# Muscarinic receptor signalling in Neuro-2a cells and its effects on cell survival: Elaborating pathways involved in neurodegenerative disorders

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## ABSTRACT

This study describes the effect of signalling through muscarinic acetylcholine receptors on the induction of the transcription factor EGR1, which has been implicated in long-term synaptic plasticity, memory consolidation and on a cellular level, the process of apoptosis or programmed cell death.

In Neuro-2a mouse neuroblastoma cells, the cholinergic agonist carbachol led to induction of EGR1, with a mean EC50 of 2.2 μM, and this induction was completely blocked by the muscarinic antagonist atropine at 1 μM. Relative antagonist potency was found to be highest for the M3-selective antagonist 4-DAMP, as compared to the M1-selective antagonist pirenzipine and the M2-selective antagonist AFDX-116. Pertussis toxin, effectively an M2/M4-specific antagonist, completely blocked EGR1 induction. These experiments have given us evidence that a combination of M3 and M4 receptor subtypes are likely to be primarily responsible for EGR1 induction through muscarinic receptors.

In experiments with rotenone, a potent mitochondrial I complex inhibitor, and serum-deprivation, where essential growth factors are not provided, we found that EGR1 induction via muscarinic stimulation did not have a protective effect on cell survival in Neuro-2a cells. EGR1 induction, by itself, is probably insufficient for preventing cell apoptosis in Neuro-2a cells.

Comparing these Neuro-2a cell results with previous results from the SK-N-SH cell line indicates that the transcription factor CREB may be more influencial in the anti-apoptotic mechanism of cells than EGR1. This supports previous evidence from CREB transfection experiments in Neuro-2a cells.

In summary, this research has used a cell model to complement the current understanding of muscarinic acetylcholine receptors and their involvement in survival pathways. Expanding our knowledge of these survival pathways is important in understanding the pathophysiology of neurodegenerative disorders such as Alzheimer's disease.

**Keywords**

Neuro-2a cells, Muscarinic receptors, EGR1, cell survival, apoptosis

## INTRODUCTION

Alzheimer's disease is a neurodegenerative disorder characterized by the loss of nerve-cells that control thought, memory and language. In New Zealand, about 20,000 people over the age of 65 are affected by Alzheimer's disease. The neurotransmitter acetylcholine (ACh) is considered to have a role in learning and memory, but levels are significantly depleted during the progression of Alzheimer's disease as a consequence of the neuronal degeneration.

Research has shown that the process of apoptosis or programmed cell death plays a central role in the onset and development of neurodegenerative disorders such as Alzheimer's disease. Cholinergic signalling through muscarinic acetylcholine receptors (mAChRs), previously linked to long-term changes in synaptic plasticity which underlie memory formation,[1,2] has also been shown to also protect against apoptotic cell death.[3,4] Active research over the last few years has identified some candidate 'memory molecules' which, as specific inducible transcription factors, are able to initiate programmes of gene expression relevant to both memory consolidation and anti-apoptotic signalling.

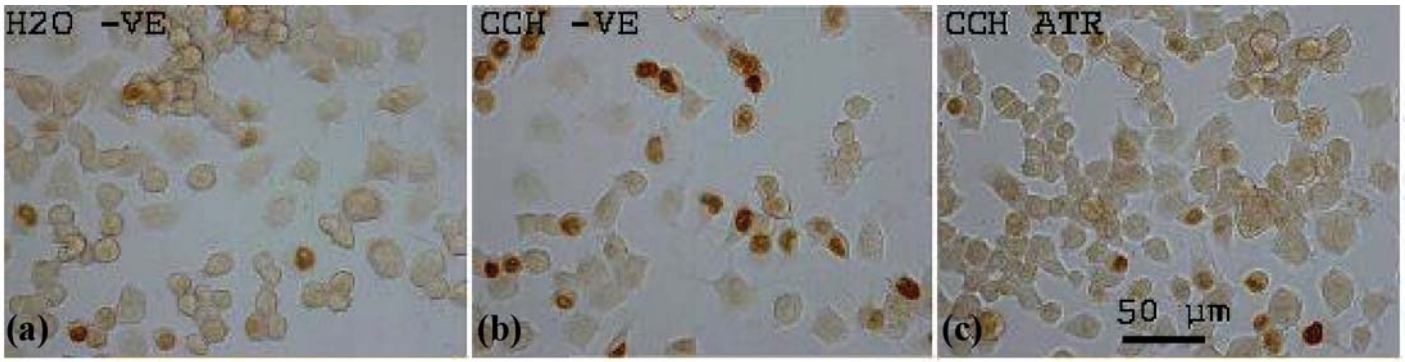
EGR1 (also known as NGFI-A, krox 24, Zif268 or Tis8) is a zinc finger transcription factor whose induction does not require prior protein synthesis.[5] Evidence for its role in memory consolidation has come from a mouse EGR1 gene knockout study, with EGR1-/- mice exhibiting an impairment in long-term memory while retaining short term memory.[6] Research using cell culture models have demonstrated muscarinic-driven EGR1 activation.[7,8] Furthermore, mAChR antagonists which cause amnesia in humans, block neuronal EGR1 induction by cholinergic agonists in rodents.[9]

Cyclic AMP response element binding protein (CREB) is another transcription factor which has emerged as a prime candidate memory molecule. Expressed constitutively in most cells, CREB becomes transcriptionally active following phosphorylation at Ser133.[10] There is compelling evidence for the involvement of CREB in initiating transcriptional programmes relevant to neuronal survival.[11-13]

Muscarinic cholinergic signalling in the SK-N-SH human neuroblastoma cell line has been shown to lead to CREB phosphorylation and EGR1 expression,[14] and to have enhanced cell survival during serum deprivation, an apoptosis-inducing condition. These effects were shown to be primarily driven through the M3 receptor (J.M. Greenwood, personal communication). In Neuro-2a cells, muscarinic cholinergic signalling has been found to induce EGR1 expression, but not CREB phosphorylation (J.M. Greenwood, personal communication).

Our experiments aim to determine the muscarinic acetylcholine receptor (mAChR) subtype(s) responsible for the induction of EGR1 in mouse Neuro-2a neuroblastoma cells using a range of pharmacological inhibitors.

**Figure 1.** EGR1 expression in Neuro-2a cells is driven through muscarinic receptors.




Immunocytochemical nuclear staining shows EGR1 expression is stimulated by cholinergic agonist carbachol (100 µM, b). EGR1 expression is similar to basal levels (H2O, a) on addition of muscarinic antagonist atropine (1 µM, c) -VE refers to absence of atropine

Furthermore, we aimed to ascertain whether EGR1 induction driven through mAChR signalling leads to a protective response.

## METHODS

These methods are based on those used by Greenwood and Dragunow (2002).

### Cell Culture

Mouse Neuro-2a neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, no CCL-131). They were cultured in 75 $cm^2$ flasks at 37°C in a humidified atmosphere (5% $CO_2$/95% air) in MEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mM sodium pyruvate. Cells grown to confluency were harvested with 0.05% trypsin in phosphate-buffered saline (PBS), washed with media and plated 18-24 h prior to carbachol addition. Cells were counted using a haemocytometer, and plated at $10^4$ cells/well in 96-well plates for immunocytochemistry and cell-based ELISA, and at $5 \times 10^3$, $10^4$ and $2 \times 10^4$ cells/well in 96-well plates for survival-viability assays, with a constant plating volume of 0.1 mL. Where indicated, atropine sulphate, pirenzipine dihydrochloride, 4-DAMP methiodide, and AFDX-116 were added 5 min prior to addition of carbachol, while pertussis toxin was added 18 h prior to carbachol addition. Human SK-N-SH neuroblastoma cells were obtained from the ATCC.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde. Primary antibody incubation was overnight at 4°C. Dilutions of rabbit polyclonal antibodies were 1/1000 for both EGR1 (Santa Cruz Biotechnology sc-189) and cleaved caspase 3 (Cell Signaling Technology 9661). This was followed by incubation with biotinylated goat anti-rabbit antibody (Sigma) and ExtrAvidin peroxidase (Sigma), each at 1/250 dilution. Antibody binding was visualised by 3,3'-diaminobenzidine (DAB) staining.

### Cell-based ELISA

Quantification of immunostaining was attained using cell-based ELISA. In brief, cells were fixed in 4% paraformaldehyde. Endogenous peroxidase activity was quenched using 0.6% $H_2O_2$ in PBS/Triton, followed by blocking with 10% goat serum in PBS/Triton for 1h. Primary antibody incubation (EGR1 at 1/2000 dilution) was overnight at 4°C, followed by peroxidase-conjugated secondary anti-rabbit antibody (Chemicon) at 1/200 dilution. Peroxidase conversion of the substrate o-phenylenediamine dihydrochloride (OPD) was determined by absorbance measurement at 490 nm (A490) corrected for light scatter at 650 nm. Measurements of A490-A650 were averaged for at least 3 replicate wells and were normalised to control ($H_2O$) values. The data were normalised to a percentage scale and best-fit sigmoidal curves were plotted, using Graphpad Prism to calculate EC50 values and relative potencies of the antagonists.[15]

### Cell survival Assays

For the serum starvation experiment, all wells were washed twice with serum-free medium 18-24 h after plating. Water or carbachol (100 µM) diluted in serum-free medium or serum containing 10% medium (full serum medium) was added (100 µL) to respective wells. For the rotenone toxicity experiment, rotenone (1 µM) or 0.01% DMSO (vehicle) was added together with water or carbachol (100 µM), 18-24 h after plating. For both experiments, plates were then incubated for 22 h. Alamar blue (10µL/well, Serotec), an indicator of cell metabolism, was added and incubation resumed for 2 h. Conversion of the alamar blue substrate was determined by excitation at 544nm and measurement of fluorescent emission at 590nm. Measurements of E590 were averaged for at least 3 replicate wells.

## RESULTS

### Muscarinic signalling leads to EGR1 induction in Neuro-2a cells, most likely driven through M3 and M4 receptors.

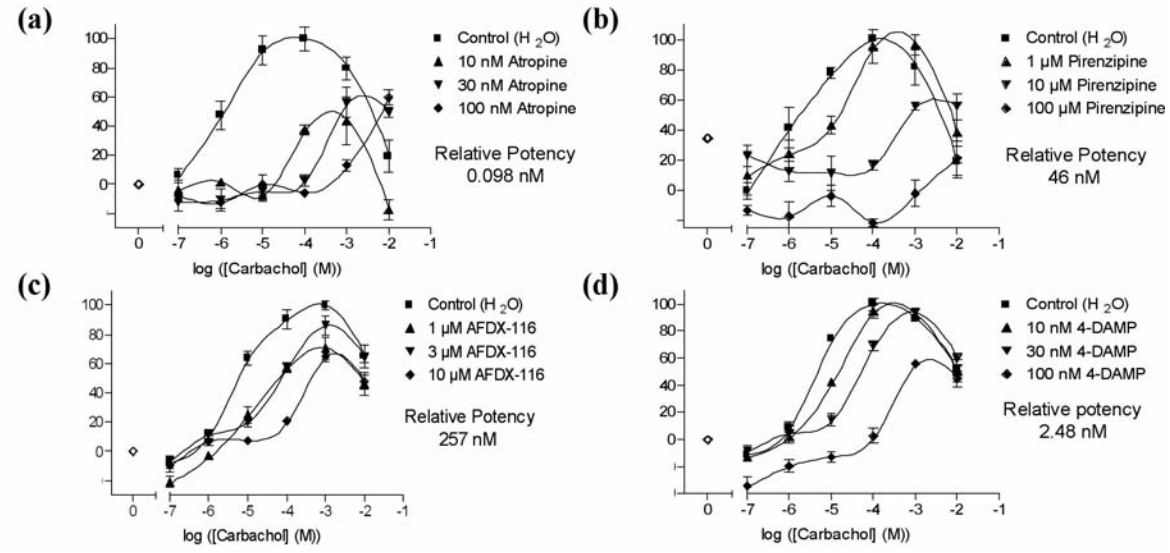
To investigate the effects of muscarinic receptor activation on EGR1 induction, immunocytochemistry and cell-based ELISA were performed on Neuro-2a mouse neuroblastoma cells following treatment with the cholinergic agonist carbachol (100 µM). An increase in nuclear EGR1 immunostaining was observed 60 min after carbachol treatment (Figure 1).

Carbachol dose-response curves for EGR1 induction in Neuro-2a cells were generated by cell-based ELISA. Cells were fixed 60 min after carbachol treatment. Induction of EGR1 had a high sensitivity to carbachol concentration with a mean $EC_{50}$ value of 2.2 µM (95% CI: 0.71 - 6.64 µM, n = 4). The muscarinic antagonist atropine (at 1 µM) completely blocked EGR1 induction when added 5 min prior to carbachol, indicating that carbachol was driving these responses through muscarinic receptor activation (Figure 1). Pre-treatment with atropine (at 10 nM, 30 nM, 100 nM) produced right shifts in the carbachol dose-response curves, consistent with competitive inhibition of muscarinic receptors (Figure 2a). The relative antagonist potency for atropine was approximately 0.098 nM.

Treatment with the M1-muscarinic antagonist pirenzipine (at 1 µM, 10 µM, 100 µM) produced right shifts in the carbachol-dose response curves, but relative potency was calculated to be significantly lower than atropine at 46 nM (Figure 2b). Similar right shifts were seen for the M2-muscarinic antagonist AFDX-116 (at 1 µM, 3 µM, 10 µM), with a relative potency of 257 nM (Figure 2c). The above results suggest that neither M1 nor M2 muscarinic receptors are significantly involved in muscarinic-driven EGR1 induction.

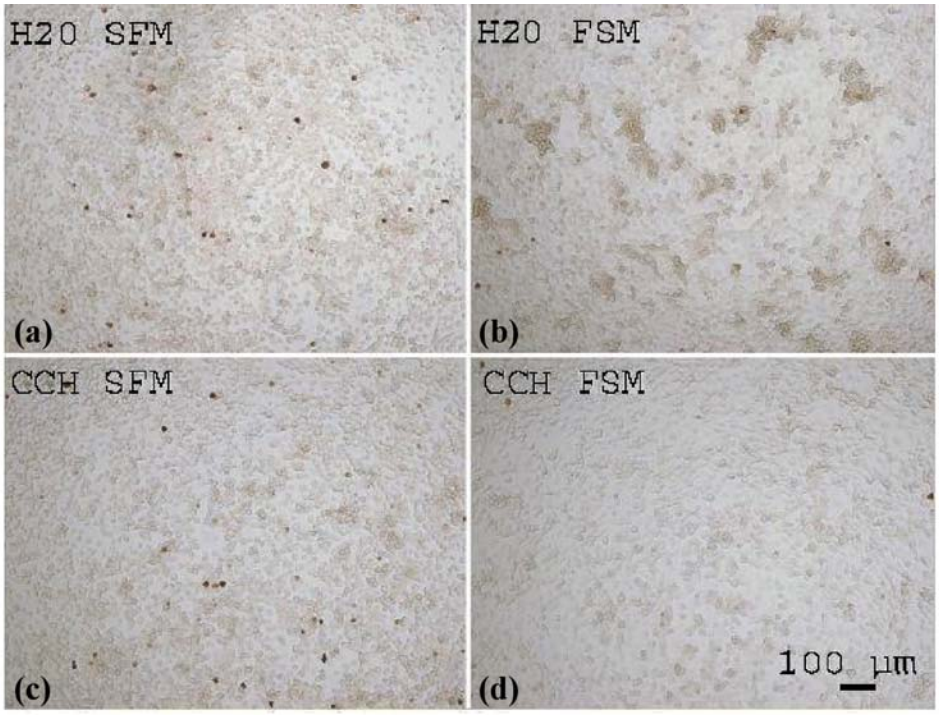
The M3-muscarinic antagonist 4-DAMP (at 10 nM, 30 nM, 100 nM),

**Figure 2.** Carbachol dose-response curves for EGR1 in Neuro-2a cells, in the presence of muscarinic antagonists.



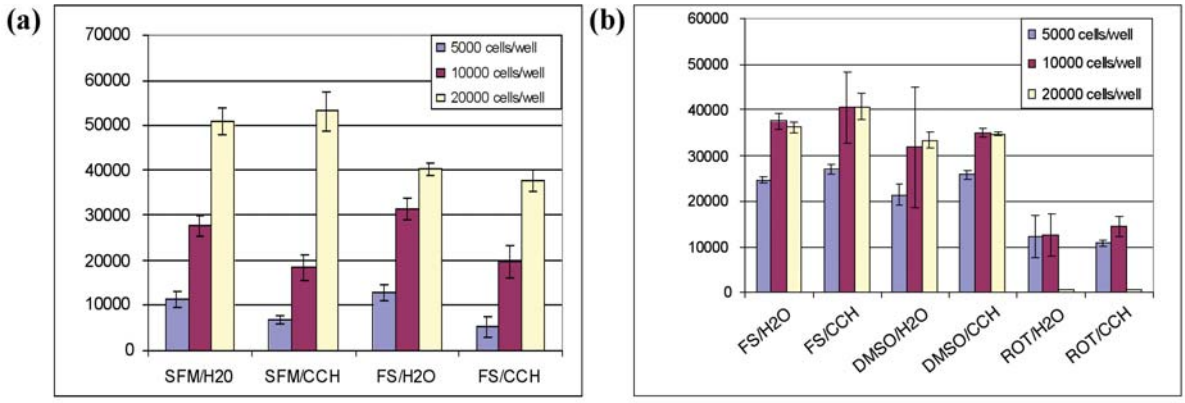

The M3-selective muscarinic antagonist 4-DAMP was the most potent (2.48 nM) selective antagonist. However, none of the selective antagonists had a potency comparable to that of non-selective antagonist atropine (0.098 nM). Values shown represent the mean ±SEM.

**Figure 4.** Serum starvation induces apoptosis in Neuro-2a cells.



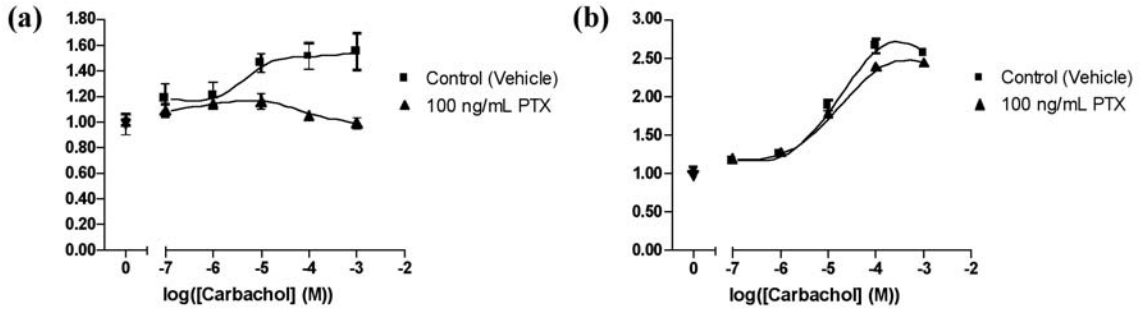

Immunocytochemical DAB staining for cleaved caspase 3, a marker of cell apoptosis. Note the presence of apoptotic cells (dark staining) in the serum free medium (SFM, a and c), but not in the full serum medium (10% serum, FSM, b and d) condition.

**Figure 5.** Carbachol treatment does not enhance cell survival of Neuro-2a cells cultured in stressful conditions.




Wells in serum free medium conditions that were also treated with carbachol had similar fluorescence readings to wells treated with H2O (a). This means cell viability was similar in both conditions which suggests that treatment with carbachol (CCH) did not have a protective effect on cell survival. This observation was similar in the rotenone experiment (b). FS represents full serum medium (10% serum). Values shown represent the mean ± s.d.

**Figure 3.** Pertussis toxin (PTX) inhibits EGR1 induction by carbachol in Neuro-2a cells.




EGR1 induction by carbachol is inhibited in Neuro-2a cells (a) but not in SK-N-SH cells (b). This suggests EGR1 expression in Neuro-2a cells may be driven through M2/M4 receptor subtypes. Values shown represent the mean ±SEM.

produced right shifts in the dose response curves with a relatively high potency of 2.48 nM (Figure 2d), which suggests that the M3 receptor subtype is one of the receptors that can lead to EGR1 induction in Neuro-2a cells.

Overnight treatment with pertussis toxin blocks the coupling of the Gi/Go proteins with M2 and M4 muscarinic receptors, rendering them inactive. Pertussis toxin (at 100 ng/mL) inhibited EGR1 induction by carbachol (Figure 3a) suggesting that M2/M4 receptor subtypes are involved in EGR1 induction in N2a cells.

A common feature of the carbachol dose-response curves is that at high concentrations of carbachol, after the peaks of the dose-response curves, there seems to be a decrease in EGR1 expression (Figure 3). This feature appears to be independent of the addition of the muscarinic antagonists.

### Muscarinic cholinergic signalling and EGR1 induction has no effect on cell survival in Neuro-2a cells.

To investigate the effects of muscarinic cholinergic signalling on cell survival in Neuro-2a cells, we used an alamar blue fluorescence assay to measure cell metabolic activity. To induce apoptosis in these cells, we cultured them overnight in serum-free medium, thus depriving them of essential growth factors required for cell growth and survival.

Levels of cleaved caspase 3 (a marker of cell apoptosis), visualised by DAB immunostaining, were higher in serum-free medium cultured cells, as compared to full-serum cultured cells (Figure 4). This indicates that serum-free medium induces apoptosis. Treatment with carbachol did not appear to have any protective effect (Figure 4).

Cells (at 5,000 and 10,000 cells/well) cultured in serum-free conditions have slightly lower fluorescence readings than full-serum cultured cells (Figure 5a). This shows they have slightly lower viability. All wells were washed with serum-free medium, and this might have influenced the viability of the full-serum cultured cells (Figure 5a). In the serum-free medium wells, cells (at 5,000 and 10,000 cells/well) treated with carbachol had lower fluorescence readings than control ($H_2O$-treated) cells (Figure 5a). This indicates that carbachol had a deleterious effect on cell metabolism and cell survival. This deleterious effect was not seen at 20,000 cells/well.

As a further test to determine if carbachol treatment had any protective effects in Neuro-2a cells, we used a mitochondrial I complex inhibitor, rotenone to create a stressful environment for cell growth. Rotenone toxicity is most significant at 20,000 cells/well (Figure 5b). In the rotenone-treated wells, wells that were also treated with carbachol had similar fluorescence readings to wells treated with H2O (Figure 5b). This suggests that treatment with carbachol did not have a protective effect.

Wells with 20,000 cells/well had relatively low fluorescence readings in both the serum starvation and the rotenone toxicity experiments (Figures 5a and 5b).

## DISCUSSION

In this study we report the induction of EGR1 by mAChR activation in Neuro-2a neuroblastoma cells. The carbachol dose-response curves show that the M3-selective muscarinic antagonist 4-DAMP was the most potent (2.48 nM) selective antagonist. Pertussis toxin was shown to inhibit EGR1 expression. This suggests M2/M4 receptor involvement and taken together with the AFDX-116 data (M2-selective antagonist), implicates M4 receptors in muscarinic-driven EGR1 induction. None of the selective antagonists had a potency comparable to that of the non-selective antagonist atropine (0.098 nM). This implies that blocking cellular signalling through a single receptor subtype was unable to completely inhibit EGR1 expression. These findings collectively suggest that a combination of M3 and M4 receptor subtypes are likely to be primarily responsible for muscarinic-driven EGR1 induction.

The decline in EGR1 expression, after the peaks of the dose-response curves, was apparent irrespective of the presence of selective antagonists. This may be the result of carbachol binding to a receptor to which it has lower affinity, leading to the downregulation of EGR1. It is known that EGR1 is very far downstream of the receptor, and this feature reinforces that many other factors may influence the levels of EGR1 in these cells.

In the alamar blue fluorescence assays, which used serum starvation and rotenone treatment to induce apoptosis, treatment with carbachol did not appear to have any protective effect. It was noted that cells plated at a higher density (20,000 cells/well) had low viabilities under both experimental conditions. This may be an endogenously-initiated downgrading of cellular metabolism at this higher density. It is possible that this downregulation of cellular metabolism potentiates the toxic effect of rotenone, resulting in the extremely low cell viability at 20,000 cells/well (Figure 5b). This awaits further analysis.

These experiments have shown that muscarinic stimulation and EGR1 induction do not have a protective effect on cell survival in Neuro-2a cells. EGR1 induction is probably, on its own, insufficient for preventing cell apoptosis in Neuro-2a cells. In Neuro-2a cells, muscarinic cholinergic signalling has been found to induce EGR1 expression, but not CREB phosphorylation (J. Greenwood, personal communication). Research using the SK-N-SH cell line has shown that treatment with carbachol induces CREB phosphorylation and EGR1 expression.[14] Carbachol treatment also has a protective effect in this cell line. The results of the Neuro-2a and SK-N-SH cell lines, if taken together, suggest that CREB phosphorylation may be more influencial in the anti-apoptotic mechanism of cholinergic signalling than EGR1 induction. This supports evidence from CREB transfection experiments in Neuro-2a and PC12 cells that showed CREB overexpression produced anti-apoptotic effects.[13]

In the SK-N-SH cell line, the protective effects of cholinergic signalling were shown to be driven through the M3-muscarinic receptor (J. Greenwood, personal communication). In the Neuro-2a cell line, we have found that if collectively stimulated, M3 and M4 receptors do not increase cell survival. It is possible that M4-receptors drive a pro-apoptotic response

that negates the effects of an anti-apoptotic M3-driven response. This is relevant to Alzheimer's disease as M3 and M4 receptors are relatively well preserved in the brains of patients that had suffered from this disease.

Our experiments have shown that muscarinic signalling leads to EGR1 expression in Neuro-2a cells, most likely driven through M3 and M4 receptors, but muscarinic signalling and EGR1 induction have no protective effects. It has produced evidence complementary to past research that EGR1 has a less significant role in the anti-apoptotic mechanism than CREB phosphorylation. The experiments have generated some interesting questions on the density-dependent effects of stressful conditions on cell survival, which may undergo further research.

Currently, most of the drugs used in the treatment of Alzheimer's disease are modulators of acetylcholine signalling (acetylcholinesterase inhibitors). These drugs have many adverse effects because they are non-specific in their action. In our research we have successfully used a cell model to complement the current understanding of the involvement of muscarinic acetylcholine receptors in cell survival pathways. Elaborating these survival pathways is important in understanding the pathophysiology of neurodegenerative disorders such as Alzheimer's disease, which may, in turn, result in more effective pharmacological therapies.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