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Originally published at:
Brunner, P; Sözer-Topcular, N; Jockers, R; Ravid, R; Angeloni, D; Fraschini, F; Eckert, A; Müller-Spahn, F; Savaskan, E (2006). Pineal and cortical melatonin receptors MT1 and MT2 are decreased in Alzheimer's disease. European Journal of Histochemistry, 50(4):311-316.
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Abstract

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.
Pineal and cortical melatonin receptors MT$_1$ and MT$_2$ are decreased in 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 (Ekmeckioğlu 2006). To date, two G-protein coupled melatonin membrane receptors, MT$_1$ and MT$_2$, have been cloned in mammals, and the newly purified MT$_1$ protein belongs to the family of quinone reductases (Ekmeckioğlu 2006; Nosjean et al., 2000). Whereas little is known about the MT$_1$-mediated effects of melatonin, MT$_1$ and MT$_2$ are involved in different physiological effects and in the antinociceptive, 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 cerebellum,
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, 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 control cases. Consecutive, coro-

<table>
<thead>
<tr>
<th>Case (NBB)</th>
<th>Age</th>
<th>Gender</th>
<th>pmd</th>
<th>BS</th>
<th>ApoE</th>
<th>OC:</th>
<th>PG:</th>
</tr>
</thead>
</table>
| 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 | 4/3 | ++ | ++ | ++
| 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).

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.
nal, 12 um-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 multiform 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 gland is controlled by the SCN innervating the
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 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

![Figure 2. MT₁ and MT₂ in the occipital cortex. (A) MT₁ immunoreactive pyramidal cell in a control case. (B) MT₁ 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 MT₁ immunoreactive cells in columnar organisation. (E) Decreased MT₁ 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.](image-url)
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 MT1 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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