# Influence of melatonin on the development of functional nicotinic acetylcholine receptors in cultured chick retinal cells

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## Abstract

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The influence of melatonin on the developmental pattern of functional nicotinic acetylcholine receptors was investigated in embryonic 8-day-old chick retinal cells in culture. The functional response to acetylcholine was measured in cultured retina cells by microphysiometry. The maximal functional response to acetylcholine increased 2.7 times between the 4th and 5th day *in vitro* (DIV4, DIV5), while the Bmax value for $[^{125}I]$-$\alpha$-bungarotoxin was reduced. Despite the presence of $\alpha$8-like immunoreactivity at DIV4, functional responses mediated by $\alpha$-bungarotoxin-sensitive nicotinic acetylcholine receptors were observed only at DIV5. Mecamylamine (100 µM) was essentially without effect at DIV4 and DIV5, while dihydro-ß-erythroidine (10-100 µM) blocked the response to acetylcholine (3.0 nM-2.0 µM) only at DIV4, with no effect at DIV5. Inhibition of melatonin receptors with the antagonist luzindole, or melatonin synthesis by stimulation of D4 dopamine receptors blocked the appearance of the $\alpha$-bungarotoxin-sensitive response at DIV5. Therefore, $\alpha$-bungarotoxin-sensitive receptors were expressed in retinal cells as early as at DIV4, but they reacted to acetylcholine only after DIV5. The development of an $\alpha$-bungarotoxin-sensitive response is dependent on the production of melatonin by the retinal culture. Melatonin, which is produced in a tonic manner by this culture, and is a key hormone in the temporal organization of vertebrates, also potentiates responses mediated by $\alpha$-bungarotoxin-sensitive receptors in rat vas deferens and cerebellum. This common pattern of action on different cell models that express $\alpha$-bungarotoxin-sensitive receptors probably reflects a more general mechanism of regulation of these receptors.

## Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed pentameric transmembrane proteins acting as key molecules in cholinergic transmission at synapses, including the retina (1), where they play an important role in synaptogenesis

(2). They are composed of combinations of subunits encoded by at least 12 different genes, and two main classes have been identified: the α-bungarotoxin-sensitive receptors made of α7, α8, α9, or α10 subunits, which can form homomeric or heteromeric receptors, and the α-bungarotoxin-insensitive receptors made of α2-α6 and ß2-ß4 subunits, which only form heteromeric receptors (3).

The α-bungarotoxin-sensitive nAChRs present in chick retina are composed of α7 and/or α8 subunits (1), whereas the α-bungarotoxin-insensitive channels in the chick retina include a variety of subtypes composed of α2, α3, α4, or α6 and ß2, ß3 or ß4 subunits, in the presence or absence of α5 or ß3 subunits (4). Chick amacrine and ganglion cells contain α3, α7, α8, ß2, and ß3 subunits (5,6), whereas bipolar cells appear to express only the α8 subunit (6). Some subunits (α3 and α8) can be detected as early as on embryonic day 5 (7), well before the appearance of choline acetyltransferase, the acetylcholine-synthesizing enzyme, which appears around day 6.5 (8).

The light-dark cycle modulates the function of many tissues, including central and peripheral cholinergic synapses. The number and the response of nAChRs located on sympathetic nerve terminals of rat vas deferens (9) present a diurnal rhythm (10). Nicotine administration affects rat locomotor activity during the day but not during the night, and the density of $[^{125}I]$-α-bungarotoxin binding sites in rat hypothalamus is lower at the end of a 12-h dark period than during the light period (9). Acetylcholine-induced release of neurotransmitter from sympathetic nerve terminals of the vas deferens and cerebellum slices of the rat is higher in the dark phase than in the light phase of the day. In both cases the effect of acetylcholine is mediated by nAChRs sensitive to α-bungarotoxin (11,12). Melatonin, the hormone released by the pineal gland during the dark phase of the day, is responsible for the

diurnal variation of nAChR-mediated responses. This rhythm is abolished in animals maintained under conditions of constant lighting (13), and in animals treated with propranolol (11), conditions which impair the nocturnal surge of melatonin. Melatonin replacement restores the response to cholinergic agonists in both cases.

Cultured cells from embryonic chick retina have been used to study neurodevelopment (14) and melatonin production by photoreceptors (15). Melatonin has a paracrine function in the retina and is produced by photoreceptors since embryonic day 7. In the early stages melatonin is synthesized tonically; the rhythmic synthesis of melatonin only starts at day 20 (16). Light-induced inhibition of melatonin biosynthesis is mediated by light-induced release of dopamine, which acts on D4 dopamine receptors expressed on the membrane of cells that synthesize melatonin (14). Accordingly, melatonin synthesis by photoreceptors is inhibited by quinpirole, a D4 dopamine receptor agonist. On the other hand, melatonin, by acting on MT2 melatonin receptors, blocks dopamine synthesis. The mutual antagonism between melatonin and dopamine is observed *in vivo* and in cultured retina.

In the present study, the influence of melatonin on the developmental pattern of functional nAChRs was investigated in a culture of embryonic chick retinal cells in which the synthesis of melatonin is not rhythmic (15,17). The functional response of nAChRs was determined by microphysiometry, (18). Firstly, the activity of α-bungarotoxin-sensitive and non-sensitive nAChRs was pharmacologically characterized and then the effect of melatonin on the nAChR-mediated response was tested.

## Material and Methods

### Culture of chick retinal cells

Fertilized chicken eggs were obtained from a local hatchery and incubated at 38ºC

in a humidified atmosphere. Primary cultures of retinal cells were prepared at embryonic day 8 as described (19). Briefly, retinas were dissected and treated with trypsin and the cells dissociated in Dulbecco's modified Eagle's medium (DMEM). The cells were plated ($1.76 \times 10^4$ cells/mm$^2$) and incubated in DMEM containing 5% fetal calf serum, 80 U/ml penicillin and 80 µg/ml streptomycin at 37ºC for 4 to 8 days in an atmosphere of 5% $CO_2$ plus air. The medium was replaced every other day. The cultures were tested after 4 (DIV4), 5 (DIV5), 6 (DIV6) or 8 (DIV8) days *in vitro*.

**Immunocytochemistry**

At DIV4 and DIV6, retinal cells, plated onto chamber slide systems (Lab Tek, Christchurch, Caterbury, New Zealand), were fixed for 10 min with 2% paraformaldehyde in 0.1 M sodium phosphate-buffered saline (PBS), pH 7.4. Cells were incubated overnight with 10 nM of a monoclonal antibody against the α3 or α8 subunit (mAb 305 and mAb 315, respectively, a kind gift by Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA, USA). The secondary antibody was an anti-rat IG antibody tagged with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate that was diluted 200 times. All antibodies were diluted in 0.3% Triton-X 100 in PBS. No immunoreactivity was observed after elimination of the primary antibodies or after incubation with normal rat serum.

**Microphysiometry**

Retinal cells, seeded onto 12-mm transwell inserts (Costar, Corning, NY, USA), and incubated for 4, 5, 6, or 8 days at 37°C in 5% $CO_2$, were loaded into the sensor chambers of the Cytosensor microphysiometer (Molecular Devices, Palo Alto, CA, USA), a silicon-based biosensor system which continuously monitors the extracellular pH sur-

rounding cells in culture, and reports receptor activation by measuring increases in extracellular acidification rate occurring in response to agonist stimulation (18). The chambers were perfused at a rate of 100 µl/min with bicarbonate-free DMEM (sodium bicarbonate replaced by 44 mM sodium chloride, 37°C, pH 7.4). Each measurement cycle consisted of a 2-min period, 110 s of pump on and 10 s of pump off (Figure 1). The rate of chamber acidification was determined as the slope of a linear least-square fit to the pH-time data and normalized to its baseline using the Cytosoft program (Molecular Devices).

Cells were exposed to acetylcholine for 14 s. The pump was on for 4 s, allowing the entry of the drug into the chamber, and off for the last 10 s, and the slope of the acidification rate was calculated. Responses to increasing concentrations of acetylcholine were measured after an equilibration period of 50 min. It is interesting to note that the acidification rate of retina cells was constant in the absence of the agonist. A 40-min washout period between exposures to acetylcholine was allowed to elapse in order to avoid desensitization. Atropine (1 µM) was perfused during the entire experiment in order to block muscarinic receptors.

The following nAChR antagonists were used: α-bungarotoxin, mecamylamine and dihydro-ß-erythroidine (DHßE). Alpha-bungarotoxin was incubated for 60 min before the addition of acetylcholine. Mecamylamine (100 µM) was applied during the entire experiment, and a control was run in parallel. In the case of DHßE a complete concentration-response curve for the antagonist was obtained. After measuring the response to 250 µM acetylcholine in the absence of the antagonist, a series of successive 40-min incubations with increasing concentration of DHßE (10-100 µM) was performed and the response to 250 µM acetylcholine was measured again.

When appropriate, the cultures were pre-

treated with the antagonist of melatonin receptors luzindole or the D4 dopamine receptor agonist quinpirole for 48 h, at the concentrations specified in Results.

### Binding assays

$[^{125}I]$-Tyr$^{54}$-$\alpha$-bungarotoxin ($[^{125}I]$-$\alpha$-bungarotoxin) binding was conducted in intact cells at DIV4 and DIV5. $[^{125}I]$-$\alpha$-bungarotoxin was incubated for 1 h in DMEM (37ºC, pH 7.4), and the cells were then washed with cold buffer, left overnight in 0.1 M NaOH and counted for radioactivity in a Packard 2300 TR liquid scintillation spectrophotometer (Springfield, IL, USA). In association assays $[^{125}I]$-$\alpha$-bungarotoxin (4-10 nM) was incubated for 2.5-120 min, and equilibrium was reached at 60 min both on DIV4 and DIV5. Therefore, 60 min was the time chosen for saturation studies. Specific binding was calculated as the difference in binding obtained in the absence and presence of 1 mM (-)-nicotine. Nonspecific binding accounts for 20-50% of total binding.

### Protein assay

Protein content was estimated by the method of Bradford (20) using bovine serum albumin as the standard.

### Drugs and chemicals

Acetylcholine bromide, $\alpha$-bungarotoxin, 2-benzyl-N-acetyltryptamine (luzindole), (-)-nicotine hydrogen tartrate, trypsin, mecamylamine hydrochloride, quinpirole, and DHßE were purchased from Research Biochemicals International (Natick, MA, USA). Fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate were supplied by Jackson Laboratories (West Grove, PA, USA). Atropine was purchased from Merck (Rio de Janeiro, RJ, Brazil), and $[^{125}I]$-$\alpha$-bungarotoxin (138-146 Ci/mmol) from NEN™ Life Science Products, Inc. (Boston,

MA, USA). Gibco-BRL (Grand Island, NY, USA) supplied DMEM and penicillin-streptomycin.

### Statistical analysis

Concentration-response curves and binding saturation curves were fitted by nonlinear equations (Graph-Pad software package; GraphPad, San Diego, CA, USA). The pD2 values reported correspond to the negative logarithm of the $EC_{50}$ (effective concentration that induced half-maximal increase in extracellular acidification rate). The pD2 values were calculated for each dose-response curve, and the values are reported as the mean ± SEM of the number of cultures.

For binding assays, the maximal number of binding site (Bmax) and the apparent equilibrium dissociation constant (Kd) values, reported as means ± SEM, were calculated from three independent assays by fitting non-linear saturation curves for one binding site model. Data were analyzed statistically by the Student $t$-test or ANOVA followed by the Newman-Keuls test, when appropriate. Values of $P < 0.05$ were considered statistically significant. All analyses were performed using the Graph-Pad software package (GraphPad).

## Results

### $\alpha$3- and $\alpha$8-like immunoreactivity

Confocal microscopy confirmed the presence of $\alpha$3- and $\alpha$8-like immunoreactivity in cultured retinal cells (Figure 1). These subunits seemed to be distributed throughout the cell, with $\alpha$3 labeling especially pronounced in the perikarya and proximal dendrites, and $\alpha$8 preferentially labeling processes and varicosities. Both subunits were detected in DIV4 and DIV6 cultures, indicating that no developmental stage-related change had occurred in the cellular distribution of $\alpha$3- and $\alpha$8-like immunoreactivity.

**Chick retinal cell culture development and the response to acetylcholine**

Acetylcholine (30 μM-2 mM) induced a concentration-dependent increase in extracellular acidification rate in retinal cells after DIV4, DIV5 and DIV6. The maximal increase in extracellular acidification rate was significantly higher ($P < 0.001$) at DIV5 and DIV6 when compared to DIV4 and DIV8 (Figure 2). On the other hand, the apparent affinity was not dependent on the number of days *in vitro*, since the pD2 values for DIV4 ($3.40 \pm 0.05$, N = 7), DIV5 ($3.57 \pm 0.10$, N = 11) and DIV6 ($3.44 \pm 0.05$, N = 7) were not significantly different ($P > 0.05$).

In order to determine which receptor subtype mediates the acetylcholine-induced response, selective nAChR antagonists were used. Mecamylamine, a non-competitive antagonist of heteromeric channels, had no effect on DIV5 cultures (N = 3; data not shown), and only a very high concentration of mecamylamine (100 μM) caused a small reduction ($15 \pm 5\%$, $P < 0.05$, N = 4) in the response to 2 mM acetylcholine in DIV4 cultures (data not shown).

DHßE, an antagonist that blocks heteromeric receptors in chick retina (21), inhibited in a concentration-dependent manner (10-100 μM) the acetylcholine-induced response at DIV4 (Figure 3; $IC_{50}$ = 16.9 μM). The response at DIV5 was not sensitive to DHßE (Figure 3).

Alpha-bungarotoxin, an antagonist that blocks receptors containing α7 or α8 subunits, presented a different profile. In DIV4 cultures, 10 nM α-bungarotoxin did not modify either the maximal response to or the apparent affinity for acetylcholine (control: pD2 = $3.40 \pm 0.05$; α-bungarotoxin: pD2 = $3.40 \pm 0.20$), but in DIV5 cultures α-bungarotoxin caused a sharp reduction in the response to acetylcholine (Figure 4), and no change in pD2 values (control: pD2 = $3.4 \pm 0.05$; α-bungarotoxin: pD2 = $3.4 \pm 0.20$). The concentration-response curve at DIV5

in the presence of α-bungarotoxin was similar to the response at DIV4 in the presence or absence of α-bungarotoxin (Figure 4).

**[$^{125}$I]-α-bungarotoxin binding sites**

[$^{125}$I]-α-bungarotoxin binding to DIV4 and DIV5 intact cells was saturable and fitted a curve modeling one binding site. Bmax and Kd were higher at DIV4 (Bmax = $233.10 \pm 31.29$ fmol/mg protein; Kd = $17.74 \pm 4.46$ nM, N = 4) than at DIV5 (Bmax = $86.94 \pm 8.86$ fmol/mg protein; Kd = $5.90 \pm 1.62$ nM, N = 4; Figure 5).

**The effect of melatonin on the acetylcholine-induced response depends on the developmental stage of cultured retinal cells**

The effect of melatonin on the development of the acetylcholine-induced response in cultured retinal cells was evaluated by blocking melatonin receptors with 100 μM
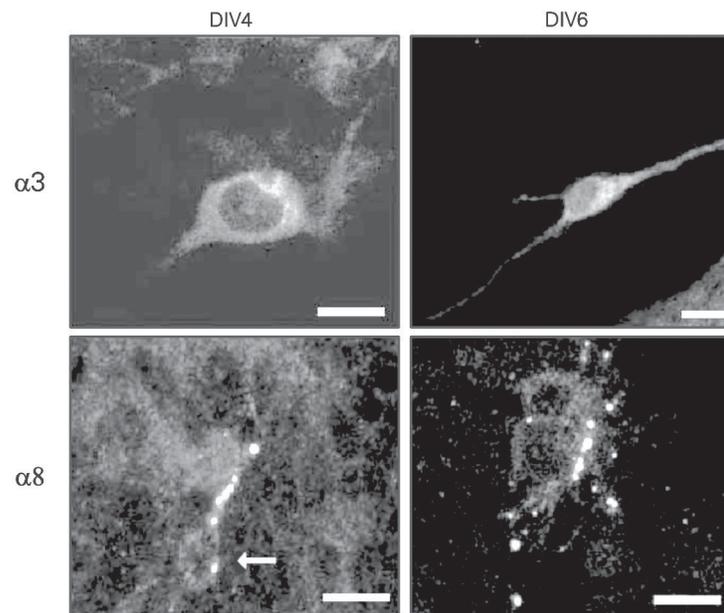


Figure 1. Confocal laser scanning images of retinal cells from 4th and 6th day *in vitro* (DIV4 and DIV6) cultures labeled with antibodies against α3 and α8 subunits, and visualized with tetramethylrhodamine isothiocyanate. α3-like immunoreactivity can be seen in the perykarion and proximal processes, and α8 in a varicose process. The arrow points to the somata of cells labeled with the α8 monoclonal antibody. Scale bar: 10 μm.
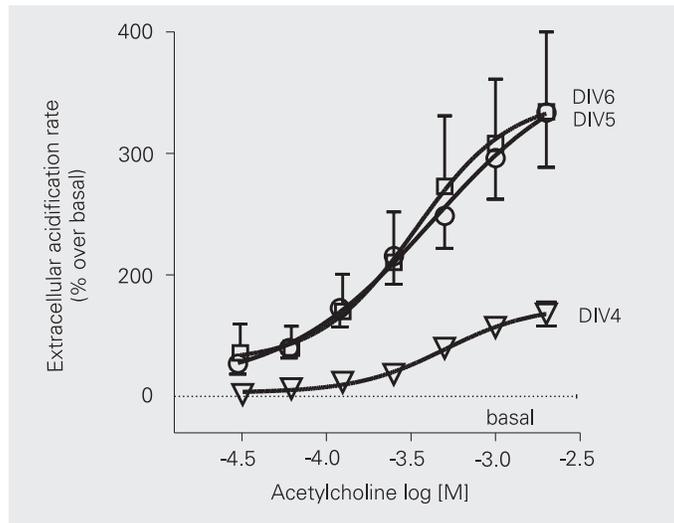
Figure 2. Acetylcholine-induced increase in extracellular acidification rate of retinal cells isolated from 8-day-old embryos and cultured for 4 (DIV4, triangles), 5 (DIV5, circles) and 6 (DIV6, squares) days *in vitro*. Cells (4 x 10^6) were exposed to increasing concentrations of acetylcholine for 10 s. The interval between subsequent concentrations was 40 min in order to avoid desensitization, and only one concentration-response curve was obtained for each well. Data are reported as means ± SEM for 5-13 independent cultures. The curves were fitted according to a sigmoid concentration-response curve.
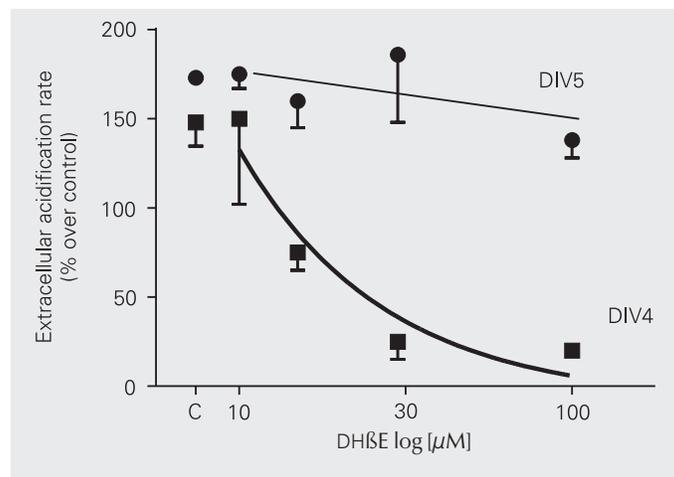


Figure 3. Dihydro-ß-erythroidine (DHßE) blocks the acetylcholine-induced increase in extracellular acidification rate of retinal cells in culture. Retinal cells cultured for 4 (DIV4, squares) or 5 (DIV5, circles) days *in vitro* were incubated in the absence or in the presence of different concentrations of the antagonist. DHßE inhibited the effect of 250 µM acetylcholine at DIV4 ($IC_{50}$ = 16.9 µM) in a concentration-dependent manner but not at DIV5. C = control. Data are reported as means ± SEM for 3-5 independent experiments.
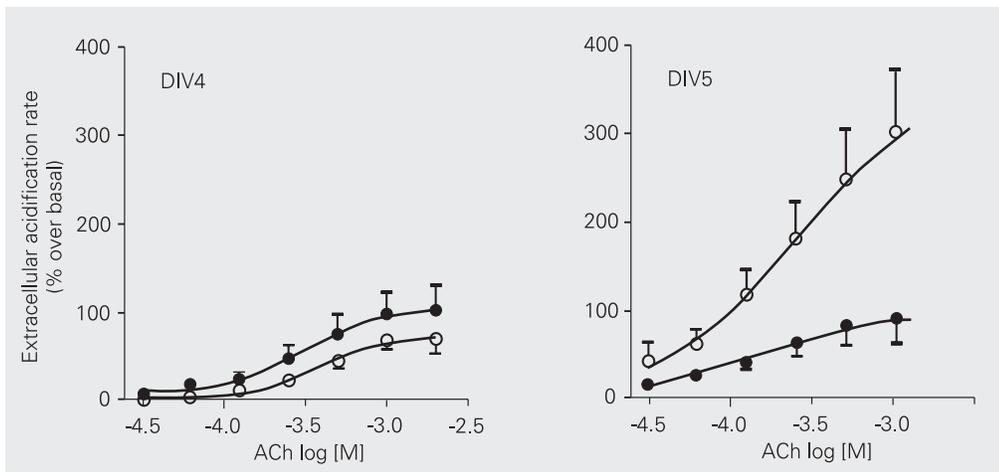


Figure 4. Blockade of acetylcholine-induced increase in extracellular acidification rate of retinal cells in culture by $\alpha$-bungarotoxin. Retinal cells cultured for 4 (DIV4, left panel) or 5 (DIV5, right panel) days *in vitro* were stimulated with acetylcholine (ACh) in the presence (filled symbols) or absence (open symbols) of 10 nM $\alpha$-bungarotoxin and incubated for 1 h. Data are reported as means ± SEM. $\alpha$-Bungarotoxin inhibited the response in DIV5 cultures (N = 4, P < 0.01) but not in DIV4 cultures (N = 5) (two-way ANOVA).

luzindole since DIV2 or by inhibiting melatonin production by stimulating dopamine D4 receptors with 10 µM quinpirole since DIV3.

Pretreatment with luzindole (48 h) reduced the acetylcholine-induced increase in acidification rate at DIV5 from 516 ± 84 to 121 ± 85% (Figure 6B), while no significant difference was observed at DIV4 (63 ± 4.9 to 150 ± 41%; Figure 6A). The number of cells in culture estimated from the protein content was not modified (0.57 ± 0.04 mg/ well in the absence and 0.51 ± 0.05 mg/well in the presence of luzindole). The $pD_2$ values for acetylcholine in retinal cultures pretreated or not with luzindole were not changed either at DIV4 ($pD_2$ value without luzindole = 4.09 ± 0.19; with luzindole = 3.98 ± 0.53) or DIV5 ($pD_2$ value without luzindole = 3.32 ± 0.21; with luzindole = 3.28 ± 0.59). Incubation of the DIV4 retinal cultures with α-bungarotoxin did not modify the maximum response to acetylcholine in the presence or absence of luzindole, while it reduced the magnitude of the response to acetylcholine by DIV5 cells in the absence, but not in the presence, of luzindole pretreatment. The reduction by luzindole in the acetylcholine-induced maximal acidification rate at DIV5 was dependent on the concentration (Figure 6C). In these experiments each well was incubated with one concentration of luzindole for 48 h, and only the effect of 1 mM acetylcholine was tested. The maximal reduction of the acetylcholine-induced response was 86%, and the $IC_{50}$ value for luzindole was 1.05 µM.

The dopamine D4 agonist, quinpirole (10 µM), was added to DIV3 cultures, and the cells were set up for microphysiometric recording at DIV5. The increase in extracellular acidification rate induced by 1 mM acetylcholine was 81.7 ± 0.5% (N = 3) smaller in cultures incubated with quinpirole when compared to cultures that were not incubated with the D4 dopamine receptor agonist. Therefore, quinpirole impairs the increase in the acetylcholine-induced effect that was observed at DIV5.
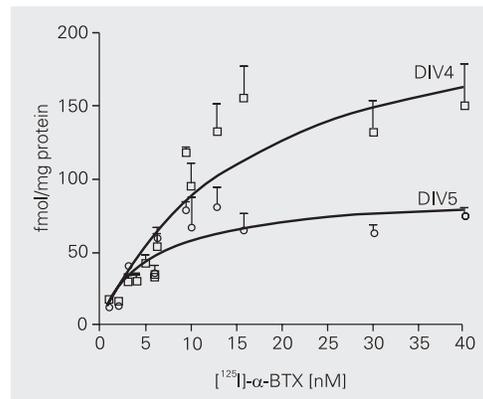


Figure 5. [$^{125}$I]-α-bungarotoxin (BTX) binding saturation curves for retinal cultures. Nonspecific binding was determined with 1 mM nicotine. Four-day (DIV4, squares) and 5-day (DIV5, circles) *in vitro* cultures were incubated for 60 min at 37ºC. Data are reported as means ± SEM for 3 independent experiments.
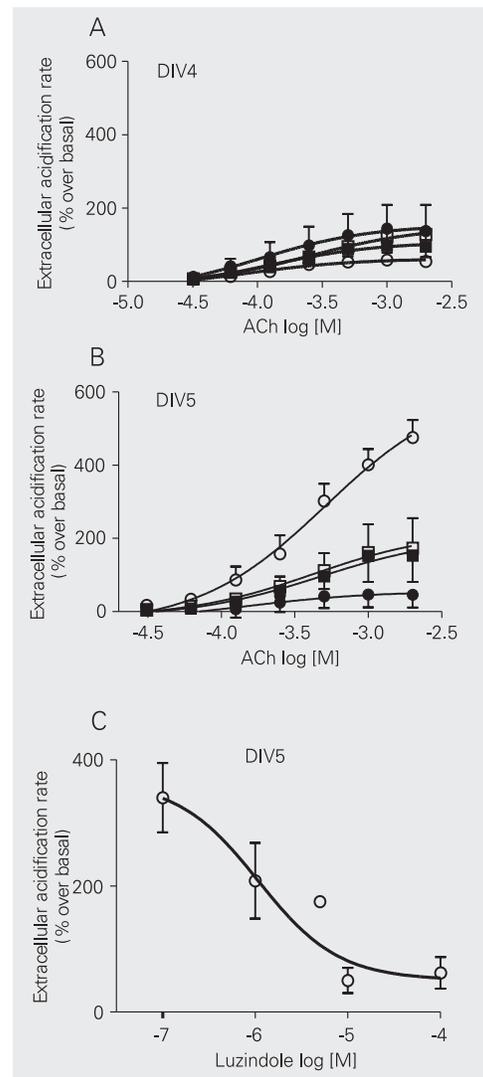


Figure 6. Pretreatment with luzindole (panels A and B - 100 µM; panel C - 0.1 to 100 µM) for 48 h inhibited the α-bungarotoxin-sensitive component of acetylcholine (ACh)-induced increases in extracellular acidification rate by retinal cultured cells. The effects on 4-day (DIV4) and 5-day (DIV5) *in vitro* cultures are shown in *panels A* and *B*, respectively. Open and filled symbols represent the effect in the absence and presence of 10 nM α-bungarotoxin, respectively. Squares and circles indicate the effect in cultures pretreated with 100 µM luzindole and untreated controls, respectively. *Panel C* shows a concentration-response curve for DIV5 cultures pretreated with luzindole (48 h) stimulated with 1 mM acetylcholine ($IC_{50}$ = 1.05 µM). Each culture was pretreated with only one luzindole concentration. Data are reported as means ± SEM (N = 3-5).

## Discussion

The present results demonstrate that, despite the immunostaining of α3- and α8-like subunits observed since DIV4, acetylcholine activated α-bungarotoxin-sensitive nAChRs of retinal cells only after the 5th day of culture. The appearance of the response of α-bungarotoxin-sensitive nAChRs was accompanied by a reduction in the number of [$^{125}$I]-α-bungarotoxin binding sites. Melatonin produced by cultured retinal cells is essential for the appearance of the α-bungarotoxin-sensitive response. Inhibition of both retinal melatonin receptors and melatonin production impaired the development of the α-bungarotoxin-sensitive response.

### Pharmacological characterization of functional nAChRs in retinal culture

The functional response of nAChRs to stimulation was measured by microphysiometry, a method that has been validated for several receptor-transduction pathways involving muscarinic (22), noradrenaline, adenosine-5'-triphosphate (23), and serotonin (24) receptors. In some cases the record of the variation of extracellular acidification rate was more sensitive than the measurement of second messengers (24). In the present study, we characterized the microphysiometric response of nAChRs to acetylcholine in the presence or absence of different selective antagonists. In all experiments the muscarinic receptors were blocked with atropine.

The acetylcholine-induced increase in extracellular acidification rate was enhanced 2.7 times between DIV4 and DIV5, and no further change in acetylcholine-induced response was observed between DIV5 and DIV6. Selective antagonists for different nAChR subtypes were used to investigate which nAChR subtypes mediate the acetylcholine-induced effect. Alpha-bungarotoxin blocked the acetylcholine-induced response in DIV5 and DIV6, but not in DIV4 cultures.

Neither mecamylamine nor DHßE reduced the acetylcholine-induced increase in extracellular acidification rate in DIV5 retinal cells, indicating that at that stage the response to acetylcholine was mainly mediated by α-bungarotoxin-sensitive receptors. These antagonists were effective in DIV4 cultures.

Mecamylamine, known as a ganglionic antagonist, is a broad spectrum nAChR antagonist which preferentially blocks the α3ß4 nAChR subtype (25), but may also inhibit noncompetitively transfected human α4ß2 nAChRs (21). This antagonist blocks CA1 rat hippocampal interneuron α3ß4-containing nAChRs at 10 μM (25) and blocks rat cerebellum glutamate receptors at a ten times higher concentration (26). In several systems, including rat cerebellum (26) and 3-6-day-old chick retina (27), α-bungarotoxin-insensitive nAChRs evoke a calcium-dependent release of glutamate. In retinal cells in culture for 4 days a very high dose of mecamylamine (100 μM) was necessary to block the acetylcholine-induced response, suggesting that this antagonist was probably blocking glutamatergic receptors. Therefore, in spite of the presence of α3-like immunoreactivity in DIV4 cultured retinal cells, we cannot suggest that the acetylcholine-induced increase in extracellular acidification rate is being mediated by α3ß4-containing acetylcholine receptors, which are highly sensitive to mecamylamine.

DHßE, the broad spectrum non-α7 and non-α8 nAChR antagonist, is a pharmacological tool for the study of non-α-bungarotoxin-sensitive receptors (28). In cells stably transfected with α4ß2-nAChRs, the Ki value for DHßE varies from 20 nM (29) to 3.5 μM (21), whereas the functional responses were fully blocked by 100 nM in rat α4ß2 receptors expressed in HEK293 cells (29) or presented an IC$_{50}$ value of 1.5 μM in human α4ß2 nAChRs expressed in human epithelial cells (21). Native α4ß2-nAChRs expressed in rat brain (30,31) and α4ß4-nAChRs expressed in 1-day-old chick retina (20) bind

DHßE with nM affinity. In rat striatal neurons DHßE inhibits $\alpha4\beta2$ nAChR-induced dopamine release in the µM range (32). In addition, human and rat $\alpha2\beta2$ nAChRs expressed in *Xenopus* oocytes are less sensitive to DHßE than $\alpha4\beta2$ nAChRs (33,34). A quantitative comparison of dose-inhibition curves for acetylcholine-induced current in $\alpha2\beta4$- and $\alpha4\beta2$-containing receptors expressed in *Xenopus* oocytes (RNA ratio 1:1) showed a significantly lower sensitivity in $\alpha2\beta4$-containing receptors ($IC_{50} = 30$ µM) than in $\alpha4\beta2$-containing receptors (9.6 µM) (28). In our study, DHßE inhibited the acetylcholine-induced increase in extracellular acidification rate in DIV4 cultures with an $IC_{50}$ value of 38 µM. This value is much higher than that observed for the classical $\alpha4\beta2$ nAChRs, but is in the range observed for $\alpha2\beta4$-containing nAChRs. Both $\alpha2$ and $\beta4$ units are known to be expressed in embryonic chick retina (4,19). Therefore, in DIV4 cultures acetylcholine stimulated $\alpha$-bungarotoxin-insensitive receptors with a pharmacological profile, considering the minimal effect of mecamylamine and the $IC_{50}$ of DHßE similar to that of $\alpha2\beta4$ nAChRs.

In DIV5 cultured retinal cells acetylcholine promoted a more efficient response, which was blocked by $\alpha$-bungarotoxin, known to antagonize $\alpha7$- and $\alpha8$-containing receptors (6). Alpha8-nAChR is the predominant subtype in embryonic and posthatched chick retina (35) and, in addition, isolated $\alpha7$- and $\alpha7\alpha8$-containing receptors have also been detected (6,35). The Kd values of $\alpha$-bungarotoxin at $\alpha8$-binding sites in embryonic (6) and post-hatched chick retina (36) are 17.4 and 5.5 nM, respectively, while for DIV4 and DIV5 cultured retinal cells the Kd values determined in the present study were 17.9 and 5.9 nM, respectively. Therefore, similar changes in Kd values were observed between equivalent developmental phases *in vivo* and *in vitro*. The coincidence between the appearance of an $\alpha$-bungarotoxin-sensitive response and the decrease in

Kd may be considered evidence that the receptor with a higher affinity is the functional one. Another important consideration is that a reduction in the number of $\alpha$-bungarotoxin binding sites was observed between DIV4 and DIV5 cultures, confirming previous reports (37). Chronic exposure of $\alpha$-bungarotoxin-sensitive nAChRs to agonists such as nicotine is classically associated with up-regulation of the number of receptors determined in binding assays (for a review, see Ref. 38). This was named the paradox of nicotinic acetylcholine up-regulation by nicotine. The mechanism underlying this desensitization is not well understood, but it is known that it does not require protein synthesis and that it is supposed to be a result of recruitment or stabilization of the receptors at the membrane surface (for a review, see Ref. 39). Therefore, it was not a surprise that the reduction in the number of $\alpha$-bungarotoxin binding sites was accompanied by the appearance of the functional response between DIV4 and DIV5 retinal cultures.

## Effect of melatonin on the development of the $\alpha$-bungarotoxin-sensitive acetylcholine-induced response

Melatonin has been shown to modulate the expression and the activity of $\alpha7$ nAChRs in sympathetic nerve terminals of rat vas deferens (10,12,13) and cerebellum (11). The release of noradrenaline in the vas deferens or of glutamate in the cerebellum is increased by melatonin. The cultured chick retina cells were found to be an interesting model to evaluate whether the effect of melatonin on $\alpha$-bungarotoxin-sensitive nAChRs is a more general mechanism since the cells develop a robust response mediated by these receptors between the 4th and the 5th day in culture.

Melatonin is continuously synthesized by cultured retinal cells (15), and this synthesis can be blocked by stimulating D4 dopamine receptors with quinpirole (14). On the other

hand, the effect of melatonin on chick retina is blocked by luzindole, a classical inhibitor of melatonin G-protein-coupled receptors. In order to test if the development of the response mediated by acetylcholine-induced α-bungarotoxin-sensitive nAChRs depends on melatonin produced by the cells in culture, we blocked the melatonin receptors with luzindole, or inhibited melatonin synthesis with quinpirole. Both strategies resulted in the inhibition of the development of the greater α-bungarotoxin-sensitive response to acetylcholine in DIV5 cultures, while they had no effect on the smaller α-bungarotoxin-insensitive response observed at DIV4. This result agrees with the functional responses observed in the rat vas deferens (13) and in the cerebellum (11), where it was shown that melatonin increases the functional response to stimulation of α-bungarotoxin-sensitive acetylcholine receptors.

We have shown that in cultured chick retina the α-bungarotoxin-insensitive nAChRs are active before the sensitive ones, and that the development of the functional nAChRs sensitive to α-bungarotoxin is dependent on the production of melatonin. The data obtained with the embryo retinal cells in culture are somewhat similar to those observed in rat sympathetic nerve terminals and in the cerebellum (11,12). In all cases melatonin only interferes with nAChRs sensitive to α-bungarotoxin. Since melatonin is a key hormone in the chronophysiology of vertebrates, this common pattern probably reflects a more general mechanism of regulation of α-bungarotoxin-sensitive nAChRs.

## Acknowledgments

## References

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