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Expression and functional characterization of a melatonin-sensitive receptor in *Xenopus* oocytes**

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Abstract Melatonin (MEL) plays a central role in the regulation of seasonal cycles and in the control of circadian rhythms in mammals. Functional MEL-sensitive receptors were expressed in *Xenopus laevis* oocytes following injection of poly (A)$^+$ RNA from rat brain. Administration of 0.1–100 μmol/l MEL to voltage-clamped oocytes (holding potential: −70 mV) elicited oscillatory inward currents (reversal potential: −24 mV) which could be blocked by 9-anthracenecarboxylic acid and caffeine. After preincubation with pertussis toxin (PTX) the MEL response disappeared. The expressed MEL-sensitive receptor probably activates Ca^{2+}-dependent chloride currents via a PTX-sensitive G protein and the phosphoinositol pathway.

Key words: Melatonin; *Xenopus* oocyte; Ca^{2+}-activated chloride current; G protein; IP_3; *Xenopus laevis*

1. Introduction

Melatonin (MEL) plays a central role in the regulation of seasonal cycles and in the control of circadian rhythms in mammals [1]. The distribution pattern of MEL receptors in the central nervous system of different species has been studied intensively over the last few years [2]. MEL binding sites were found in the pars tuberalis and the nucleus suprachiasmaticus in all tested mammals including humans [3]. Additional binding sites for MEL were detected in the parietal cortex of the rabbit and in the hippocampus of the bovine, ovine, rabbit and several primates [4–7]. In humans, MEL receptors were additionally found in the cerebellum and in the frontal cortex [8,9].

The MEL receptors are functionally coupled to G-proteins which inhibit the activity of the adenylyl cyclase [10]. The activation of MEL receptors causes a reduction of the forskolin-stimulated cAMP level in the cells [2,7]. Recently cloned MEL receptors from *Xenopus laevis* melanophores and from mammalian pars tuberalis and hypothalamus of different mammals also inhibit, after their stable expression in a mammalian cell line, the forskolin-induced cAMP elevation [11].

There are several hints indicating that MEL binds to receptors with different structural and pharmacological properties. Barrett et al. [12] isolated a solubilized Mel receptor/G-protein complex which was structurally different from the pars tuberalis solubilized complex. Furthermore, it has been shown that MEL is linked to the phosphoinositol second messenger pathway [13].

The majority of the investigations are based on data from binding studies and molecular cloning experiments. There are, however, only a few electrophysiological studies that have investigated the way in which MEL affects neuronal cells [7]. Studies on the nature of the pharmacological and electrophysiological activity of MEL in nervous tissue are hampered by the complexity of the central nervous system. For this reason we investigated by electrophysiological techniques the action of MEL-sensitive receptors on *Xenopus* oocytes, previously microinjected with mRNA from rat brain. This expression system allows the analysis at the level of single cells under controlled condition.

2. Materials and methods

2.1. Materials

Melatonin, forskolin and caffeine were purchased from Sigma and 9-AC from Merck. 2-[^{125}J]iodomelatonin (1600–2000 Ci) was obtained from Amersham (Germany). PTX was a gift from Prof. A. Schmidt, Westfälische Wilhelms-Universität, Institut für Infektiologie, Münster.

2.2. Autoradiography

To determine the pattern of melatonin binding sites, brains of adult rats were rapidly removed, frozen in isopentan on dry ice at −30°C. Sections (20 μm) were cut on a cryostat at −20°C and stored at −80°C until processed for autoradiography as described previously [14]. Briefly, sections were preincubated in Tris-BSA buffer (50 mmol/l Tris-HCl, 4 mmol/l $CaCl_2$, 0.05% BSA, pH 7.4 at 25°C) and then incubated in the same buffer containing 100–120 pmol/l 2-[^{125}J]iodomelatonin in the presence or absence of 0.1 μmol/l unlabeled MEL. After 90 min sections were washed in Tris-BSA buffer and Tris buffer without BSA at 4°C for 15 min and dipped in ice cold distilled water for 15 s. Afterwards they were air dried and exposed for 15 days to a ^{3}H-film. Films were developed according to the instruction of the manufacture (Amersham). Adjacent sections were stained with cresyl violet and examined by light microscopy for morphological correlation of binding sites.

2.3. Expression system

Total RNA was isolated from total brain of adult rats using a guanidine/LiCl method [15]. Poly(A)$^+$ RNA was prepared by column chromatography on oligo-(dT) cellulose according to the manufacturer's instructions (Pharmacia). The poly(A)$^+$ RNA was dissolved in distilled water at a concentration of 1 mg/ml and stored at −20°C until injection. Small parts of the ovary were taken from adult

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Abbreviations: MEL, melatonin; G protein, guanyl nucleotide binding protein; cAMP, cyclic adenosine 5′-monophosphate; PTX, pertussis toxin; IP_3, inositol 1,4,5-triphosphate; DMSO, dimethyl sulfoxide; 9-AC, 9-anthracenecarboxylic acid; Tris, Tris(hydroxymethyl)aminomethane; BSA, bovine serum albumine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane sulfonic acid.

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Xenopus laevis under anaesthesia (5 mmol/l ethyl *m*-aminobenzoate; Sandoz). Stage V or VI oocytes [16] were manually dissected and each injected with 50 ng poly(A)$^+$ RNA. The injected oocytes were maintained under tissue culture conditions in a modified Barth medium composed of (in mmol/l): NaCl 88, $CaCl_2$ 1.5, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.8, HEPES 5; pH 7.4; temperature 20°C. The medium was supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml). For some investigations follicular cells surrounding the oocytes were removed by manual dissection with forceps.

2.4. Electrophysiology

The electrophysiological investigations were performed from day 3 to day 7 after injection of poly(A)$^+$ RNA. Membrane currents were measured using a standard two microelectrode voltage clamp technique. The current and voltage electrodes were filled with 2 mol/l KCl (resistances: 1 to 2 MΩ). Unless otherwise noted, membrane currents were measured at a holding potential of −70 mV. The control bath fluid was a salt solution composed of (in mmol/l): NaCl 115, KCl 2, $CaCl_2$ 1.8, HEPES 10; pH 7.2. Forskolin (1 μmol/l), 9-AC (2 mmol/l) and caffeine (1 mmol/l) were dissolved in the control bath solution. 10 mmol/l MEL was dissolved in DMSO and this stock solution was diluted immediately prior to experiments with the control bath solution to give final concentrations of MEL of 0.1–100 μmol/l (DMSO concentration: 0.0001%–0.1%). The solutions were administered extracellularly to the oocytes using a concentration clamp technique [17]. To test the effects of PTX on the MEL response, mRNA-injected oocytes, tested for MEL sensitivity, were incubated for 17 h in a modified Barth medium in which PTX was dissolved at a concentration of 5 μg/ml.

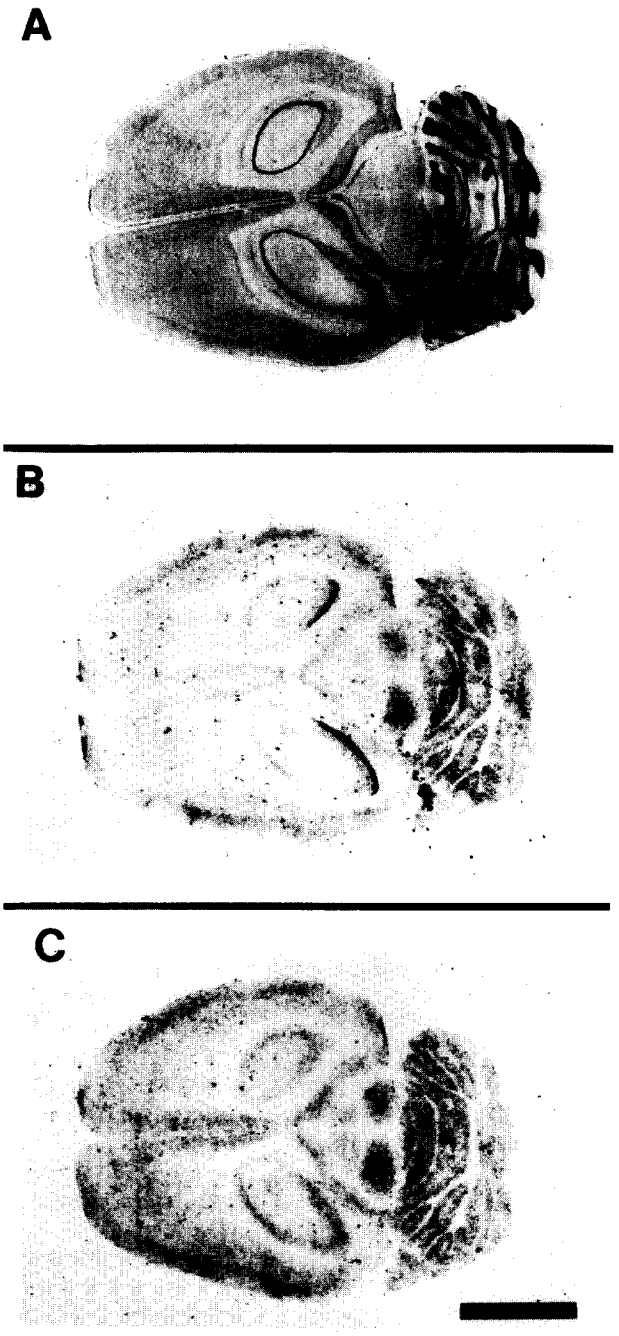

Fig. 1. Autoradiography of 2-[^{125}J]iodomelatonin binding sites in the rat brain. A: Cresyl violet stained horizontal section. B: Specific binding with 2-[^{125}J]iodomelatonin. C: Unspecific binding in the presence of 0.1 mol/l unlabelled MEL. Bar: 5 mm.

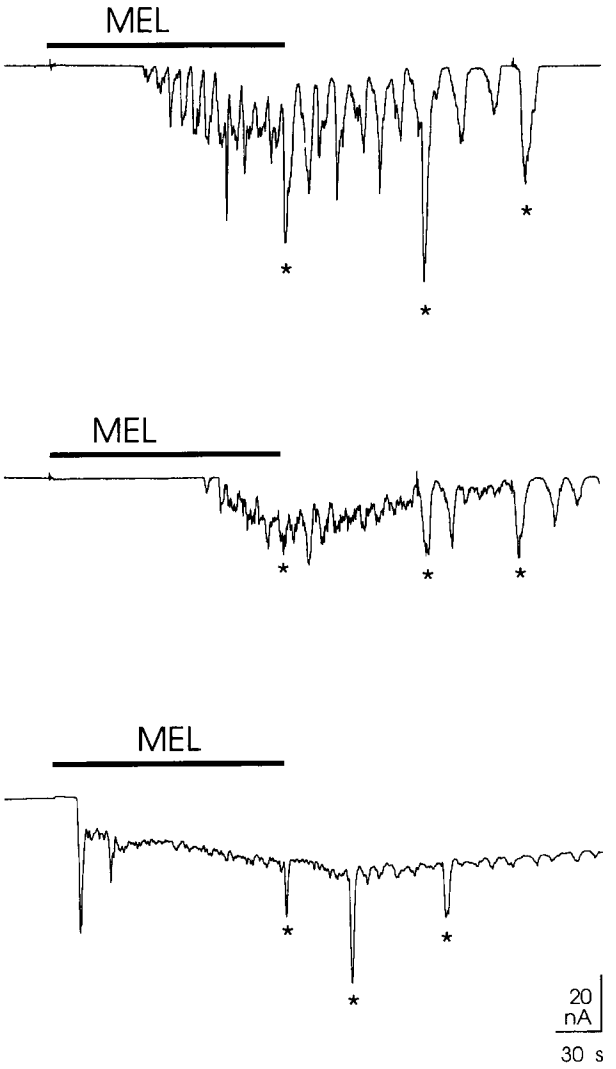

Fig. 2. Membrane currents of three different oocytes (*Xenopus laevis*) elicited by administration of 10 μmol/l MEL. Recordings 4 days after injection of poly (A)$^+$-RNA from rat brain. Administration is marked by horizontal bars. Holding potential: −70 mV. Inward current: downward deflection. Asterisk: stream evoked inward current [21].

3. Results

3.1. Pattern of MEL binding sites in the rat brain

The distribution of specific [^{125}I]MEL binding sites was re-

stricted to a few sites in the rat brain. Intense labelling was observed only in the pars tuberalis and in the suprachiasmatic nuclei (data not shown) and additionally in the hippocampus (Fig. 1). Although the resolution of the autoradiography may not be sufficient to detect the MEL binding sites on the cellular level, the localization of the binding sites in the hippocampus seems to be restricted to the subicular region. The nonspecific binding was near the background.

3.2. *Expression of melatonin-sensitive receptors in microinjected oocytes*

Oocytes of 20 different donors were injected with rat brain poly $(A)^+$-RNA from 10 different batches. Injection of the RNA into the oocytes led to the functional expression of various ligand operated ion channels. The pharmacological

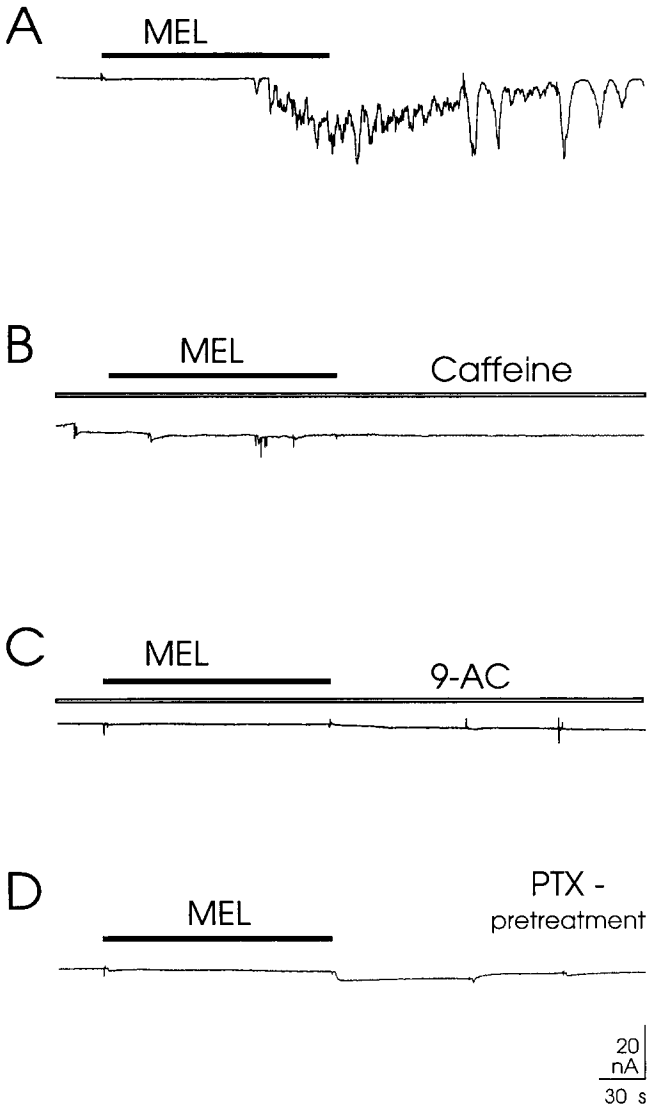

Fig. 3. Membrane currents of a single oocyte (*Xenopus laevis*) elicited by MEL (A; control reaction) and effects of caffeine (B), 9-AC (C) and PTX pretreatment (D) on the MEL response. Recordings 4 days after injection of poly $(A)^+$-RNA from rat brain. Concentration of the substances: MEL 10 μmol/l, caffeine 1 mmol/l, 9-AC 2 mmol/l and PTX 5 μg/ml (incubation time of PTX: 17 h). Administration of substances are marked by horizontal bars. Holding potential: −70 mV. Inward current: downward deflection.

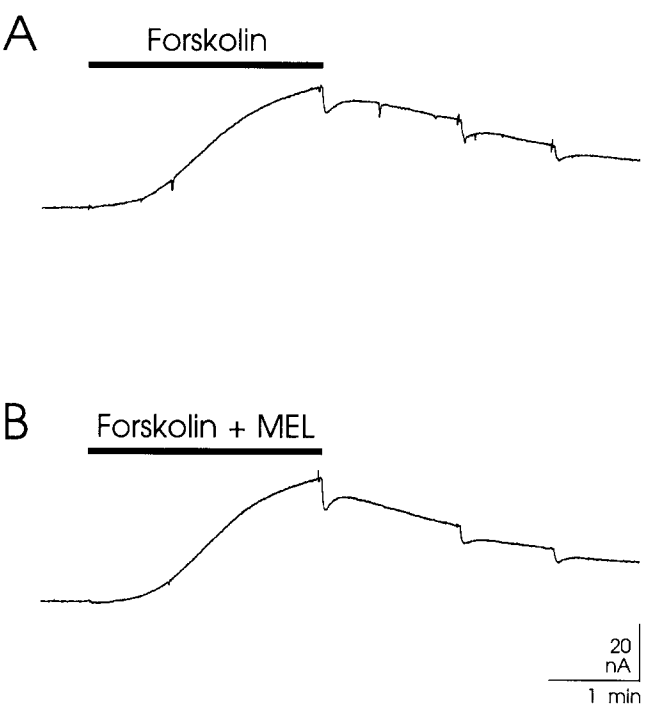

Fig. 4. Membrane currents of a single oocyte (*Xenopus laevis*) elicited by forskolin (A) and interaction of forskolin and MEL with simultaneous administration (B). Recordings 4 days after injection of poly $(A)^+$-RNA from rat brain. Concentration of the ligands: MEL 100 μmol/l, forskolin 1 μmol/l. The oscillatory inward currents of the oocytes evoked by MEL were blocked by 2 mmol/l 9-AC in the background. Administration is marked by horizontal bars. Holding potential: −70 mV. Inward current: downward deflection.

screening demonstrated that the injected oocytes expressed receptors sensitive to the neurotransmitters gamma-aminobutyric acid, glutamate, dopamine and serotonine (data not shown; [18,19]).

The administration of MEL to the RNA-injected oocytes evoked oscillatory inward currents (Fig. 2; $n = 62$), whereas native oocytes or water-injected oocytes did not respond to either MEL or other transmitters ($n = 30$). The time course of expression of MEL-sensitive receptors in oocytes following mRNA injection was similar to that observed for other receptors [20]. Maximum reponses occurred between 3 and 5 days following mRNA injection. Original recordings from three RNA-injected oocytes are shown in Fig. 2. The delay between application of MEL and onset of the current response showed some degree of variability. In most cases the MEL response occurred within 20 to 30 s, but in some cells it took more than 60 s before the current oscillations started to rise. The oscillations persisted for several minutes, sometimes for more than 1 h, regardless of whether the MEL application continued or had been stopped. With wash-out of MEL or control solution during current oscillations the so called 'stream evoked' inward current appeared (asterisk in Fig. 2; [21]). The response to MEL was dose dependent. The minimum concentration needed to evoke MEL responses was 0.1 μmol/l, maximum responses appeared with applications of 100 μmol/l MEL. The MEL response persisted even after removal of the outer follicular layers ($n = 5$). The response was not caused by the solvent DMSO since application of DMSO (0.1%) alone did not elicit membrane currents ($n = 18$).

3.3. Electrophysiological characterization of the MEL-induced inward currents

The reversal potential of the MEL-induced oscillatory inward currents was found to be −24 mV ($n=6$) which corresponds to the equilibrium potential for Cl^- ions in oocytes [22]. The oscillatory current could be based on the Ca^{2+}-activated Cl^- currents that can be evoked in *Xenopus* oocytes via the phosphoinositol-dependent second messenger pathway [22]. To test for this, the chloride channel blocking substance 9-AC and caffeine, which inhibits IP_3-mediated release of intracellular calcium [23], were applied before, during and after MEL application. As shown in Fig. 3, both substances completely blocked the MEL response (Fig. 3B,C; $n=3$). With preincubation of mRNA-injected oocytes in a Barth medium containing PTX the MEL response disappeared (Fig. 3D; $n=12$).

3.4. Effects of MEL on the cyclic AMP pathway

It has been reported that in the cells of the pars tuberalis MEL-sensitive receptors are functionally coupled to a G protein, which causes a decrease in the cyclic AMP level [2,7,10]. To test whether the expressed MEL-sensitive receptors are additionally able to influence the cyclic AMP level in oocytes, the effects of MEL on the action of forskolin, an unspecific activator of the adenylyl cyclase, was tested. The application of forskolin to mRNA-injected oocytes evoked a slow onset, smooth and long lasting outward current (Fig. 4A; $n=6$). The current has a reversal potential of about −105 mV ($n=3$) which is close to the equilibrium potential of potassium ions in oocytes [22]. These data are in line with investigations of Greenfield et al. performed on oocytes, demonstrating that forskolin increased the potassium conductance via elevation of the cyclic AMP level [24]. The effect of MEL on the forskolin-induced response was tested by simultaneous application of both MEL and forskolin. The oscillatory inward currents evoked by MEL were blocked by continuous administration of 2 mmol/l 9-AC. There was no effect of 9-AC on the forskolin-induced outward current ($n=4$). The amplitude of the forskolin responses (17 ± 4 nA; mean ± S.E.M.) was not significantly changed with simultaneous application of 100 μmol/l MEL (18 ± 5 nA; $n=6$; Fig. 4B).

4. Discussion

The present experiments demonstrate that injection of mRNA from rat brain into the heterologous *Xenopus* oocyte expression system results in the functional expression of a receptor sensitive to MEL. This MEL-sensitive receptor was determined to be exogenous since native oocytes or water-injected oocytes were non responsive to MEL. Defolliculated mRNA-injected oocytes maintained their sensitivity to MEL, indicating that the MEL-sensitive receptor is integrated into the plasma membrane of the oocyte. This is further supported by the fact that follicle cells loss their functional integrity after a cultivation period of more than 3 days [25].

The MEL receptors are coupled to G proteins and their activation often leads to the inhibition of the adenylyl cyclase, resulting in a decrease in the cAMP level of the cells [2,7,10]. Recently it has been reported that MEL is also able to influence the phosphoinositide breakdown in chicken brain slices [13]. Our experiments showed that the MEL-sensitive receptor expressed in oocytes did not inhibit the increase in the cAMP level induced by 1 μmol/l forskolin. The expressed receptor is obviously not coupled to an inhibitory G protein which reduces the activity of the adenylyl cyclase. The current responses to application of MEL may be interpreted as to be due to the activation of a PTX-sensitive G protein eliciting a Ca^{2+}-dependent Cl^- current. This hypothesis is supported by the findings that (i) the MEL response disappeared after PTX pretreatment of the oocytes, (ii) the current disappeared after incubation with caffeine, which inhibits IP_3-mediated release of intracellular calcium, (iii) the equilibrium potential of the current was near to that of chloride, and (iv) the current was abolished after application of the chloride channel blocker 9-AC. Therefore, the expressed MEL-sensitive receptor may represent a receptor type which is obviously linked to G proteins activating the phospholipase C and subsequently the phosphoinositol second messenger pathway of the cells. This receptor type may belong to the putative ML_2 subtype proposed by Popova and Dubokovich [13].

To our knowledge there is only one report in literature using *Xenopus* oocytes to express MEL receptors. Fraser et al. [26] injected mRNA from the pars tuberalis of bovine pituitary into oocytes and reported that MEL reduced AlF_4-induced current oscillations. This was shown for the RNA-injected oocytes as well as for native oocytes. However, the described effects were observed at a concentration of 1 mmol/l MEL. In the present experiments the expressed MEL-sensitive receptors derived from RNA of total rat brain are able to induce oscillatory current responses in a concentration range of MEL which is near to the physiological concentration measured in mammals. The current response could be evoked at a minimum concentration of 100 nmol/l MEL, whereas the physiological concentration in mammals is in the range of 1 nmol/l [1].

The higher concentration of MEL needed to evoke detectable ion currents in *Xenopus* oocytes may be due to the fact that the relative amount of specific mRNA and, as a consequence, the amount of functional MEL receptors expressed in oocytes are low. This is in line with the fact that specific [^{125}I]MEL binding sites in the rat brain, as examined in adjacent sections by in vitro autoradiography, are restricted to a few sites (Fig. 1; see also [2]).

The oocyte expression system is found to be a suitable tool to investigate new agonists and antagonists for the MEL-sensitive receptor and its specific subtype presented here. Furthermore, this expression system can be used as part of a cloning strategy to identify and isolate the cDNA for this MEL-sensitive subreceptors.

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