FEBS Letters 471 (2000) 56–60 FEBS 23500

Ligand interaction with the purified serotonin transporter in solution and at the air/water interface

V. Faivre[a], P. Manivet[b], J.C. Callaway[c], M.M. Airaksinen[d], H. Morimoto[e], A. Baszkin[a], J.M. Launay[b], V. Rosilio[a,*]

[a] *Laboratoire de Physico-Chimie des Surfaces, UMR CNRS 8612, 5 rue J-B. Clément, 92296 Châtenay-Malabry, France*
[b] *C.R.C. Bernard 'Pathologie Expérimentale et Communications Cellulaires', Service de Biochimie, IFR 6, Hôpital Lariboisière AP-HP, 2 rue Ambroise Paré, 75010 Paris, France*
[c] *Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 6, 70211 Kuopio, Finland*
[d] *Department of Pharmacology and Toxicology, University of Kuopio, P.O. Box 6, 70211 Kuopio, Finland*
[e] *National Tritium Labelling Facility (NTLF), University of California, 1 Cyclotron Road, Berkeley, CA 94720, USA*

Received 1 February 2000; received in revised form 9 March 2000

Edited by Guido Tettamanti

Abstract The purified serotonin transporter (SERT) was spread at the air/water interface and the effects both of its surface density and of the temperature on its interfacial behavior were studied. The recorded isotherms evidenced the existence of a stable monolayer undergoing a lengthy rearrangement. SERT/ligand interactions appeared to be dependent on the nature of the studied molecules. Whereas an unrelated drug (chlorcyclizine) did not bind to the spread SERT, it interacted with its specific ligands. Compared to heterocyclic drugs, for which binding appeared to be concentration-dependent, a 'two-site' mechanism was evidenced for pinoline and imipramine.

Key words: Serotonin; Transporter; Surface pressure; Protein monolayer; Serotonin transporter–drug interaction

1. Introduction

In nerve cells, platelets and gut enterochromaffin cells, serotonin (5-hydroxytryptamine, 5-HT) is taken up by a high-affinity transporter, serotonin transporter (SERT), a member of a large superfamily of homologous transporters that are predicted by hydropathy analysis to contain 12 transmembrane-spanning domains (TMDs), and that use the biomembrane Na^+ and Cl^- gradients as a source of energy ([1] for review). SERT is most closely related to transporters specific for the other biogenic amines dopamine and norepinephrine. These three monoamine transporters are the molecular targets of important psychoactive drugs, including cocaine, antidepressants and amphetamines. Apart from expression [2] and site-directed mutagenesis [3] studies, we took advantage of our previous purification to homogeneity of SERT from human platelets [4] to further characterize this purified SERT using biophysical techniques. It was thought that they may provide new insights into the mechanism and regulation of the electroneutral transport of 5-HT.

*Corresponding author. Fax: (33)-1-4683 5312.
E-mail: veronique.rosilio@cep.u-psud.fr

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; RTI (cocaine congener), 3β-(4-iodophenylpropene-2β-carboxylic acid methyl ester); MDMA, 3,4-methylene dioxymethamphetamine (ecstasy)

2. Materials and methods

2.1. Materials

Imipramine, chlorcyclizine, cocaine, pinoline and 3,4-methylene dioxymethamphetamine (MDMA) were purchased from Sigma (Saint Louis, MI, USA), [^{3}H]MDMA from Amersham (Buckinghamshire, UK), [^{3}H]imipramine, [^{3}H]paroxetine and [^{125}I]3β-(4-iodophenylpropene-2β-carboxylic acid methyl ester) (RTI) from New England Nuclear (Boston, MA, USA). Indalpine and paroxetine were a gift from Dr. V. Mutel (Hoffman-LaRoche, AG., Basel, Switzerland). [^{3}H]Pinoline was synthesized by two of us (J.C. and H.M. at the NTLF). Human platelet membranes and purified SERT, as well as membranes from human brain cortex were prepared as previously described [4,5]. Ultrapure water was obtained by osmosis from a MilliRO6 Plus Millipore apparatus and then double-distilled from permanganate solution in an all-Pyrex apparatus. Sodium chloride (150 mmol/l) and sodium phosphate (10 mmol/l) used to prepare buffer solutions (pH adjusted to 7.40) were Normapur from Prolabo (Paris, France). All glassware was cleaned by a sulfochromic mixture and then abundantly rinsed with distilled water.

2.2. Binding experiments

[^{3}H]MDMA, [^{3}H]imipramine, [^{3}H]paroxetine, [^{125}I]RTI and [^{3}H]pinoline assays were performed as previously reported in [2,4–7]. Prism software (GraphPad, San Diego, CA, USA) was used to analyze all kinetic, saturation and competition data.

2.3. Surface tension measurements

The surface tension measurements at equilibrium (at constant area) were performed by the Wilhelmy plate method as previously described in [8,9]. The interfacial behavior of the SERT protein at the interface was inferred from the change in surface tension after protein deposition onto the buffer subphase. Any tested ligand was dissolved into the buffer and its adsorption at the air/water interface was assessed by measuring the decrease in surface tension with time. The surface pressures of a SERT monolayer (π_P) and of a considered ligand (π_L) were deduced from the $\pi = \gamma_0 - \gamma$ relationships, where γ_0 stands for the surface tension of the buffer solution and γ is the surface tension observed in the presence of a spread protein or an adsorbed ligand monolayer. The experiments were conducted in an enclosed chamber and the cell was thermostated at a chosen temperature. All reported surface tension values were means of at least three measurements. The accuracy of the measurements was estimated to be ± 0.2 mN/m.

2.4. SERT/ligand interactions at the air/water interface

SERT molecules were spread to form a monolayer (1.5 μg/cm^2) onto a ligand-containing subphase and the surface tension was recorded as a function of time until it reached equilibrium (π_T). When a ligand was surface inactive, an additional surface pressure (π_{PL}) was observed as its molecules interacted with the protein monolayer ($\pi_T = \pi_P + \pi_{PL}$). When a drug was surface active, surface pressure changes due to its adsorption at the free air/solution interface, π_L, had to be taken into account. In the presence of the SERT monolayer, the reduction of the available space at the interface would lead to a $\pi_{L'}$ value lower than π_L. Thus, the interaction between a surface

PII: S0014-5793(00)01362-4

active specific ligand and the SERT could be described by the equation:

$$\pi_T = \pi_P + \pi_{L'} + \pi_{PL} \quad (1)$$

where $\pi_{L'}$ and π_{PL} are the unknown quantities. An approximation was made by considering that $\pi_{L'} = \pi_L$. In this case, the surface pressure π_{PL} resulting from the specific interaction between the protein and its ligands was underestimated.

The ability of a ligand to induce conformational rearrangements of the SERT were inferred from the values of first rate kinetic constants obtained by means of Eq. 2 which describes interfacial events following protein spreading [10]:

$$\ln\frac{(\pi_{eq}-\pi_t)}{(\pi_{eq}-\pi_0)} = -kt \quad (2)$$

where $\pi_{eq} = \pi_P + \pi_{PL}$, π_t is the surface pressure change at time t, π_0 the initial surface pressure, and k is the kinetic constant.

3. Results

3.1. Interfacial behavior of the SERT

As previously reported, the purified SERT may be spread at the air/water interface of a buffer solution and forms a monolayer [9]. Its interfacial behavior was found to be dependent on the surface density (Fig. 1). At 21.5°C, the surface pressure increased rapidly at low surface coverages until 1.5 μg/cm^2 and then above this surface density, only a slight increase in the surface pressure was observed. The effect of temperature, shown in the inset to Fig. 1, clearly indicates that the higher the temperature, faster the rate at which the maximum surface pressure was reached. In addition, the adsorption profiles were different one from another. Whereas at 35°C the π–time curve resembled the profile of a globular protein adsorbed at the air/water interface [11], at 21.5°C, the initial increase in the surface pressure (A), was followed by a short equilibrium step (from A to B) and then by a slow pressure increase (from B to C) which lasted several hours. However, at both temperatures, the π values after 20 h following SERT deposition converged to an almost identical value. The stability of the SERT monolayer was checked by performing successive compression–expansion–compression cycles under dynamic conditions, using a Langmuir trough. They yielded identical π–A profiles (data not shown).

3.2. SERT/ligand interactions

3.2.1. Binding experiments in solution. Extending our previous report [4], in Table 1 are the K_d values summarized for each considered ligand applied to different SERT-containing materials. From these data, it is clear that the binding efficiency splits into two groups, one with K_d values below 10 nM

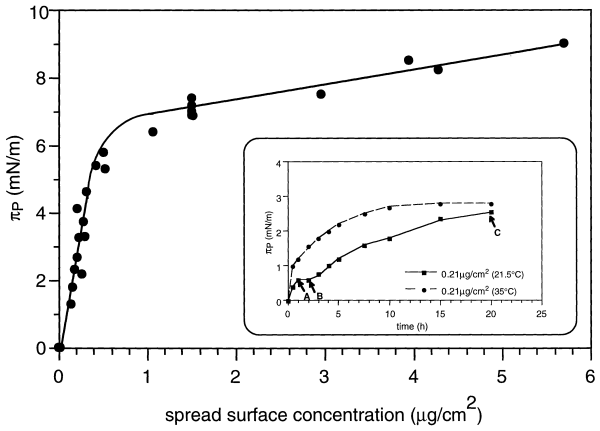

Fig. 1. Surface pressure (π_P) of the SERT as a function of its surface density at 21.5°C. Inset: surface pressure (π_P) of the SERT as a function of time and at two temperatures (■: 21.5°C and ●: 35°C).

(paroxetine, imipramine and indalpine) and another one with K_d values above 100 nM (cocaine or RTI, MDMA and pinoline).

3.2.2. Binding at the air/water interface. The interaction of SERT with two specific ligands (paroxetine and indalpine) and with the unrelated ligand (chlorcyclizine, an anti-histaminic) was also studied and the results are shown in Fig. 2. It is apparent that specific interactions with SERT depended both on the ligand concentration in solution and on the affinity of the ligand for the transporter. The response to the ligand presence in the subphase was also selective. When SERT was spread onto a paroxetine solution (8×10^{-5} M corresponding to $\pi_L = 7.2$ mN/m), an increase in the surface pressure lasted for several hours. Conversely, the adsorption of chlorcyclizine (1.3×10^{-5} M corresponding to the same $\pi_L = 7.2$ mN/m) to a SERT monolayer led to negative values of π_{PL}. Since no specific interaction was expected in this case, π_{PL} should be considered insignificant. The observed negative values are related to the surface activity of the ligand and to the fact that the ligand contribution to the change in surface pressure, $\pi_{L'}$, was taken to be equal to π_L. The increase in surface pressure observed after several hours was most probably due to the rearrangement of the chlorcyclizine–SERT mixed monolayer. A similar behavior was observed in the reverse situation where a SERT specific ligand (indalpine) was injected beneath a non-specific protein (bovine serum albumin) monolayer (the results are not shown here). The interaction of pinoline (a surface active molecule at a concen-

Table 1
K_d values for ligand binding to the SERT in brain, platelets and in solution, and kinetic constants (k) for SERT/ligand interaction at the air/solution interface ($k_{SERT} = 0.5176 \pm 0.046$ h^{-1})

Ligands	K_d in brain (nM)	K_d in platelets (nM)	K_d purified SERT (nM)	k purified SERT (h^{-1})
Imipramine	1.25 ± 0.08	1.54 ± 0.15	1.73 ± 0.13	11.64 ± 1.54
Paroxetine	0.08 ± 0.01	0.25 ± 0.07	0.18 ± 0.06	2.41 ± 0.37
Indalpine	0.96 ± 0.15	1.20 ± 0.29	1.31 ± 0.23	3.14 ± 0.44
Pinoline	572 ± 73	175 ± 18	217 ± 17	0.55 ± 0.20
RTI	217 ± 23	446 ± 54	511 ± 52	–
cocaine	–	–	–	0.27 ± 0.15
MDMA	2067 ± 139	1887 ± 169	> 1 mM	–
Chlorcyclizine				0.22 ± 0.04

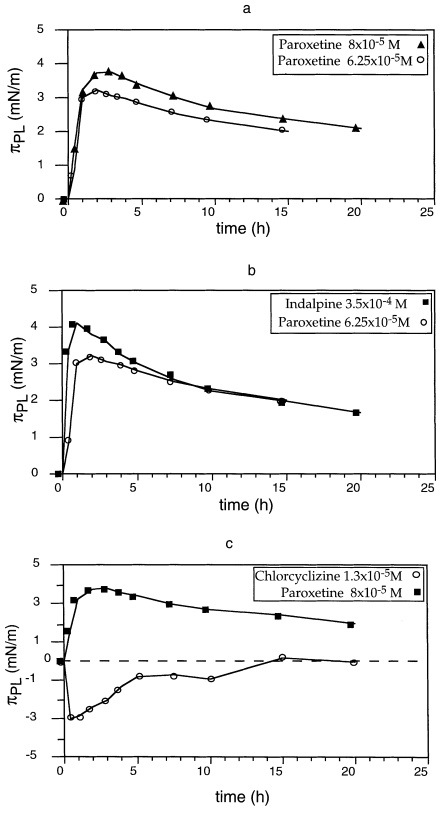

Fig. 2. Interaction of SERT with heterocyclic molecules. Effect of (a) ligand concentration, (b) ligand affinity for the transporter and (c) ligand specificity, on the surface pressure response.

tration above 10^{-5} M) with SERT has been studied as a function of pinoline concentration (Fig. 3). At the low surface coverage (0.5 μg/cm^2), no specific interaction could be evidenced even at high pinoline concentrations in the subphase. At the higher surface coverage (1.5 μg/cm^2), a triphasic behavior was observed and depended on the concentration of the ligand. An increase in the surface pressure corresponding to a specific interaction occurred, reaching a maximum at 5×10^{-5} M of pinoline. Above this threshold value, the decrease in π was observed until it reached a zero π value at about 10^{-4} M. A similar mechanism to that with pinoline was observed also with imipramine at its high solution concentration (5×10^{-4} M). Cocaine interacted with the transporter monolayer only at high concentrations and the increase in surface pressure was limited to 2 mN/m, above 5×10^{-4} M. In the range of the studied concentrations, 5×10^{-5}–5×10^{-3} M, no binding of MDMA was detected.

4. Discussion

The cloning of rat, mouse and human SERT revealed proteins sharing a common, putative 12 TMD structure (highly hydrophobic domains). In this model, the extended N- and C-terminals were assigned to the cytoplasm, because a hydrophobic N-terminus displaying the characteristics of a signal peptide was missing. The cytoplasmic regions contained putative phosphorylation sites which were used for the regulation of transport activity [12]. In addition, a long putative extracellular loop containing *N*-glycosylation sites was conserved between TMDs 3 and 4. The predicted 12 α-helices of the SERT confer to it an ability to spread at the air/water interface from a buffer solution and to form a stable monolayer. The interfacial behavior of the protein has been shown to be temperature, concentration and time-dependent. These results strongly suggest that protein rearrangement after the deposition lasted several hours until an equilibrium was reached. Indeed, Graham and Phillips [10] have demonstrated that the surface pressure of an adsorbed protein layer may increase for more than 10 h even if its surface concentration did not change. Also Tronin et al. [13] showed that protein molecules injected beneath a protein monolayer exchanged with those of the monolayer to a very limited degree. Most probably, the presence of macromolecules deposited at the interface hindered adsorption of molecules of the same species dissolved in the subphase. This would indicate that progressive rearrangement of protein molecules rather than their dissolution in the subphase and subsequent adsorption at the interface accounted for the lengthy times necessary to level off surface pressure values (B–C section in the inset to Fig. 1).

Specific binding is based on molecular recognition of ligand–protein complementary sites. These complementary sites may only exist if a protein is capable of preserving its conformation. As the SERT was capable of binding most of its ligands, the results of the present work clearly demonstrate that the protein spread from a buffer solution onto a buffer subphase maintained, at least partially, its original conformation. However, it should be stressed that whereas binding experiments provided information on the affinity of the protein for a ligand through the determination of the K_d values, the measured surface tension variations accounted rather for SERT conformational changes induced by ligand binding. At the air/solution interface, the arrangement of SERT is controlled by its inflexible structure and by the hydrophobic/hydrophilic constrains of the environment. The transport function is, obviously, suppressed. However, we have shown here that, at the surface density of 1.5 μg/cm^2, SERT molecules were confined into a favorable conformation that enabled the specific binding of ligand molecules.

From our previous work [9] and also from the above reported results, it was apparent that the 5-HT and MDMA did not produce any surface pressure change. These two molecules are also the only two tested molecules that SERT is capable to transport. Indeed, MDMA ('ecstasy') and PCA, as well as

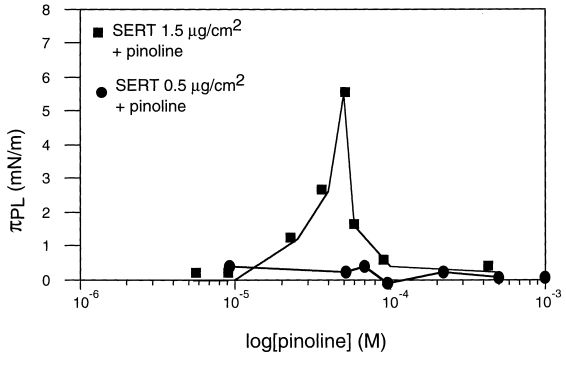

Fig. 3. Interaction of SERT with pinoline. Influence of SERT surface density and of pinoline bulk concentration on the change in surface pressure.

non-neurotoxic amphetamines, have been found not only to competitively inhibit 5-HT transport into human platelets with high affinity, but also to stimulate previously accumulated [^{3}H]5-HT efflux from these platelets by reversed plasma membrane transport [14]. The finding that both amphetamine-mediated processes were Na^+-dependent and imipramine-sensitive was consistent with a model in which amphetamines were transported by SERT. This in turn implicates that SERT is a 5-HT–amphetamine exchange system. Recently, targeted gene disruption has shown that SERT is a main target of action of MDMA because the locomotor-enhancing effect of MDMA was totally absent in mice lacking SERT [7]. Moreover, SERT has been shown to co-transport one Na^+ and one Cl^- ion with each 5-HT molecule per transport cycle accompanied by counter-transport of one internal K^+ ion [1]. It follows, therefore, that the results reported in this study may be attributed both to the alteration of SERT transport function in the conditions used and to the absence of the K^+ ions in the experimental medium.

The detection of SERT interaction with ligands at the air/solution interface required higher ligand concentrations than those usually reported to induce pharmacological responses. The reduced accessibility of a ligand to its binding site may be attributed both to steric hindrance at high protein surface densities and an unfavorable orientation of SERT molecules at the air/water interface. It seemed, therefore, more accurate to relate the effect of the ligands to the kinetics of surface pressure changes, i.e. to the first rate constants, calculated from Eq. 2. The k values for the studied ligands are presented in Table 1. It is interesting to note that for all studied ligands, the k values were invariable with bulk concentration and remained in the limits of the calculated standard deviations. The obtained k values clearly show that indalpine, paroxetine and imipramine interact with SERT differently than cocaine and pinoline. Interestingly, this discrimination of the ligands in the two groups is the same as that deduced from binding experiments (K_d values in Table 1). Moreover, for the two heterocyclic ligands (paroxetine and indalpine), the k values were close one to another. Since these two molecules are known to bind to the same site of the SERT [15], it was reasonable to assume that the extent and the rate of SERT conformational changes were related to the accessibility of the ligand binding site at the interface and to the protein ability to rearrange after the ligand binding. Our results show also that whereas binding of heterocyclic molecules (indalpine, paroxetine) was solution concentration-dependent, a more complex behavior was characteristic of pinoline and imipramine.

The inhibitory mechanisms of heterocyclic and tricyclic ligands at SERT are poorly understood as yet. These molecules bind to the transporter protein with high affinity, thereby preventing substrate translocation, but without being translocated themselves. The existence of distinct binding sites for these two classes of antidepressants on the transporter protein, involving complex interactions between these sites as well as with the substrate site, has also been inferred from the independently carried out pharmacological studies. For the heterocyclic molecules, the surface tension measurements allowed not only to observe a ligand effect on the conformational changes of the transporter, but also to compare this effect for antidepressant drugs such as paroxetine and indalpine. The concentration of indalpine in the subphase had to be 5.6 times higher than that of paroxetine to generate the same surface pressure (Fig. 2). Interestingly, it should be noted that for the 5-HT uptake, a similar ratio is obtained in binding experiments carried out in solution ($[K_{i,indalpine}/K_{i,paroxetine}] = 5$) [4]. The extent of conformational changes in SERT seems, therefore, to be related to the inhibitory effect of the studied drugs.

The triphasic behavior observed with imipramine (increase in surface pressure up to a threshold concentration followed by a π decay) may be explained by the existence of two different affinity states for binding of tricyclic molecules to SERT [16], one of them being strictly Na^+-dependent [17]. It is also interesting to note that when pinoline interacted with SERT, a similar triphasic behavior was observed (Fig. 3). We interpret this finding by the existence of two binding locations for the ligand on the protein, as already suggested by Airaksinen et al. [18]. It may be assumed that a conformational protein change occurs only when each pinoline molecule interacts with two locations. Thus, whereas at low concentrations, pinoline easily interacted with the transporter, at the concentrations above the threshold value (Fig. 3), protein sites appeared to be shielded by accumulated pinoline molecules that hindered further access to SERT. This resulted in the decrease in surface pressure up to a steady state value, an onset of saturation of all binding sites.

All together, our biophysical data ([9] and present report) assert unambiguously direct interactions between the human SERT and various ligands, and may explain the functioning of the transporter in the cell membrane. Indeed, the absence of any detectable conformational change for the two tested carried molecules (5-HT and MDMA) would favor the hypothesis of an electroneutral transport instead of a channel-like mechanism (see [1] for review). The transporter conformation in the cell membrane would naturally allow transport of molecules such as 5-HT or MDMA. Conversely, the Na^+-dependent binding of the other ligands (especially of tricyclic and heterocyclic antidepressants) would induce transporter conformational changes and, thus, inhibit transport.

References

[1] Nelson, N. (1998) J. Neurochem. 71, 1785–1803.
[2] Eshelman, A.J., Carmolli, M., Cumbay, M., Martens, C.R., Neve, K.A. and Janowsky, A. (1999) J. Pharmacol. Exp. Ther. 289, 877–885.
[3] Penado, K.M.Y., Rudnick, G. and Stephan, M.M. (1998) J. Biol. Chem. 273, 28098–28106.
[4] Launay, J.M., Geoffroy, C., Mutel, V., Buckle, M., Cesura, A., Alouf, J.E. and Da Prada, M. (1992) J. Biol. Chem. 267, 11344–11351.
[5] Plenge, P., Mellerup, E.T. and Laursen, H. (1991) Eur. J. Pharmacol. 206, 243–250.
[6] Pähkla, R., Rägo, L., Callaway, J.C. and Airaksinen, M.M. (1997) Pharmacol. Toxicol. 80, 122–126.
[7] Bengel, D., Murphy, D.L., Andrews, A.N., Wichems, C.H., Feltner, D., Heils, A., Mössner, R., Westphal, H. and Lesch, K.P. (1998) Mol. Pharmacol. 53, 649–655.
[8] Rosilio, V., Boissonnade, M.M., Zhang, J., Jiang, L. and Baszkin, A. (1997) Langmuir 13, 4669–4675.
[9] Dalençon, F., Rosilio, V., Manivet, P., Launay, J.M. and Baszkin, A. (1997) Colloids Surf. B 9, 197–203.
[10] Graham, D.E. and Phillips, M.C. (1979) J. Colloid Interface Sci. 70, 403–414.
[11] Birdi, K.S. (1989) in: Lipid and Biopolymer Monolayers at Interfaces (Birdi, K.S., Ed.), p. 325, Plenum Press, New York.
[12] Ramamoorthy, S. and Blakely, R.D. (1999) Science 285, 763–766.

[13] Tronin, A., Dubrovsky, T., Dubrovskaya, S., Radicchi, G. and Nicolini, C. (1996) Langmuir 12, 3272–3275.
[14] Rudnick, G. and Wall, S.C. (1993) Mol. Pharmacol. 43, 271–276.
[15] Agnel, M., Esnaud, H., Langer, S.Z. and Graham, D. (1996) Biochem. Pharmacol. 51, 1145–1151.
[16] O'Riordan, C., Phillips, O.M. and Williams, D.C. (1990) J. Neurochem. 54, 1275–1280.
[17] Jess, U., Betz, H. and Schloss, P. (1996) FEBS Lett. 394, 44–46.
[18] Airaksinen, M.M., Callaway, J.C., Nykvist, P., Rägo, L., Kari, E. and Gynther, J. (1993) in: Melatonin and the Pineal Gland: from Basic Science to Clinical Application (Touitou, Y., Arendt, J. and Pevet, P., Eds.), pp. 83–86, Excerpta Medica, Amsterdam.