

Pineal and cortical melatonin receptors MT₁ and MT₂ are decreased in Alzheimer's disease

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The pineal hormone melatonin is involved in physiological transduction of temporal information from the light dark cycle to circadian and seasonal behavioural rhythms, as well as possessing neuroprotective properties. Melatonin and its receptors MT₁ and MT₂, which belong to the family of G protein-coupled receptors, are impaired in Alzheimer's disease (AD) with severe consequences to neuropathology and clinical symptoms. The present data provides the first immunohistochemical evidence for the cellular localization of the both melatonin receptors in the human pineal gland and occipital cortex, and demonstrates their alterations in AD. We localized MT₁ and MT₂ in the pineal gland and occipital cortex of 7 elderly controls and 11 AD patients using immunohistochemistry with peroxidase-staining. In the pineal gland both MT₁ and MT₂ were localized to pinealocytes, whereas in the cortex both receptors were expressed in some pyramidal and non-pyramidal cells. In patients with AD, parallel to degenerative tissue changes, there was an overall decrease in the intensity of receptors in both brain regions. In line with our previous findings, melatonin receptor expression in AD is impaired in two additional brain areas, and may contribute to disease pathology.

Key words: Melatonin receptors; MT₁; MT₂; pineal gland, cortex; human; Alzheimer's disease.

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The pineal hormone melatonin, secreted in a diurnal pattern during darkness, provides a circadian and seasonal signal to the organism in vertebrates (Reiter 1993; Savaskan 2002). Light signals to the retina are transduced via the retino-hypothalamic pathway to the suprachiasmatic nucleus (SCN) of the hypothalamus which drives the synthesis of melatonin in the pineal gland as a hormonal message encoding the duration of darkness (Stehle *et al.*, 2003). Apart from the pineal gland, melatonin is synthesized as well in the retina, gut and Harderian gland, and its synthesis in these organs also shows a daily rhythm. The pineal gland seems to be the main source of melatonin contributing to peripheral plasma melatonin levels (Vanecek 1998). Besides its seasonal and circadian regulatory functions, melatonin is involved in a variety of physiological responses including vasoactive, visual, oncostatic and neuroimmunological properties (Reiter *et al.*, 2003, 2001; Reiter 1991; Savaskan 2002), and has high antioxidative and neuroprotective effects (Pappolla *et al.*, 2000; Reiter *et al.*, 2003, 2001).

Melatonin acts through specific plasma membrane receptors (Ekmekcioglu 2006). To date, two G-protein coupled melatonin membrane receptors, MT₁ and MT₂, have been cloned in mammals, and the newly purified MT₃ protein belongs to the family of quinone reductases (Ekmekcioglu 2006; Nosjean *et al.*, 2000). Whereas little is known about the MT₃-mediated effects of melatonin, MT₁ and MT₂ are involved in different physiological effects and in the antinoceptive, anxiolytic, antineophobic, and locomotor activity modulatory effects of melatonin (Uz *et al.*, 2005). In addition to rodent tissues, where the expression patterns of both receptors have been well studied, some studies have addressed melatonin receptor expression in human CNS and found mRNA for both receptors in the

Table 1. Demographic and immunohistochemical data of elderly controls (C) and Alzheimer's disease (AD) patients. Pmd: post-mortem delay in minutes; BS: Braak staging (BS); ApoE: apolipoprotein E allele differentiation. NBB: Netherlands Brain Bank autopsy numbers. OC: Occipital cortex; PG: Pineal gland. F: female, M: male.

Case (NBB)	Age	Gender	pmd	BS	ApoE	OC:		PG:	
						MT1	MT2	MT1	MT2
C: 1. 02-008	62	M	418	0	3/3	+	++	+++	+++
2. 03-033	61	F	410	0	3/2	+++	+++	+++	+++
3. 02-024	75	F	330	1	4/2	++	++	+++	++
4. 02-087	71	M	460	1	3/3	+++	+++	+++	+++
5. 01-104	77	F	330	1	4/3	+++	+++	+++	+++
6. 03-006	91	F	320	3	3/2	+++	+++	+++	++
7. 03-002	51	M	464	0	3/3	++	++	++	++
AD: 1. 01-095	81	F	330	6	4/4	+	+	++	+
2. 02-002	92	M	210	4	3/3	-	-	++	++
3. 02-061	76	F	645	5	4/4	+	+		++
4. 01-125	77	F	510	6	4/4	++	++	++	++
5. 02-021	64	F	220	6	4/3	++	++	++	++
6. 01-124	86	F	320	6	4/4	+	++	+	+
7. 01-141	69	F	420	5	4/2	++	++	++	++
8. 02-047	83	F	427	5	4/3	+	+	+	+
9. 02-085	79	F	255	5	3/3	+	+	++	+
10. 01-129	91	F	220	6	3/3	+	+	++	+

Staining intensities of the MT1 and MT2 immunoreactivities: (-): no immunopositive cellular structures; (+): slight, single immunopositive cellular structures; (++): moderate, at least more than 5 immunopositive cellular structures on each section; (+++): high immunoreactivity, almost all cells are immunoreactive on each section (in OC in the layers II-V, in PG pinealocytes).

cerebellum, hippocampus, cortex, thalamus and retina (Al-Ghoul *et al.*, 1998; Mazzucchelli *et al.*, 1999; Reppert *et al.*, 1995). Using immunohistochemistry, we have previously shown the cellular localisation of MT₁ and MT₂ in the human hippocampus, retina and in cerebrovascular tissues (Savaskan *et al.*, 2005, 2002a, b, 2001). The expression patterns of both receptors were found to be differentially altered in patients with Alzheimer's disease (AD), a progressively disabling neurodegenerative disorder that is the major cause of dementia in the elderly population. Nocturnal melatonin levels are selectively decreased in AD patients and AD-related sleep-wake cycle disturbances are associated with melatonin secretion rhythm disorders (Pappolla *et al.*, 2000; Swaab 2003; Wu & Swaab 2005).

Although the distribution of melatonin receptors have been well studied in rodent brain, detailed mapping of the receptors is still missing in humans. Therefore the aim of the present study was to provide immunohistochemical evidence for the cellular distribution of both melatonin receptors MT₁ and MT₂ in the pineal gland and the occipital cortex of elderly controls and AD patients. Since both brain regions are heavily affected during the disease pathology, the results may provide additional clues for the involvement of melatonin system in aging and neurodegeneration.

Materials and Methods

Human Tissue

Occipital cortex and pineal gland of 7 elderly control cases without neurological or psychiatric disorders (4 female and 3 male; mean age: 69.7±4.9; mean post-mortem delay: 390.3±23.7 minutes) and 10 patients with AD (9 female and 1 male; mean age: 79.8± 2.8; mean post-mortem delay: 355.70±45.56 minutes) were included in the study. The demographic data of the cases is demonstrated in Table 1. Heart failure or pneumonia was the cause of death in all subjects. Paraffin-embedded and paraformaldehyde-fixed tissue samples were kindly provided by the Netherlands Brain Bank. Sample collection was according to ethics committee criteria and the Declaration of Helsinki in 1975. The diagnosis of AD was performed according to the NINCDS-ADRDA criteria (McKhann *et al.*, 1984) and confirmed by post-mortem neuropathological examination. For the staging of the various pathological hallmarks of AD the neuropathological Braak staging (Braak&Braak 1991), and, as a major risk factor for sporadic AD, apolipoprotein E (ApoE) allele frequency were determined for each case. Braak stages 4-6 show advanced neuropathological changes and correspond to AD. The predominant ApoE alleles were 4/4 and 4/3 in AD cases, and 3/3 and 3/2 in con-

trol cases. Consecutive, coronal, 12 μ m-thick, serial tissue sections were used for the immunohistochemical staining. Every tenth section in each series was included in the study and at least ten sections were used for the staining procedure.

Immunohistochemistry

The observed antigens, MT₁ and MT₂, were visualized by peroxidase staining using the peroxidase substrate 3-amino-9-ethylcarbazole. The staining method has been previously reported in detail (Savaskan *et al.*, 2005, 2004, 2002a, b, 2001). The experimentally determined optimum concentration for the primary antibodies was 1:150 both for MT₁ and MT₂. The concentration of the secondary antibody was 1:100. The samples were counterstained with Mayer's hemalum. The experiments were performed in duplicate. Adjacent sections to MT₁- or MT₂-stained tissue samples were stained simultaneously to serve as control samples, using the same procedure with the exception that primary antibodies were omitted. All sections were assessed for localization and intensity of specific immunoreactivity on a semiquantitative scale of +/+++ by two blind observers using a Zeiss Axiolab microscope.

Results

Pineal gland

The predominant cells of the pineal gland, the pinealocytes, which produce melatonin, were immunoreactive for both MT₁ and MT₂ (Figure 1 A, C, F). The pineal gland was subdivided by the connective tissue septa into lobules and pinealocytes. Cells with large, light and round nuclei located within the lobules, displayed a granular, perinuclear immunoreactivity for the two antigens. MT₁ and MT₂ were located both in cell somata and in cellular processes compactly filling up the lobules. Immunoreactive cellular processes framed the lobular border. All controls showed high immunoreactivity for both receptors independent of age, post-mortem delay, Braak staging or ApoE allele type. In some sections acervuli cerebri or calcium-containing concretions were found within the pineal parenchyma which were free of immunoreactivity.

The immunoreactivities for MT₁ and MT₂ were distinctly decreased in AD cases both in cell somata and cellular processes (Figure 1 B, D). Thus, the pineal gland showed clear changes with reduced

number of pinealocytes within the lobules and a reduced network of cellular processes displaying slight immunoreactivity. The calcification was increased in most AD cases. However, there was no AD case missing MT₁ or MT₂ immunoreactivity. The degree of the decrease in the immunoreactivities did not correlate with age, post-mortem delay, Braak staging or ApoE allele type.

Occipital cortex

Cells in the cortical layers II to V displayed MT₁ and MT₂ immunoreactivity (Figure 2 A, B, D). The outermost of the six cortical layers (the molecular layer or layer I), and the deepest layer (the multi-form layer or layer VI), contained no MT₁ or MT₂ immunoreactive cells besides a few cells of the second layer scattering into the layer I. Some pyramidal-shaped and non-pyramidal cells of layers II to V, on the other hand, showed a distinct granular, cytoplasmic and perinuclear immunoreactivity for MT₁ and MT₂ (Figure 2 A, B, D). Interestingly, not all pyramidal and non-pyramidal cells were found to express the receptors and in some sections MT₁ and MT₂ immunoreactive cells formed descending cellular columns throughout several layers (Figure 2 B, D). The layers of the occipital cortex were not clearly subdivided, and pyramidal and non-pyramidal cells were mixed within different layers. Apical dendrites of the pyramidal neurons and some cellular processes were also immunoreactive for both receptors.

Similar to the pineal gland, immunoreactivities for MT₁ and MT₂ were decreased in the AD cases, both in pyramidal and non-pyramidal cells (Figure 2 C, E). In general, less cells were stained for the receptors in the AD cortex. The decreased intensity of the MT₁ or MT₂ immunoreactivities did not correlate with age, post-mortem delay, Braak staging or ApoE allele type.

Discussion

The present data provides the first immunohistochemical evidence for the

cellular localizations of the two melatonin receptors MT₁ and MT₂ in the human pineal gland and occipital cortex, and demonstrates decreased expression of the receptors in AD.

The pinealocytes of the pineal gland are the production site of melatonin and, as shown in the present study, the main cells expressing both MT₁ and MT₂. The rhythmic melatonin secretion in the pineal

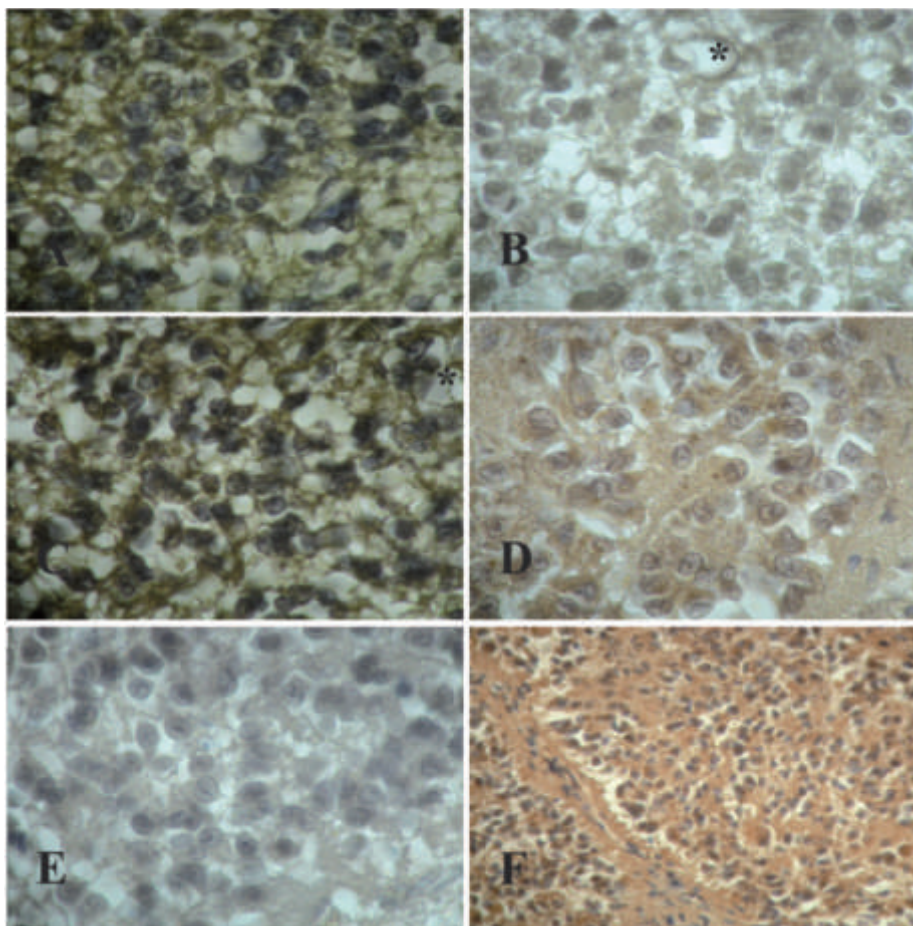


Figure 1. MT₁ and MT₂ in the pineal gland. (A) The cell somata and cellular processes of the pinealocytes are immunoreactive for MT₁ (red-brown deposits) in a control case. (B) MT₁ immunoreactive structures in an AD case. The density of the immunoreactivity and the cell count of the cells are distinctly decreased (star indicates calcification). (C) MT₂ immunoreactive pinealocytes in a control case (star indicates calcification). (D) Decreased MT₂ immunoreactivity in an AD case. (E) Control section stained simultaneously according to the same procedure, with the exception that the primary antibody (for MT₁) was omitted. (F) Lower magnification micrograph of the pineal gland demonstrating the lobular structure of the tissue, stained for MT₁. (A-E) Original magnification x242. (F) Original magnification x60.

gland is controlled by the SCN innervating the gland via the dorsomedial hypothalamic nucleus, the upper thoracic intermediolateral cells columns of the spinal cord and the superior cervical ganglia (Wu&Swaab 2005). The sympathetic stimulus is crucial for pineal melatonin secretion and a circadian rhythm of β 1-adrenergic receptors has been found in human pinealocytes (Oxenkrug *et al.*, 1990). In turn, pineal melatonin elicits two distinct, separable effects on the SCN through its receptors, i.e. acute neuronal inhibition and phase shifting (Liu *et al.*, 1997). As shown in the present study melatonin may also act locally via melatonin receptors on pinealocytes.

The pineal gland shows age- and AD-related changes which is reflected by continuously decreasing melatonin levels (Wu&Swaab 2005). The acervuli cerebri or calcium-containing concretions in the pineal gland, which were commonly observed in our series, increase with age, however, there is no clear evidence that pineal calcification affects pineal metabolism (Wu&Swaab 2005). Thus, in the elderly, there are no obvious degenerative changes

in the pineal gland (Pardo *et al.*, 1990). Similarly in our control series, the pineal gland consisted of a compact tissue of pinealocytes with no clear degenerative alterations. On the other hand, the opposite was the case in the AD series: the pineal gland showed a distinct decrease in pinealocytes and their cellular processes indicating cell loss. We have also observed more calcium concretions within the gland. This is in contrast to previous findings reporting no alternation in calcium deposition in the pineal gland in AD (Friedland *et al.*, 1990), nor in pineal weight or pineal total protein content (Wu *et al.*, 2003). Thus, the pineal cells and afferent fibers were found to be clear of AD-related changes, i.e. neurofibrillary tangles, the accumulation of neurofilaments, tau, hyperphosphorylated tau or amyloid deposition (Pardo *et al.*, 1990). However, loss of melatonin rhythmicity has been found to occur already in early AD neuropathology, possibly related to the severe degenerative changes in the SCN (Wu&Swaab 2005), and, in addition, to the dysfunction of pineal noradrenergic regulation and the depletion of pineal serotonin by increased

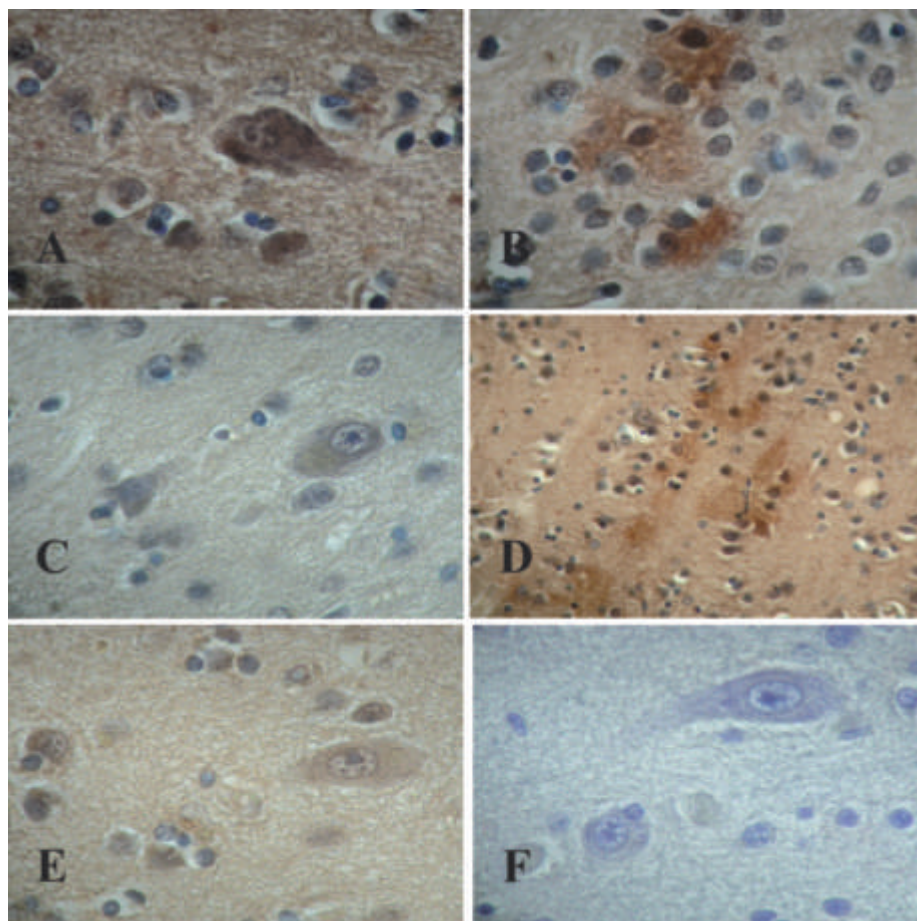


Figure 2. MT₁ and MT₂ in the occipital cortex. (A) MT₁ immunoreactive pyramidal cell in a control case. (B) MT₂ immunoreactive non-pyramidal cells in columnar organisation. (C) Decreased MT₁ immunoreactivity in pyramidal and non-pyramidal cells in an AD case. (D) Lower magnification micrograph of the cortex with MT₂ immunoreactive cells in columnar organisation. (E) Decreased MT₂ immunoreactivity in pyramidal and non-pyramidal cells in an AD case. (F) Control section omitting the primary antibody (for MT₁). (A-C, E, F) Original magnification x242. (D) Original magnification x60.

monoamine oxidase A activity (Wu *et al.*, 2003). The AD pineal glands in our series show obvious degenerative changes which are clearly distinguishable from control cases. Moreover, MT₁ and MT₂ expression on pinealocytes are severely affected suggesting that parallel to degenerative changes in the pineal gland the melatonin receptor system may be impaired during the disease course. The alterations in receptor expression may be also a consequence of altered melatonin, since changes in pineal melatonin levels have been shown to autoregulate the density of its receptors (Guerrero *et al.*, 2000).

Melatonin binding in the cerebral cortex can be detected very early in the human fetus (Yuan *et al.*, 1991) and several studies have demonstrated the presence of MT₁ mRNA in the human cortex, including the occipital cortex (Mazzucchelli *et al.*, 1999; Uz *et al.*, 2005). Compared to frontal and temporal cortex the level of the MT₁ messenger was highest in the parietal and occipital cortex (Mazzucchelli *et al.*, 1999). In other mammalian species, the relative abundance of mRNA in various cortical regions was found to correspond well with

melatonin receptor density in the same areas (Bittman&Weaver 1990; Stankov *et al.*, 1992). However, direct comparison with expression of the receptor protein could not be performed because of extremely low specific binding obtained in *in vitro* ligand-binding experiments (Mazzucchelli *et al.*, 1999). The authors consider that this may suggest low expression, or, alternatively, a limited number of specialized neurons may express melatonin receptors in the various cortical regions of the human brain, implying discrete differences in function (Mazzucchelli *et al.*, 1999). Also in our series, not all pyramidal and non-pyramidal cells in the occipital cortex were positive for MT₁ or MT₂, and, in some sections, melatonin receptor positive cells were found to be organized in columns of a group of cells. Together with previous findings, these results may be indicative for localization of both melatonin receptors in subgroups of cortical cells with a specific function. Indeed, the presence of MT₁ proteins has been demonstrated in areas related to dopaminergic behaviours including the prefrontal cortex (Uz *et al.*, 2005).

In the present study both MT₁ and MT₂ were located on pyramidal and non-pyramidal cortical cells. There is growing evidence that G protein-coupled receptors such as MT₁ and MT₂ co-localized on the same cell are organized as homo- and heterodimers and this oligomerization may have important consequences for receptor function (Levoye *et al.*, 2006). The formation of MT₁ and MT₂ homodimers and MT₁/MT₂ heterodimers occurs at physiological expression levels and the formation of MT₁/MT₂ heterodimers in native tissues is suggested by the co-expression of MT₁ and MT₂ in many melatonin-sensitive tissues (Levoye *et al.*, 2006). In line with this observation, our results may indicate co-localization of the two melatonin receptors in human cortical cells.

Both MT₁ and MT₂ were decreased in AD. We have previously shown that both receptors are impaired in the AD hippocampus (Savaskan *et al.*, 2005, 2002a), a region highly implicated in AD pathology. Whereas MT₂ was found to be decreased in AD hippocampus, the expression of MT₁ was increased. Therefore, the AD-related alterations in melatonin receptors may be region-specific, with an overall loss of expression in the pineal gland and occipital cortex. In conclusion, both G protein-coupled melatonin receptors MT₁ and MT₂ are expressed in the pineal gland and occipital cortex on the same cellular structures, and the intensity of both receptors decreases during AD pathology.

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