	60.23
Methysergide	61.82	Haloperidol	55.03
Ritanserin	100.00	Sulpiride	50.05
Pirenperone	85.44	Imipramine	12.53
Pipamperone	89.95	Prazosin	95.09
Spiperone	71.37	Serotonin	20.90

[3H]-spiperone (2nM) binding was studied as described in "Material and Methods " in the presence of 10 μM of different serotonin related compounds. 100 % of competition was defined by 10 μM of ritanserin, the highest affinity substance.

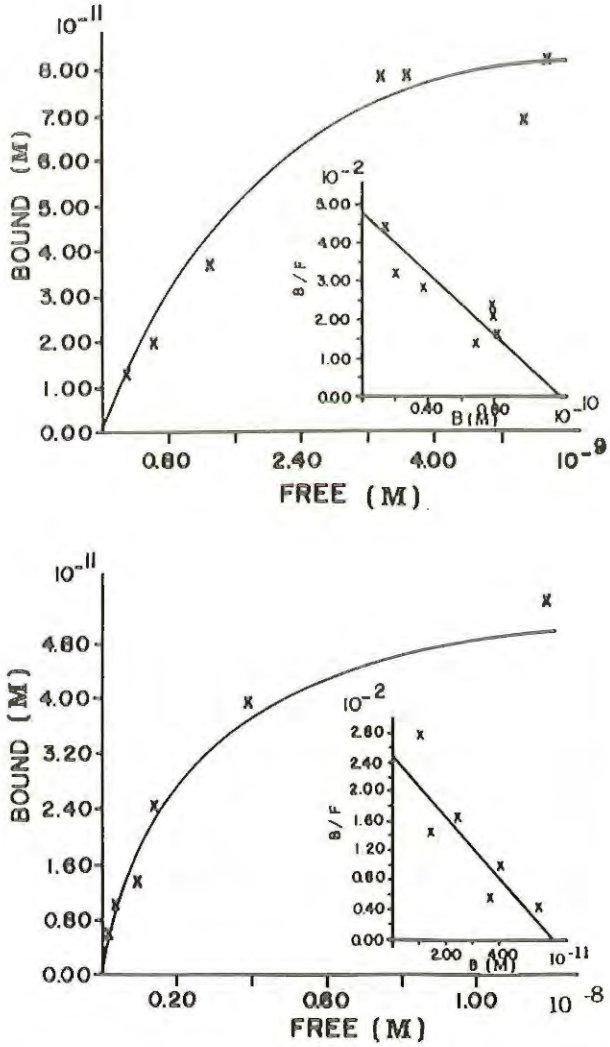

Fig. 2. Saturation curve and Scatchard analysis (in the inset) of [^{3}H]-spiperone binding on platelet membrane of one individual from the young healthy (upper panel) and the elderly healthy (lower panel) groups. The binding was carried out in 0.2-10 nM of [^{3}H]-spiperone. The plots which were obtained from triplicate determinations, represented the specific binding of [^{3}H]-spiperone , the data were analysed by using LIGAND computer program and the line of the best fit was derived from Enzfitter computer program. The results of the experiments were shown and provided Kd values of 2.60and 2.38 nM and Bmax vaules of 78.57 and 35.77 fmol / mg protein in the upper and lower panels respectively.

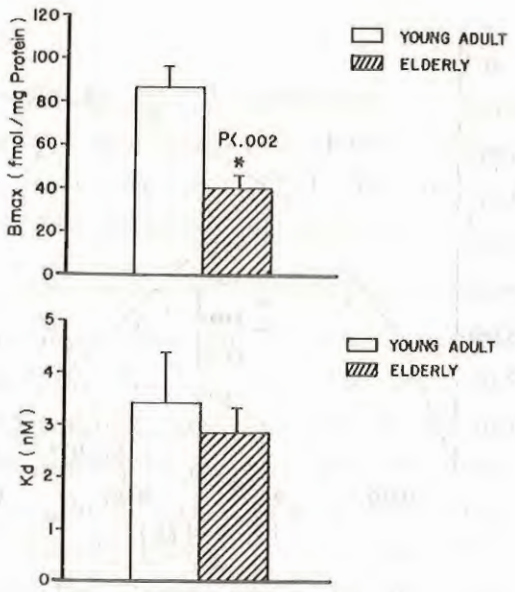

Fig. 3. Comparison of dissociation equilibrium constant (Kd) and the maximal number of receptor (Bmax) values of the young and elderly healthy groups. The mean values of Kd and Bmax were shown in the upper and lower panels of the figure respectively. The Kd and Bmax values were obtained from the saturation experiments.The data were obtained from 6-10 subjects in each group.

DISCUSSION

Identification of serotonin binding sites on human platelets

The presence of 5-HT_2 serotonin receptor sites on the human platelets membrane has demonstrated by using [^{3}H]-spiperone as radioligand. This experiment revealed that only one binding site with a Kd of 3.41$\pm$0.95 nM and a Bmax of 86.59$\pm$9.09 f mol/mg protein. [^{3}H]-spiperone also labeled serotonin recognition sites in addition to D_2 dopamine receptor (29). Peroutka and Snyder (30) concluded that at least two distinct serotonin membrane recognition sites are present in the central nervous system. The sites labeled by [^{3}H]-5-HT were designated "5-HT_1 receptor" and those labelled by [^{3}H]-

spiperone were designated "5-HT_2 receptor". The classification was based primarily on pharmacological test and the affinity of serotonin for this site in the frontal cortex, which was about 30 times greater than that of dopamine, the other neurotransmitters known to have high affinity for [^{3}H]-spiperone. A further indication that [^{3}H]-spiperone specific to serotonergic sites was examined by Seeman's group (31). Their finding was in agreement with the work of Leysen's group (29) on rat frontal cortex that the IC_{50} for serotonin was 4.5 μM, which was much lower than for dopamine (23 μM) or for epinephrine and norepinephrine (100 μM). The binding of [^{3}H]-spiperone to calf frontal cortax was tested in the presence of different concentration of serotonin analogs and the inhibition was found to be the competition type. [^{3}H]-Spiperone can bind to multiple sites which appear to be present regardless of the brain region examined, including brain are where binding appeared to be primarily dopamine (eg. rat striatum) or serotonergic (eg. frontal cortex) in nature. By using the specific compounds which have high affinity for 5-HT_2 receptors or D_2 dopamine receptor single population to serotonergic and dopaminergic [3H]-spiperone binding sites can be selectively labeled (32). Ketanserin, a 5-HT_2 antagonist, appeared to be a particularly selective agent with respect to differentiate between two 5-HT serotonin receptors, it primarily displayed high binding affinity for 5-HT_2 receptor and was inactive at 5-HT_1 receptor (33). Consequently, ketanserin reprensents a tool of quantitatively discriminate D_2 dopamine and 5-HT_2 serotonin receptors in [3H]-spiperone binding in vitro assays (34). Therefore, in this study, [^{3}H]-spiperone was selected for using as a radioligand and ketanserin was used for non-specific binding drug.

Concerning the saturation experiment, the Kd and the Bmax values of [^{3}H]-spiperone binding in human platelet membrane are similar to those found by others (Table 2).The Kd value of the serotonin receptor site in human platelet is 1-3 folds higher than 5-HT_2 serotonin receptor sites that reported from previous studies in brains However it is close to the value reported from the intact human platelet by using [^{3}H]-spiperone as the radioactive ligand

(34), by using [3H]-ketanserin (25), by using [3H]-LSD in human platelet (35) , by using [3H]-LSD in cat platelet (36) and by using [125I]-LSD in human platelet (37).

The density of this receptor site (Bmax) in human platelet is 3-53 folds less than those reported in rat frontal cortex,rat posterior cortex (32), human frontal cortex (38), rat motor cortex layer 5A , rat claustrum (39). The Bmax of this 5-HT2 receptor sites is close to previous study from intact human platelet (24) as shown in Table 3, and also is close to the values obtained from mouse frontal cortex (40),calf frontal cortex and hippocampus (31), and in rat thalamus (32), as shown in Table 2.

Table 2. Summary of [^{3}H]-spiperone binding to 5-HT2 serotonin receptor in mammaian membrane.

Membrane	Kd (nM)	Bmax (fmol/mg protein)	Reference
Calf frontal cortex	0.57	75	31
Calf hippocampus	0.4	52	31
Rat frontal cortex	1.2	666	32
Rat posterier cortex	2.3	258	32
Rat thalmus	0.7	98	32
Mouse frontal cortex	0.76	63	40
Human frontal cortex	1.2	256	38
Rat motor cortex layer 5A	1.54	2841	39
Rat claustrum	1.86	4518	
Bovine pineal gland	1.26	193	23
Human platelet	3.41	86.5	Present st

Inhibition of [^{3}H]-spiperone binding to human platelet membrane by several serotonin related compounds.

The presence of 5-HT_2 serotonin receptor sites on human platelet membrane is also confirmed by several serotonin related compounds. Numbers of various drug classes have been shown to interact at [^{3}H]-spiperone binding sites. The binding of neuroleptic agents to serotonin site has neen investigated. In general, neuroleptic agents display a higher affinity for 5-HT_2 sites than 5-HT_1 site. Nevertheless, their affinity for dopamine sites is usually higher than their affinity for 5-HT sites. In our experiment, the neuroleptics ie. haloperidol, chlopromazine and sulpiride demonstrated relatively low affinity to compete with [^{3}H]-spiperone binding. Serotonin, in general displays a low affinity for 5-HT_2 serotonin binding site.

Table 3. Radioligand binding studies in platelet membrane.

Radioligand	Membrane	Kd (nM)	Bmax	Reference
[^{3}H]-spiperone	human platelet	2.7	1.4 pmol/10^8 platelet	24
[^{125}I]-LSD	human platelet	1.69	14.5 pmol/g protein	37
[^{3}H]-ketanserin	human platelet	1.5	33.9 pmol/mg protein	25
[^{3}H]-LSD	human platelet	0.53	57.1 fmol/mg protein	35
[^{3}H]-LSD	cat platelet	1.02	86.0 fmol/10^8 platelet	36
[^{3}H]-spiperone	human platelet	3.41	86.5 fmol/mg protein	Present study

In this experiment, many agents used to indicate 5-HT_2 recepto sites on human platelet membrane. The rank order of potencies was shown that the serotonergic antagonists, ritanserin, pipamperone, pirenperone, ketanserin, spiperone, and methysergide exhibited higher affinity for [^{3}H]-spiperone binding sites than chlorpromazine, haloperidol, sulpiride (D_2 antagonists), serotonin (5-HT_{1A} agonist) and imipramine (serotonin uptake inhibitor). This result is in agreement with the studies by Janssen (33) .

Compare the values of receptor density and dissociation equilibrium constant between young healthy adults and elderly subjects.

This experiment found significantly decreased ($p < 0.001$) in maximun number of [^{3}H]-spiperone binding (Bmax) on platelet membrane in elderly compared to normal healthy adult subjects (39.96 ± 5.42 and 86.59 ± 9.09 f mol/mg protein for elderly and young healthy subjects , respectively).There was no significant difference in Kd value between these two groups (2.84 ± 0.48, and 3.47 ± 0.95 nM for the elderly and young adult subjects, respectively). This result is compatible with the previous reports for example: a reduction of 5-HT_2 receptors in aging rats (11), 5-HT_1 receptors in human brain (41-43) and non-specific 5-HT binding in human brain (40,44). In addition, the decrease in 5-HT_2 serotonin was observed in living human brain of eldery subjects which were studied by positon emission tomography (PET) (45). Many aged-related changes in dopamine system also occur with norepinephrine. (46).The effect of age on serotonergic neurons have also been studied (46), however it is less extensive than catecholamines also the resulting reports are inconsistent.

Platelets are often used as a model of the serotonin neuron and parallel changes in imipramine binding in brain and platelet of the depressed patients with the decreased serotonin and 5-hydroxyindolacetic acid (5-HIAA) levels in brain and CSF (22). Recent data reported by Biegon's group (25) showed an increase in 5-

HT_2 serotonin receptor number on the platelets of depressive patients.

In the present study, the result shows the reduction of 5-HT_2 serotonin receptor on platelet membrane of elderly subjects. However the mechanism(s) by which alteration of 5-HT_2 receptor in elderly is not known. Several possibilities may speculate , for example, an increase turnover of serotonin receptor , decrease in receptor synthesis, changes in the metabolism of 5-HT then resulted in the alteration of receptor number in platelet membrane. It is possible that 5-HT_2 binding in aging subjects reflects a similar change in brain serotonergic neuron, thus platelet will be an excellent model for the study of aging processes and other processes of the living human brain.

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