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Involvement of an Altered 5-HT$_6$ Receptor Function in Behavioral Symptoms of Alzheimer's Disease

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Abstract. We studied the hypothesis that disturbances in 5-HT$_6$ receptor function in the temporal cortex may contribute to clinical symptoms of Alzheimer's disease (AD). 5-HT$_6$ density and 5-HT levels were significantly decreased in a cohort of AD patients prospectively assessed for cognitive/behavioral symptoms. cAMP formation after stimulation with the selective 5-HT$_6$ receptor agonist E-6801 was significantly lower ($p < 0.01$) in AD (170.02 $\pm$ 27.53 pmol/mg prot.) compared to controls (823.33 $\pm$ 196.67). In addition, the ratio cAMP formation after stimulation with E-6801/5-HT$_6$ receptor density was significantly lower ($p < 0.01$) in AD (6.67 $\pm$ 0.83) compared to controls (16.67 $\pm$ 3.33). Splitting these results by sex, 5-HT$_6$ receptor activation was significantly lower ($p < 0.01$) in AD females compared to males (121.67 $\pm$ 30.02 vs. 231.67 $\pm$ 34.17 pmol/mg prot). 5-HT$_6$ density and 5-HT levels were significantly correlated ($p \leqslant 0.01$) in both controls and AD patients, although in AD, this correlation was lost in females. Psychosis factor was the best predictor of reduced 5-HT levels or adenylate cyclase activity after E-6801 stimulation, the former result being due to females. It may be suggested that psychotic symptoms may be related to a dysregulation of 5-HT$_6$ activation by 5-HT in the temporal cortex. These results are discussed in terms of purported influence of sex and therapeutical approaches to psychosis in AD.

Keywords: Adenylate cyclase, gender, neocortex, psychosis

INTRODUCTION

The serotonergic 5-HT$_6$ receptor was first identified from rat striatal mRNA [30,33] and the human receptor was subsequently identified [19]. The human 5-HT$_6$ receptor is coupled to the stimulation of adenylate cyclase and have a widespread distribution in the CNS [9,30, 38]. Immunohistochemical and ultrastructural studies for 5-HT$_6$ receptors suggest a postsynaptic and mainly dendritic localization [10,38], where they can participate in 5-HT-mediated control of the discharge of neurons innervated by serotonergic terminals [11].

The affinity of some antidepressants and atypical antipsychotics for 5-HT$_6$ receptors together with its localization in the CNS, have prompted interest in the involvement of 5-HT$_6$ receptors in psychiatric disorders. In addition, a role for 5-HT$_6$ receptors in memory and learning processes has also been suggested in animal studies (see review by Woolley et al. [39]). There is a particular interest in the possible implication of 5-HT$_6$

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receptor dysfunction in Alzheimer's disease (AD). AD is a chronic progressive disorder characterised by dementia but often featuring behavioral and psychological syndromes referred to as BPSD (behavioral and psychological symptoms of dementia, [16]). The extent and nature of BPSD in AD [15] includes: aggressive behavior, overactivity, depression or psychosis. It is notable that behavioral syndromes in AD may have a sex-related differential expression [12], i.e., female sex appears to be a risk factor for delusions [17].

In a previously published work from our group [8], and in agreement with another work by Lorke et al. [22], we found significant reductions in 5-HT$_6$ receptor density in cortical areas of AD patients, and these reductions were found to be related to some of the behavioral symptoms of the illness. Continuing with that work, we studied the hypothesis that disturbances not only in 5-HT$_6$ receptor expression but also in function may contribute to the clinical symptoms of AD. In addition, we checked sex-related effects. We assayed 5-HT$_6$ receptor function in terms of cAMP formation after 5-HT$_6$ receptor stimulation in the temporal cortex of AD patients with respect to cognitive impairment and BPSD prospectively assessed in a patient group [15]. Activation of these receptors by 5-HT was evaluated by measuring 5-HT levels.

MATERIALS AND METHODS

Drugs used

Superpure H_2O water (SpS, Romil), bovine serum albumin and polyethyleneimine, 3-isobuthylmethylxanthine, creatine phosphate, creatine phosphokinase, forskolin and Mg-ATP, dithiothreitol were from Sigma-Aldrich Ltd, Germany. Bio-Rad Protein Assay Kit were from Bio-Rad, Munich, Germany. GF/B filters were from Whatman, UK and ecoscint TM from National Diagnostics, UK. Two cAMP kits were used: cAMP Biotrack Enzymeimmunoassay System (RPN225) and [^{3}H] cAMP assay (TRK 432), both from GE Healthcare, UK. All other chemicals were purchased from Panreac, USA. [^{125}I]-SB-258585 and E-6801 were generously provided by GlaxoSmithKline, Harlow, UK and Laboratorios Dr. Esteve S.A., Barcelona, Spain respectively.

Table 1
Demographic details of controls and patients with AD

	Control	Alzheimer
Gender (male/female)	11/9	10/12
Age (years)	74.75 ± 6.67	81.06 ± 1.60
range	53–99	64–89
Postmortem delay (h)	39.28 ± 5.40	48.63 ± 6.30
pH	6.28 ± 0.16	6.44 ± 0.10

Values are mean ± S.E.M. pH, standard chemical symbol, negative log of hydrogen ion concentration; S.E.M, Standard error of the mean. Values are mean ± S.E.M of the maximum number of cases available 20 (control) or 22 cases (AD) in which clinical determinations were performed. Not all the cases were available for all neurochemical determinations and the number of cases used for each determination varied (controls, $n = 13$–20, AD, $n = 15$–22). There were no significant differences between age, postmortem delay or brain pH in either control patients or those with dementia (Student's t-test, $p > 0.05$).

Patients and assessment of behavior

A total of 42 postmortem brains from individuals were included in the study, 20 elderly normal controls and 22 patients with clinical diagnosis of dementia, matched for age, gender, postmortem delay and brain pH (Table 1). Patients were an autopsied subset of subjects included in a prospective study of behavioral changes in clinically diagnosed demented patients [15]. At entry to the study, assessment and diagnoses were made using CAMDEX [31], DMS-III-R criteria [1] and NINCDS-ADRA criteria [24]. Drug histories were recorded for all patients; 21 patients were taking tranquilizers (neuroleptics, sedative-hypnotics or antidepressants). None of the patients with AD received cholinomimetics. Four behavioral and psychological syndromes were assessed using the Present Behavioral Examination (PBE) [14]: depression, overactivity, psychosis and aggressive behaviour [15]. Depression factor was the sum of 4 components: apparent sadness, gloomy thoughts, feeling like a failure, and tearfulness. The overactivity factor consisted of the sum of the highest ratings for walking, and trailing and checking. The psychosis factor was the sum of scores for hallucinations, persecutory ideas and inappropriate anxiety. Aggressive behaviour included physical aggression, aggressive resistance and verbal aggression [18]. Each component of the syndrome was scored from 0 (absent) to 2 (severe, the behaviour had occurred on half of the days or more in the previous 4 weeks). This gives a maximum score of 6 for the overactivity, psychosis and aggression factors (and 8 for the depression factor).

Tissue samples and neuropathology

For all subjects, informed consent for postmortem examination and for use of tissue for research was obtained. Selection of subjects for the study was based on tissue availability, not gender, age, or disease severity. Blocks corresponding to the temporal cortex (Brodmann area 20, BA20) were removed and stored at $-80°$C until processed. All 22 patients were found to meet CERAD criteria [27] for a diagnosis of AD and all brains were Braak stage V or V1. To partially mitigate the possible effects of cause of death on neurochemical determinations, brain pH was measured with deionised water as an index of acidosis associated with terminal coma (Table 1). Brain pH is used as an indication of tissue quality in postmortem research, with pH > 6.1 considered acceptable [2,21].

All subsequent analyses were performed blinded to clinical information. The present work has been performed in the same sample tissue samples as those presented in Garcia-Alloza et al. [8]. Not all the cases were available for all neurochemical determinations.

Serotonin measurements

5-HT and its metabolite 5-HIAA concentrations were determined by high performance liquid chromatography (HPLC) with electrochemical detection (Waters Spheribor® S10 0DS2 4.6 $\times$ 150 mm) as previously reported [23]. Tissue was homogenised in 20 vol of extraction mixture (0.4 M percloric acid; 1 mM EDTA; 0.1% metabisulphitic acid). Homogenates were centrifugated 32,500 g for 20 min. The mobile phase consisted of 80:16 (v/v) mixture of buffer ($KH_2PO_4{\cdot}2H_2O$ 0.1 M, citric acid 0.1 M, EDTA 1 mM and octanosulphonic acid 0.74 mM; pH $= 3$). 5-HT content was calculated by comparing with a 1 ng standard. The limit of detection was 1 pg/10 μl. Turnover was calculated as the ratio 5-HIAA/5-HT.

[^{125}I]-SB-258585 binding to 5-HT$_6$ receptors

[^{125}I]-SB-258585 binding was assayed essentially as previously reported [13]. Tissue samples were partially thawed and homogenized in 10 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.7) using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 35,000 g for 20 min and the resulting pellet was rehomogenized and incubated at 37°C for 15 min. Following two further centrifugations, membranes were finally resuspended (approximately 50–80 mg tissue/ml) and stored at $-80°$C until used. All determinations were carried out in duplicate. [^{125}I]-SB-258585 binding assays consisted of 320 μl of membrane suspension (corresponding to approximately to 8 mg tissue), 40 μl of unlabeled SB258585 at concentrations from 1 to 10 nM, and 40 μl of 1 nM [^{125}I]-SB-258585, to give a final concentration of 0.1 nM. Non specific binding was determined in the presence of 10 μM SB-214111. At the end of incubations, tubes were rapidly filtered under reduced pressure using a cell harvester on GF/B filters that had been pre-soaked in 0.3% v/v polyethyleneimine in ice-cold buffer. The amount of radioactivity bound to filters was measured in a Wallac liquid scintillation counter. Data were subject to Scatchard analysis to determine the number of binding sites ($B_{\max}$: fmol/mg of protein) and the dissociation constant (K_d: nM). Protein content was measured using the assay described by Bradford [3], using bovine serum albumin as standard.

cAMP levels

Endogenous cAMP levels were assayed using an enzyme immunoassay commercial kit (RPN225) following manufacturers instructions.

Adenylate cyclase assay

Membrane preparations and enzymatic activity assay was carried out as the method described by Valdizán et al. [37], from frozen hippocampal tissue. 25 μL of membrane suspension (6–12 μg protein/assay) was preincubated for 15 min on ice in 150 μL of reaction buffer (53 mmol/L N-[2-hydroxyethyl]piperazine-N'[2-ethane-sulphonic acid] pH 7.4, 0.3 mM EGTA, 5 mM $MgCl_2$, 0.1 mg/mL bovine albumin, 1 mM dithiothreitol, 0.5 mM 3-isobuthylmethylxanthine, and the nucleoside triphosphate regeneration system of 5 mM creatine phosphate, 50 units/mL creatine phosphokinase), and 20 μL of water (basal activity), forskolin (1 μM), or the specific 5-HT$_6$ agonist E-6801 (0.01 μM). The reaction was started by addition of 0.5 mM Mg-ATP and incubated at 30°C for 10 min. Reactions were stopped by boiling the tubes for 4 min. Samples were the centrifugated at 13,000 g for 5 min at 4°C. For cAMP content, 50 μL aliquots of supernatant were assayed using a commercial protein-binding assay kit (TRK432) combining the high specificity and affinity for cAMP of a high purified and stabilized binding protein with an improved charcoal separation step. Membrane protein concentrations were determined using the Bio-Rad Protein Assay Kit.

Statistical analysis

Data were analysed using SPSS for Windows, release 11.0. Normality was checked by Shapiro-Wilks's test ($p > 0.05$). Student's t-test was used in initial comparisons between control patients and patients with AD. The effects of demographic factors (age, postmortem delay and brain pH) on neurochemical variables, intercorrelation between neurochemical variables or relationships between severity of dementia (MMSE score at last interview before death), and neurochemical measures were determined by Pearson's or Spearman's correlation coefficients, according to the normality of variables. Multiple regression analysis using "stepwise" method was used to investigate possible relationships between neurochemical variables and psychiatric assessment. As individual patients may show more than one clinical symptom (depression, overactivity, psychosis and aggressive behaviour) multiple-regression indicates the strongest correlate between one of these clinical symptoms and the neurochemical variable [26]. All data was re-analyzed separately by sex.

RESULTS

There were no significant correlations between age, postmortem delay or brain pH and any of the neurochemical variables studied in either control patients or those with dementia ($p > 0.05$). Demographic details of subjects are shown in Table 1.

Mean last scores before death ($\pm$ s.e.m.) for cognitive status, measured as MMSE, was 5 ± 1.2. Mean last scores before death ($\pm$ s.e.m.) for BPSD assessed by PBE in AD patients were as follows: depression factor 3 ± 0.3 (range varies between 1-6); overactivity 3 ± 0.4 (range varies between 1–6); psychosis 2 ± 0.3 (range varies between 0–6); aggressive behavior 5 ± 0.4 (range varies between 1–6).

Serotonergic markers in Alzheimer's disease

Significant decreases in 5-HT levels (up to 46%, $p < 0.01$), 5-HIAA (up to 49%, $p < 0.001$) and the density of 5-HT$_6$ receptor binding (up to 54%, $p < 0.01$) were found in the temporal cortex of AD patients ($n = 20$, controls and 22, AD). 5-HT turnover (5-HIAA/5-HT) was similar in AD and control patients (5.03 ± 0.41 vs. 5.55 ± 0.50).

As shown in Fig. 1, 5-HT$_6$ receptor density and 5-HT levels were significantly correlated in the temporal cortex of both controls ($n = 20$, $r = 0.619$*, $p = 0.004$) and AD patients ($n = 22$, $r = 0.534$*, $p = 0.010$). In AD, this correlation was lost in females, whereas a strong correlation was observed male patients ($n = 10$; $r = 0.836$*; $p = 0.005$).

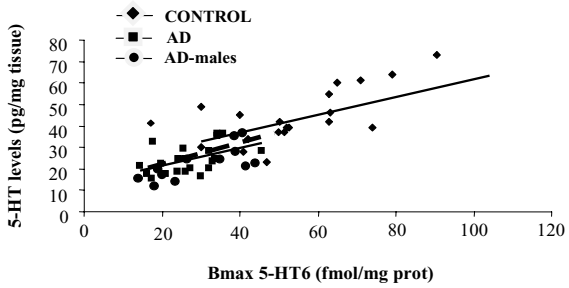

Fig. 1. Significant correlations (Pearson's correlation coefficient) between 5-HT levels and 5-HT$_6$ receptor density in the temporal cortex in control ($n = 20$, diamond symbols), total AD patients ($n = 22$, square symbols) and male AD patients ($n = 12$, circle symbols).

5-HT$_6$ receptor function

Total cAMP levels were significantly reduced ($p < 0.01$) in the temporal cortex of AD samples ($n = 16$) compared to controls ($n = 16$) (1.69 ± 0.09 vs. 2.15 ± 0.25 fmol/mg tissue).

Basal adenylate cyclase activity was similar in AD ($n = 15$) and control patients ($n = 13$) (157.51 ± 32.52 vs. 160.01 ± 42.51 pmol/mg prot). Stimulation of adenylate cyclase activity by the non-specific activator forskolin, 1 μM, yielded similar results in AD (613.33 ± 157.51 pmol/mg prot, $n = 15$) and controls (679.17 ± 186.67 pmol/mg prot, $n = 13$).

As shown in Fig. 2, cAMP formation after stimulation with the 5-HT$_6$ receptor agonist E-6801, 0.01 μM, was significantly lower ($p < 0.01$) in AD samples ($n = 13$, controls, $n = 15$, AD) (170.02 ± 27.53 vs. 823.33 ± 196.67 pmol/mg prot, 4.72 vs. 414.37% of increase). In addition, the ratio cAMP formation after stimulation with E-6801/5-HT$_6$ receptor density was significantly lower ($p < 0.01$) in AD ($n = 15$) compared to controls ($n = 13$) (6.67 ± 0.83 vs. 16.67 ± 3.33). In AD, splitting some of these results by sex, both cAMP induced by E-6801 and the ratio cAMP formation after stimulation with E-6801/5-HT$_6$ receptor density were significantly lower ($p < 0.01$) in females ($n = 8$) compared to males ($n = 7$) (121.67 ± 30.02 vs. 231.67 ± 34.17 pmol/mg prot and 5.67 ± 0.91 vs. 8.80 ± 1.33 respectively).

cAMP formation after stimulation with E-6801 was significantly correlated to 5-HT levels in controls ($n = 13$, $r = -0.615$*, $p = 0.015$), not in AD ($n = 15$, $r = -0.238$, $p > 0.05$)

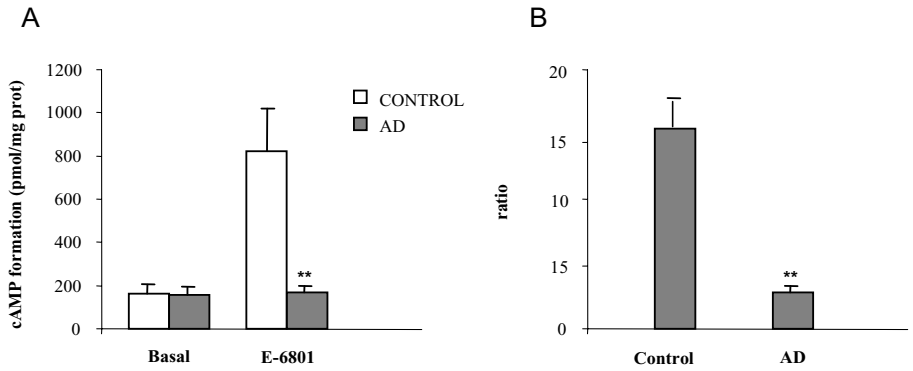

Fig. 2. A) cAMP formation and B) ratio cAMP formation after stimulation with E-6801/5-HT$_6$ receptor density, in the temporal cortex of control and AD patients ($n = 13$–15). E-6801: 5-HT$_6$ receptor agonist-induced cAMP formation. **Statistically significant, control vs. AD, $p < 0.01$, Student t-test.

Relationship with clinical symptoms of Alzheimer's disease

No significant correlations were found between 5-HT$_6$ receptor density or adenylate cyclase activity after E-6801 stimulation and cognitive status (MMSE or MMSE decline). Correlation coefficients were as follows: 5-HT$_6$ receptor density and MMSE ($n = 21$, $r = 0.120$, $p > 0.05$) and MMSE decline ($n = 21$, $r = 0.145$, $p > 0.05$); adenylate cyclase activity after E-6801 stimulation and MMSE ($n = 15$, $r = -0.072$, $p > 0.05$) and MMSE decline ($n = 15$, $r = -0.178$, $p > 0.05$).

Regarding behavioral symptoms, the best predictor for reductions in adenylate cyclase activity after E-6801 stimulation was the psychotic behavior score ($n = 13$, adjusted $r^2 = -0.513$*; $p < 0.037$). Stepwise multiple regression indicated that psychosis factor was the best predictor for reduced 5-HT levels ($n = 20$; adjusted $r^2 = 0.267$; $p = 0.028$). A more detailed analysis revealed that this result was largely due to females (females, $n = 11$; adjusted $r^2 = 0.450$; $p = 0.024$; males $n = 9$; adjusted $r^2 = 0.029$; $p > 0.05$).

DISCUSSION

In animal studies, previous works have examined the purported localization of 5-HT$_6$ receptors in serotonergic/non-serotonergic neurons [9,23]. Extensive degeneration of serotonergic neurons in the anterior raphe area following microinfusion with 5, 7-DHT produced no changes in 5-HT$_6$ mRNA levels in the nucleus accumbens, striatum or hippocampus of the rat, suggesting that 5-HT$_6$ receptors are not located in serotonergic neurons, and that these receptors do not function as autoreceptors in these regions [9]. Supporting this idea, an injection of a 5-HT$_6$ receptor antagonist was ineffective in modulating 5-HT release within the striatum [5]. However, to our knowledge, little work has been done in humans. An important finding of the current study is the correlation between 5-HT levels and 5-HT$_6$ receptors in AD males, but not in AD female patients. Even though these results could be explained simply in terms of neurodegenerative processes, it is also possible to speculate on the involvement of 5-HT$_6$ receptors in sensing the concentration of 5-HT in the extracellular milieu, as previously suggested in electron microscopy studies [4]. Therefore, it is possible that, even in situations of extensive neurodegeneration, such as AD, the expression of 5-HT$_6$ receptors is regulated by its own neurotransmitter, 5-HT. In this sense, serotonin terminal regions (cerebral cortex and striatum, substantia nigra, facial nucleus, trigeminal nucleus) are also enriched in 5-HT$_6$ receptor-like immunoreactivity [10]. However, the possibility of 5-HT$_6$ receptors mediating 5-HT release cannot be completely excluded, since concomitant administration of SB-271046 with amphetamine induced an elevation in striatal 5-HT levels, suggesting that 5-HT$_6$ receptors could somehow be modulating serotonergic neurotransmission [5]. Future studies, using double labelling techniques in human tissue, may give a clearer answer on the location/function of 5-HT$_6$ receptors.

The 5-HT$_6$ receptor has become an increasingly promising target for improving cognition, at least in animal studies, in which different 5-HT$_6$ receptor antagonists have shown their ability to improve learning and memory in several cognitive paradigms and to reverse scopolamine-induced learning deficits [25,28,29,

39]. It has been suggested that the purported 5-HT$_6$ receptor's influence on memory is mediated at least partially by increased cholinergic neurotransmission [23]. However, in our hands, neither 5-HT$_6$ receptor expression [8] nor function (present data) correlated to the cognitive status, measured as MMSE.

The high affinity of atypical antipsychotics such as clozapine for 5-HT$_6$ receptors and their possible modulation of 5-HT$_6$ expression in the hippocampus [7] have led to the investigation of 5-HT$_6$ receptors in the therapeutic properties of these drugs. In support of this hypothesis, a 5-HT$_6$ receptor polymorphism (267C/T) has been reported to affect clozapine response [40] and is a risk factor for schizophrenia [36]. However, it has also been described that an altered 5-HT$_6$ receptor density does not contribute to schizophrenia [6]. In agreement with this idea, previously reported data from our group [8] showed that 5-HT$_6$ receptor expression was not associated with psychotic symptoms in AD. A possible alteration in 5-HT$_6$ receptor function was addressed next. Even though reductions in total levels of cAMP were found, probably representing neurodegeneration, it is notable that cAMP is generated by many different receptors, and the fraction generated by 5-HT$_6$ receptors is probably very small with respect to the total receptor stimulated levels of cAMP. Therefore, cAMP formation related to 5-HT$_6$ receptor activation was measured as the amount of cAMP formation after 5-HT$_6$ receptor activation by the selective agonist E-6801 [31]. It could be argued that the decreases in E-6801 induced cAMP formation found in AD may be due to the decreases in 5-HT$_6$ receptor density. However, the amount of cAMP found after each 5-HT$_6$ receptor activation (ratio cAMP formation after E-6801/5-HT$_6$ receptor binding) was also significantly lower in AD, suggesting that in AD, the dysregulation of the serotonergic system affects not only the number of 5-HT$_6$ receptors expressed but also the signal transduction system. Even more, these parameters of 5-HT$_6$ receptor function seem to be especially affected in females. Decreases in cAMP formation after E-6801 stimulation were the best predictor for psychotic symptoms. In addition, when 5-HT levels were not correlated to 5-HT$_6$ receptor density (females), cAMP induced by E-6801 was significantly lower, and the best predictor for 5-HT levels in temporal cortex was the psychotic factor. In light of our findings, it is possible to argue that psychotic symptoms could be related to a dysregulation of the activation of 5-HT$_6$ receptors by 5-HT. There are previous studies describing that sex is related to psychosis in AD [12], and female gender appears to be a risk factor for delusions [17]. Supporting the present findings of an involvement of 5-HT$_6$ receptor function in psychotic symptoms of AD in females, it has been reported that male patients respond better to risperidone [20] and clozapine [35] than female patients.

Atypical antipsychotics are becoming the main therapeutic group used in schizophrenia treatment. However, there is little consensus as to what constitutes atypicality in antipsychotic medication [34]. The affinity for 5-HT$_6$ receptors showed by some atypical antipsychotics, such us clozapine, and the high levels of 5-HT$_6$receptors in areas where antipsychotics are thought to exert their effect, has led to the suggestion that 5-HT$_6$ receptors contribute to the therapeutic properties of these drugs. From the present results it might be suggested that, in the temporal cortex, psychotic symptoms in AD may be related to a dysregulation of the activation of 5-HT$_6$ receptors by 5-HT, at least in females. This relationship does not appear to relate to cognitive deficits in AD, but may relate to dementia-associated psychosis, or even more, perhaps to psychosis more generally. Future research will help us to fully understand the exact nature of the relationship between 5-HT and 5-HT$_6$ receptors and their relationship with the underlying mechanism for psychosis in AD.

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