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Increased melatonin 1a-receptor immunoreactivity in the hippocampus of Alzheimer's disease patients

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Abstract: The pineal secretory product melatonin has, in addition to regulating retinal, circadian and vascular functions, neuroprotective effects. Blood melatonin levels are often decreased in Alzheimer's disease (AD), a progressively disabling neurodegenerative disorder. In this study we provide the first immunohistochemical evidence for the localization of melatonin 1a-receptor (MT_1) in aged human hippocampus and a comparison of AD cases. MT_1 was localized to pyramidal neurons in the hippocampal cornu ammonis (CA)1-4 subfields. There was a distinct increase in staining intensity in all AD cases indicating an up-regulation of the receptor, possibly as a compensatory response to impaired melatonin levels in order to augment melatonin's neuroprotective effects.

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Introduction

Alzheimer's disease (AD), as the most common cause of cognitive deterioration in the elderly population, is neuropathologically characterized by progressive formation of insoluble amyloid plaques consisting of amyloid β-peptide (Aβ) and neurofibrillary tangles, particularly in the hippocampus and cerebral cortex (Mesulam, 1999; Vassar et al., 1999). Aβ is formed by proteolytic cleavage of a large transmembrane protein, the amyloid precursor protein. Aβ initiates the generation of free radicals in the central nervous system which contribute to neuronal dysfunction and loss (Reiter, 1998; Reiter et al., 1999; Pappolla et al., 2000). Furthermore, Aβ-induced oxidative stress plays a key role in AD by accelerating damage to neuronal membrane lipids, proteins and nucleic acids (Miranda et al., 2000; Pappolla et al., 2000). Therefore, there is a growing interest in the protective role of antioxidants in AD.

Melatonin is a highly effective antioxidant scavenging the hydroxyl, and possibly the peroxyl radical (Reiter, 1998; Pappolla et al., 2000). A similar detoxifying effect of melatonin is also known for hydrogen peroxide, nitric oxide and peroxynitrite (Blanchard et al., 2000; Tan et al., 2000). In addition, it augments the activity of antioxidizing enzymes such as superoxide dismutase, glutathione peroxidase and glutathione reductase and protects against glutamate excitotoxicity. Cell culture experiments have shown that melatonin also prevents Aβ-induced neurotoxicity (Pappolla et al., 1997, 2000). There appears to be some diminution in melatonin secretion with age; furthermore, reduced melatonin levels in AD patients may correlate with dementia severity (Ferrari et al., 2000; Pappolla et al., 2000). A deficiency in melatonin appears to accompany neurodegeneration in AD.

Melatonin exerts some of its functions through specific receptors belonging to the superfamily of G protein-coupled receptors (Brydon et al., 1999; Dubocovic et al., 2000). Three different melatonin receptor subtypes are known so far, Mel_{1a} (MT_1), Mel_{1b} (MT_2) and MT_3, respectively (Dubocovic et al., 2000). The molecular structure has been decoded for MT_1 and MT_2, which mediate different melatonin effects. MT_2 appears primarily to be involved in feedback regulation of circadian rhythms in the suprachiasmatic nucleus (SCN), as well as being responsible for vascular and retinal effects (Dubocovic et al., 2000). MT_1, on the other hand, has been shown to acutely inhibit neuronal firing in the SCN (Dubocovic et al., 2000). The somnogenic effects of melatonin have been also attributed to MT_1. In situ hybridization studies have confirmed that both MT_1 and MT_2 subtypes are present in the human brain (Dubocovic et al., 2000). The distribution of MT_1 is well documented for different mammalian species and nonhuman primates (Mazzucchelli et al., 1996). Because of the lack of sensitive antibodies, information on the distribution pattern of melatonin receptors at the protein level is still missing for humans. Comparative reverse transcriptase polymerase chain reaction (RT–PCR) analysis of MT_1 gene expression in human brain revealed that the receptor mRNA is present

in regions associated with higher mental functions, particularly the neocortex and the hippocampus, which are preferentially affected in the pathology characteristic of AD (Mazzucchelli et al., 1996). Therefore, MT_1 may mediate possible neuroprotective effects of melatonin in these highly affected regions.

The aim of this study is to provide immunohistochemical data for the distribution of MT_1 in human hippocampus, and, in addition, to describe possible MT_1 alterations in AD patients.

Materials and methods

To localize the MT_1 receptor protein and its possible alterations in AD, we examined the hippocampus of 11 AD patients and eight age-matched controls using immunohistochemistry (details in Table 1). In addition to the histopathologic diagnosis of AD, Braak staging and the apolipoprotein E (ApoE) allele frequency was determined for each case. Braak staging differentiates six neuropathologic stages in AD according to the distribution pattern of the neurofibrillary tangles (Braak and Braak, 1991). Stages I and II correspond to the preclinical phase of AD and these patients were classified as controls. The ascent to stage III marks the modest involvement of the hippocampus beginning with the CA1 subfield and proceeding to CA4 during the next stages (Braak and Braak, 1991). ApoE, encoded by a gene on chromosome 19, can consist of different alleles and the E4 allele of ApoE is a major risk factor for sporadic AD, promoting the precipitation of Aβ into insoluble plaques and inhibits neurite growth and dendritic plasticity (Mesulam, 1999).

Table 1. Data of controls (C) and AD cases including postmortem delays (pmd), brain weights in gram (bw), Braak staging (BS), apolipoprotein E allele differentiation (ApoE) and semiquantitative data tabulating the intensity of melatonin 1a-receptor (MT_1) immunoreactivity

Case no.	Age	Gender	pmd (min)	bw	BS	ApoE	MT_1
C							
1	89	F	260	1152	II	33	+
2	86	F	810	1168	0	43	+
3	75	M	435	1423	II	33	+
4	78	F	450	1330	II	43	–
5	72	M	270	1196	0	33	+
6	83	M	385	1300	I	33	+
7	78	M	335	1467	I	43	–
8	62	M	395	1352	0	43	–
AD							
1	85	M	295	1050	III	43	+
2	93	F	225	988	V	43	+ +
3	86	F	200	1094	V	33	+ + + +
4	83	M	315	1247	IV	33	+ +
5	63	F	295	934	VI	33	+ + + +
6	75	M	225	1140	V	44	+ + + +
7	71	F	260	1150	V	33	+ + +
8	78	F	315	1005	VI	43	+ + + +
9	82	F	195	1050	VI	43	+ +
10	72	M	315	1520	V	43	+ +
11	87	M	215	1017	VI	43	+ +

M, male; F, female; min, minutes; BS, Braak stages 0 and I/II (transentorhinal stages corresponding to the preclinical phase of AD), III/IV (limbic stages) and V/VI (isocortical stages). MT_1 staining intensity: –, no immunoreactive neurons; +, few immunoreactive neurons; + +, slight increase; + + +, almost all neurons are immunoreactive; + + + +, all neurons immunoreactive.

Paraffin embedded hippocampus samples were cut in the coronal plane with a microtome in 10 μm-thick serial sections and every tenth section was taken for immunohistochemistry. The affinity-purified specific antibody to detect MT_1 was developed against peptide 536 in the C-terminus of the receptor (Brydon et al., 1999). As this sequence has no homology with the corresponding regions of other melatonin receptors, little cross-reactivity is expected (Brydon et al., 1999). The specific antibody recognition has been investigated in detail. The optimum concentration of the primary antibody was experimentally determined to be 1:100. For each case, control sections were stained simultaneously following the same procedure as the test samples, with the exception that the primary antibodies were omitted to reveal the specificity of the primary antibody. After incubation with primary antibody, MT_1 was visualized by peroxidase staining using the substrate 3-amino-9-ethylcarbazole (ACE) which provides a red staining as reported previously (Olivieri and Miescher, 1999). All sections were assessed for intensity of immunoreactivity on a semiquantitative scale by a blinded observer (Table 1).

Results

The cytoarchitectural classification of the hippocampal subdivisions in this study follows detailed previous reports dividing it into four main subfields emanating from the dentate gyrus or CA4 and progressing through CA3, CA2 and CA1 subfields (Duvernoy, 1997). MT_1 immunoreactivity was localized to pyramidal neurons of all hippocampal subfields (Fig. 1A–D), but in controls immunoreactive neurons were predominantly present in the CA1 subfield. No MT_1 immunoreactivity was observed in non-neuronal cells. In controls, a subset of the pyramidal neurons revealed a slight immunoreactivity which was homogeneously distributed within the perinuclear cytoplasm (Fig. 1A). Three control cases did not show MT_1 immunoreactivity (Table 1). Morphologically, MT_1 immunoreactive neurons were polymorphic. In the CA1–3 subfields the immunoreactive neurons exhibited primarily triangular somata. The majority of MT_1 positive neurons in the dentate gyrus occupied the polymorphic layer immediately subjacent to the granule cell layer and displayed oval or bipolar somata.

All AD cases revealed MT_1 immunoreactivity (Table 1). There was an obvious increase in the staining intensity and in the number of MT_1 positive neurons, indicating a receptor up-regulation (Fig. 1B,D). The distribution and morphological characteristics of MT_1 immunoreactive neurons were consistent with controls. Especially the triangular neurons in the CA1 subfield were strongly immunoreactive for MT_1 in all cases corresponding to different Braak stages (Fig. 1B). In four AD cases all pyramidal neurons showed immunoreactivity (Table 1). Interestingly, these cases correspond to Braak stages V and VI marking the severest grade of neuropathology. The immunoreactivity appeared as an intense red intracellular deposit homogeneously distributed

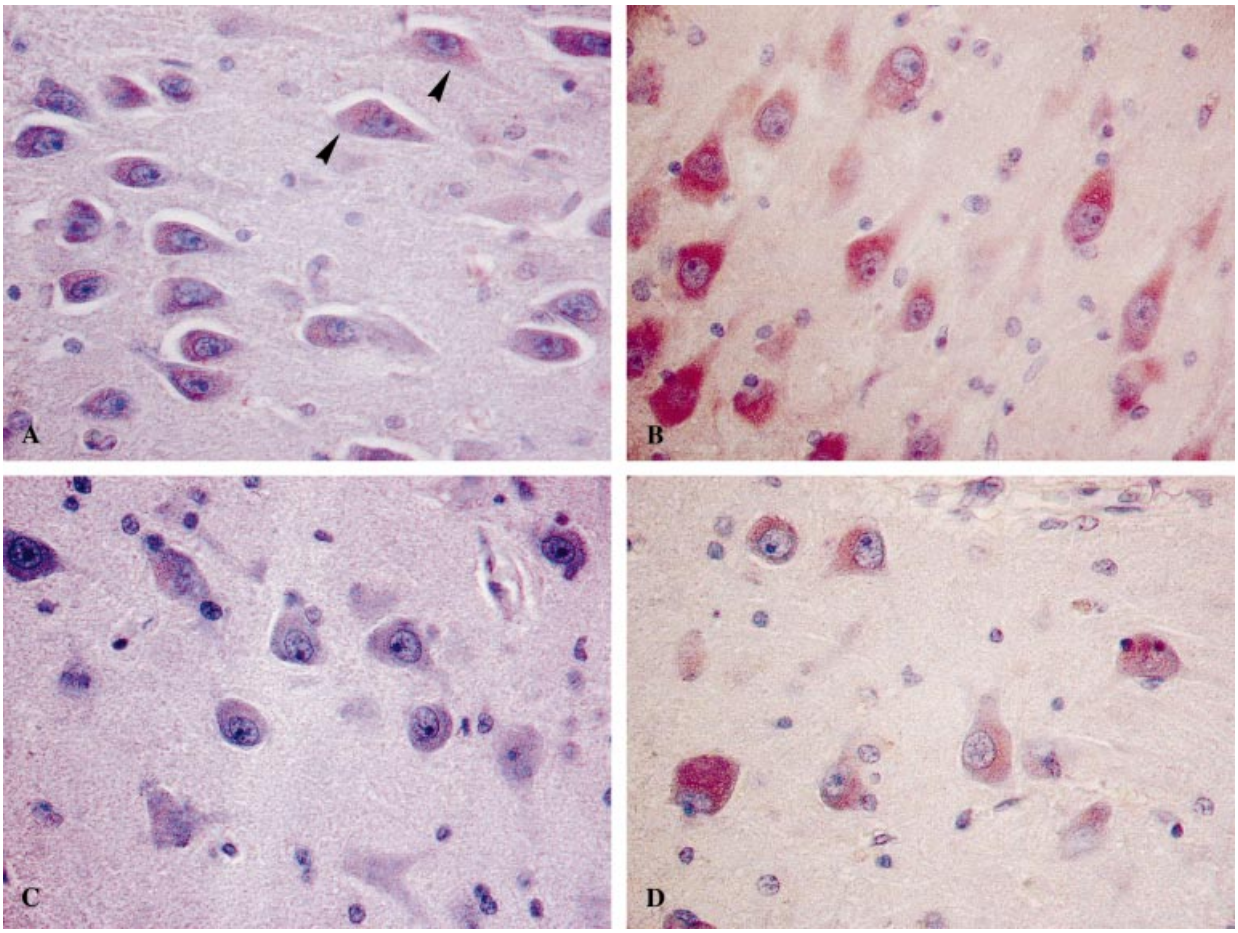

Fig. 1. Micrographs of hippocampal neurons (259× magnification for all). (**A**) Pyramidal neurons in the CA1 subfield of the hippocampus in a control case. Some neurons reveal a slight red MT_1-immunoreactivity as indicated by arrows. (**B**) The same hippocampal subfield in an AD case. There is an obvious increase in the number of MT_1 positive neurons and in MT_1 staining intensity shown as a distinctly red deposit. (**C**) Pyramidal neurons in the CA4 subfield of the hippocampus in a control case. (**D**) The same hippocampal subfield in an AD case. The pyramidal neurons are strongly immunopositive for MT_1.

within the perikaryal cytoplasm and extended into the initial parts of the primary dendrites. The increase in MT_1-staining was not restricted to those patients with the high risk ApoE 4/4 allele frequency in AD cases, but it was especially obvious in AD cases classified in Braak stages V and VI corresponding to the most advanced neuropathologic changes (Table 1). Non-neuronal cells did not show MT_1 immunoreactivity in any AD cases.

Discussion

Our results provide the first immunohistochemical evidence for the cellular localization of MT_1 in human hippocampus, and point to a prominent MT_1 increase in AD cases. MT_1 is exclusively localized to pyramidal neurons of the hippocampus, mainly in the CA1 subfield. The major efferent connections of the hippocampus emanates from CA1 (Rosene and Van Hoesen, 1977), which is the first part of the hippocampus displaying AD related pathology (Hyman et al., 1984). Our results are in accordance with previous findings demonstrating the presence of MT_1 mRNA in human hippocampus with in-situ hybridization (Mazzucchelli et al., 1996). However, the hippocampus was the brain region with the lowest level of MT_1 mRNA expression (Mazzucchelli et al., 1996). In our series, we failed to detect MT_1 immunoreactivity in three controls which may be the consequence of low expression levels in this brain region. Nevertheless, the remaining five controls showed MT_1 immunoreactivity in a subset of pyramidal neurons revealing the precise cellular localization of the receptor.

The MT_1 immunoreactivity was obviously increased in AD cases and the localization was extended to almost all pyramidal neurons. In particular, AD cases corresponding to the advanced stages of neuropathological changes as documented by Braak staging displayed a distinct increase (Table 1). In these cases all pyramidal neurons were stained. Since the hippocampus belongs to the brain region with the lowest MT_1 expression (Mazzucchelli et al., 1996), this distinct increase may reflect a disease-related alteration, probably accompanying the progression of AD related pathology. Considering the neuroprotective role of melatonin, the MT_1 increase in AD may reflect adaptation of hippocampal neurons to yield the maximum efficacy in spite of markedly reduced melatonin levels in AD patients (Reiter et al., 1999; Ferrari et al., 2000; Pappolla et al., 2000).

Melatonin is neuroprotective through various mechanisms: it protects neurons against Aβ-induced oxidative damage including increased lipid peroxidation, increased intracellular Ca^{2+} levels and apoptotic changes, besides strongly inhibiting the generation of Aβ itself (Reiter, 1998; Reiter et al., 1999; Pappolla et al., 2000). In addition, melatonin neutralizes free radicals extracellularly generated by Aβ and intracellularly generated by elevated Ca^{2+} levels (Reiter, 1998, Reiter et al., 1999; Pappolla et al., 2000). Both melatonin's direct receptor-independent scavenging effects as well as receptor-mediated influences on enzyme activities may account for its beneficial effects in AD. For example the induction of glutathione synthesis, a powerful antioxidant, by melatonin is probably receptor-mediated (Urata et al., 1999).

These neuroprotective mechanisms have been proposed as a new therapeutic strategy in AD (Pappolla et al., 2000). Although extensive clinical studies are still missing, melatonin treatment has been shown to induce a mild impairment in memory function and a substantial improvement of sleep quality in AD patients besides being an easily applicable substance rapidly crossing the blood–brain barrier and devoid of toxicity (Brusco et al., 1998). Taken together with the present data, the clinical use of melatonin during the course of AD may be advantageous. Further investigations will be necessary to reveal the alterations in levels of melatonin and its receptors in brain regions closely associated with AD pathology.

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