ERK-CREB signaling dysregulation with increasing levels of the β-Amyloid protein

Amanda Grant-Orser*[1], Fabio Canneva[2]

[1]Department of Anatomy and Cell Biology, McGill University, 3640 University Street, Montréal Québec, H3A 2B2
[2]Department of Pharmacology and Therapeutics, McGill University, McIntyre Medical Building, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada, H3G 1Y6

ABSTRACT

Introduction: Alzheimer's Disease is characterized by cognitive impairment and neurodegeneration, for which the pathogenic cause is considered to be the accumulation of the β-amyloid peptide ($A\beta_{42}$). The Swedish and Indiana mutations of the amyloid precursor protein gene ($APP_{Swe,Ind}$), found in the genetic familial form of AD, increase $A\beta_{42}$ levels due to augmented enzymatic cleavage. Previous experiments demonstrate that high levels of soluble $A\beta_{42}$ upregulate extracellular regulated kinase 1/2 (ERK1/2) and downregulate cyclic AMP-response element (CRE) binding protein (CREB) phosphorylation. Increasing levels of soluble $A\beta_{42}$ protein are hypothesized to dysregulate the ERK-CREB signalling pathway. **Methods**: Three plasmid constructs containing wild type amyloid precursor protein (APP_{Wt}), APP_{Swe} or $APP_{Swe,Ind}$, each producing increasing levels of $A\beta_{42}$, were stably transfected into eukaryotic cell lines. Immunocytochemistry was performed using the McSA1 antibody to measure the amount of Aβ protein in the $APP_{Swe,Ind}$ and APP_{Wt} cell lines. Cells were stimulated with forskolin and KCl. ERK and CREB phosphorylation were analyzed by western blot. **Results**: McSA1 staining demonstrated increased Aβ protein in the $APP_{Swe,Ind}$ cell line compared to the APP_{Wt} cell line. Upon cell stimulation at 30 minutes, the APP_{Wt} cell line demonstrated the highest levels of CREB phosphorylation and $APP_{Swe,Ind}$ demonstrated the lowest levels. ERK phosphorylation increased upon stimulation and was sustained across all four time points, but there was no significant difference in levels between the clones. **Discussion**: These results suggest that the Aβ protein has beneficial effects on CRE-regulated gene expression at physiological levels and negative consequences that mimic Alzheimer's Disease at pathogenic levels.

KEYWORDS

Amyloid precursor protein, ERK, CREB

*Corresponding author:
amanda.grant-orser@mail.mcgill.ca

Received: 3 January 2010
Revised: 18 March 2010

INTRODUCTION

Alzheimer's Disease (AD) is currently the leading cause of dementia, affecting 10% of the Canadian population over the age of 65. AD is clinically characterized by a gradual decline in cognitive abilities, and biologically by neuron death in the cortex and hippocampus *(1)*. The pathogenic cause of Alzheimer's disease has been attributed to the β-amyloid protein which exists in two isoforms: ($A\beta_{40}$) at a length of 40 amino acids and $A\beta_{42}$ at 42 amino acids. Since $A\beta_{42}$ is more hydrophobic and prone to aggregation, it seems to be the pathogenic isoform *(2)*. Aβ is cleaved from the amyloid precursor protein (APP) by the β and γ-secretases sequentially. There also exists a separate processing pathway not

linked to AD, in which APP is cleaved by the α-secretase *(3)*. Genetic variants of APP have been discovered in familial forms of AD (FAD), whereby mutations at the sites of secretase cleavage favour the production of $A\beta_{42}$. Other FAD-related mutations have been described for the genes encoding presenilin 1 and 2, the catalytic components of the γ-secretase, which also result in augmented $A\beta_{42}$ production *(4)*. Harnessing these FAD mutations has led to the manipulation and study of AD in both in vivo and in vitro models to obtain a better understanding of the cellular pathology and more effective therapeutic remedies.

Early stages of AD are characterized by the accumulation of intracellular oligomeric Aβ (iAβ). In later stages of disease, indicators of AD pathology include a wide variety of physiological and behavioural consequences including, but not limited to, abnormal neuron projections, inflammation, neurotransmitter defects, extracellular Aβ plaque deposition and intracellular neurofibrillary tangles (NFTs), which consist mainly of hyperphosphorylated microtubule-binding Tau protein *(5-9)*. While many investigations have studied the consequences of extracellular Aβ plaques, researchers have more recently focused on the significance of iAβ accumulation in the progression of behavioural impairments characteristic of the disease. Current research is also examining the roles of neuroinflammation and neurodegeneration *(10)*. This "pre-plaque" phenotype of accumulated iAβ has been shown to have downstream effects on the phosphorylation of extracellular regulated kinase 1/2 (ERK 1/2), an intracellular kinase coupled to an extracellular activation signal, and cyclic AMP (cAMP) response element binding protein (CREB), a transcription factor which binds cAMP response elements on DNA to regulate transcription *(11)*. ERK is an activation kinase of another kinase called p90RSK, which phosphorylates CREB. Upon phosphorylation, CREB acts as a transcription factor for the cAMP response element (CRE)-regulated genes. CRE-regulated genes are implicated in learning and memory, namely in the development of long term potentiation (LTP) at synapses *(12-13)*, a process involved in synaptic strength increase and which is greatly disrupted in AD.

Previous *in vivo* studies suggest that when the Aβ protein is over-produced, CRE-regulated gene expression is downregulated (11). The effect of Aβ levels on CRE-regulated gene activation has also been demonstrated *in vitro* using transiently transfected cells in the lab (14), whereby nucleic acids were introduced into the cell by non-viral methods and transiently expressed. The use of stably transfected cell lines where there is genomic integration of the introduced nucleic acids, however, would allow for a more precise assay of ERK-CREB signalling regulation and better reproducibility of previous experiments. Our hypothesis is that with increasing levels of soluble $A\beta_{42}$ protein, ERK/CREB phosphorylation and CRE-directed gene expression will be dysregulated. This dysregulation is expected to correspond to the amount of $A\beta_{42}$ produced. To test this idea, three plasmid constructs for the expression of wild type APP (APP_{Wt}), APP_{Swe} and $APP_{Swe,Ind}$ variants, each leading to increased levels of the Aβ protein, respectively, were stably transfected into eukaryotic cell lines. The consequent dysregulation of ERK-CREB signalling was evaluated by measuring ERK and CREB phosphorylation before and after chemical stimulation of the cells.

METHODS

The DNA constructs were all derived from the pIRES vector backbone in which the transgenic protein (APP variants) is expressed under the control of the human cytomegalovirus (CMV) promoter and co-translated with the enhanced green fluorescent protein (EGFP). Three transgenic proteins were used: APP_{Wt}, APP_{Swe} and $APP_{Swe,Ind}$. Stable transfections were done on PC12 cells using G418, a neomycin analog, 48 hours after transfection for selection. Cells were selected into homogenous populations of APP expression using fluorescence activated cell sorting (FACS). Immunocytochemistry was used to detect the presence of Aβ protein using the MCSA1 antibody. To test the ERK-CREB signalling pathway, cells were stimulated with KCl and forskolin at varying time points and analysed via western blot. Results were analyzed by one-way (FACS cell sorting) or two-way (cell stimulation) ANOVA tests. Differences were considered statistically significant at $p < 0.05$ (*). ($p < 0.001$ is indicated by ***). In a separate cell stimulation experiment with time points of 30 minutes, 1 hour, 2 hours and 3 hours, results were analyzed qualitatively, since $N = 1$.

RESULTS

TRANSFECTIONS

PC12 cells were successfully and stably transfected with the four clones; pIRES (control), APP_{Wt}, APPSwe and $APP_{Swe,Ind}$. All subsequent results shown are from the PC12 transfected cell lines.

POPULATION STANDARDIZATION BY FACS

Levels of transgene expression can vary with the amount of plasmid integrated into the cell genome. To ensure that the cell lines being tested expressed APP homogeneously and were comparable, EGFP was used as a second selectable marker for protein expression. FACS selected cells based on their emitted green fluorescence. Sorting resulted in a standardized population of cells with an average of over 75% EGFP positive in all cell lines (Fig. 1). The APP_{Swe} clone is shown before and after the sorting process (Fig. 2) as a qualitative example of the homogenized population of increased EGFP expression.

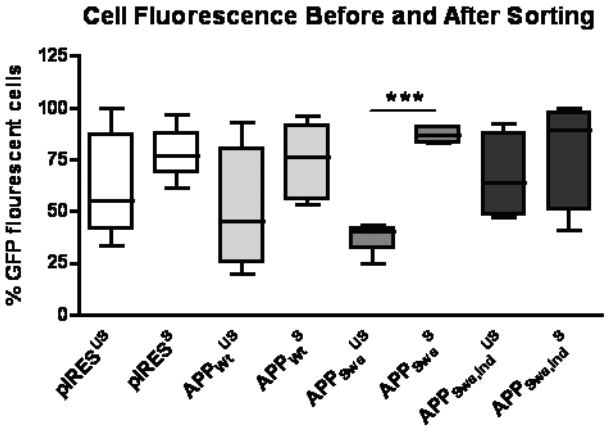

Fig. 1 Cell Fluorescence Before and After Sorting
Comparison of GFP% positive unsorted (US) and sorted (S) cells. Only the APP_{Swe} clone had a statistically significant increase in green fluorescence ($p<0.001$).

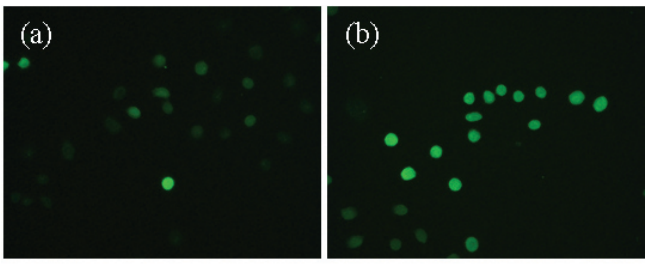

Fig. 2 APP_{Swe} clone FACS selection (**A**) before selection (**B**) after selection

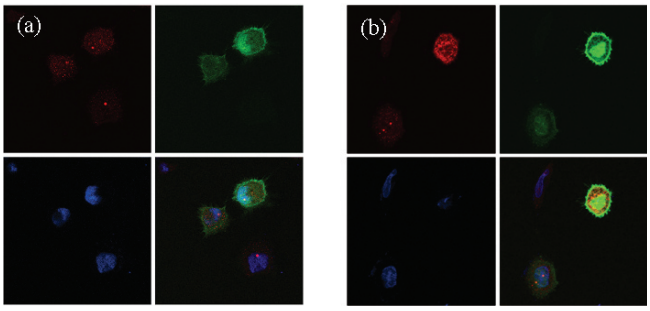

Fig. 3 ICC Confocal microscopy
Evidence for the differential expression of the Aβ protein between the (a) APP_{Wt} and (b) $APP_{Swe,Ind}$ clones.

QUALITATIVE Aβ EXPRESSION

Immunocytochemistry (ICC) was used to qualitatively analyze Aβ protein expression between the different clones. Cells were examined at 63x magnification on a confocal microscope, with Blue DAPI staining identifying cell nuclei. The primary antibody used was McSA1, which is specific for the human Aβ protein, and the secondary antibody used was Rhodamine flourophore. The negative control (no primary antibody applied) showed minimal rhodamine fluorescence, demonstrating the specificity of the secondary antibody for McSA1 (results not shown). The APP_{Wt} clone had more intense McSA1 staining than the background pIRES (control) as well as a unique peripheral pattern. McSA1 staining correlated with the intensity of EGFP fluorescence, indicating that green fluorescence correlated with transgene protein expression. Comparison of the APP_{Wt} and $APP_{Swe,Ind}$ clones (Fig. 3) demonstrated that levels of Aβ protein production were much greater in the $APP_{Swe,Ind}$ clone than in the wild-type, as evidenced by increased McSA1 staining. The peripheral staining pattern in the APP clones suggests Aβ could be contained in peripheral vesicles.

CREB PHOSPHORYLATION BEFORE AND AFTER STIMULATION

UNSTIMULATED (NI), 5 MINUTES, 15 MINUTES, 30 MINUTES

Western blot analysis was used to determine levels of phosphorylated CREB (pCREB) in PC12 cells transfected with APP_{Wt}, APPSwe and $APP_{Swe,Ind}$ clones. There was no statistically significant difference (2-way ANOVA) of pCREB levels between the clones in unstimulated cells (Fig. 4). Cells were stimulated with Forskolin and KCl. Stimulation for 5 minutes demonstrated that the presence of moderate Aβ levels, produced by the APPSwe clone, increased the cell's responsiveness to CREB phosphorylation ($p<0.05$). After 15 minutes, high levels of Aβ produced by the $APP_{Swe,Ind}$ clone appear to inhibit the phospho-

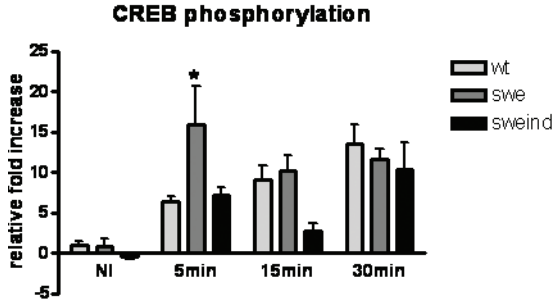

Fig. 4 CREB phosphorylation levels normalized against β-tubulin before and after stimulation
At 5 min. the rise Aβ levels caused a statistically significant increase in cell's responsiveness ($p<0.05$). At 30 min. intermediate levels of Aβ caused a progressive decrease in CREB phosphorylation levels, although results were statistically nonsignificant (NS).

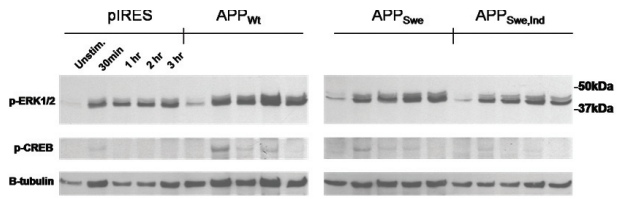

Fig. 5 Stimulation of transfected PC12 cells
pERK activation is increased upon stimulation and was sustained across all time points. pCREB increased upon stimulation and had the greatest increase in the APP_{Wt} clone. Among the APP clones, Wt had the greatest and Swe,Ind the lowest level of pCREB activity.

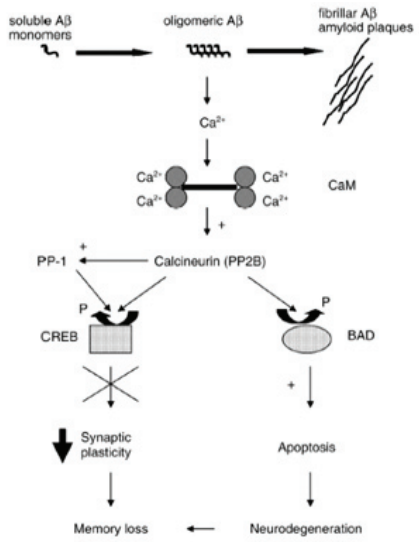

Fig. 6 PP2B signaling pathway proposed by Taglilatela et al.
Decreased CREB phosphorylation and activation of the BAD pro-apoptotic protein BAD result in the presence of high levels of oligomeric Aβ

rylation of CREB, though this result did not reach statistical significance. At 30 minutes, increasing levels of the Aβ protein (APP_{Wt}<APPSwe<$APP_{Swe,Ind}$) caused a progressive decrease in CREB phosphorylation.

UNSTIMULATED, 30 MINUTES, 1 HOUR, 2 HOURS, 3 HOURS STIMULATION

A second western blot analysis was used to determine CREB phosphorylation levels (Fig. 5), with pIRES CREB phosphorylation used as a control. Upon stimulation, CREB phosphorylation was greatest at 30 minutes for all clones; the largest increase in pCREB was evident in the APP_{Wt} clone, followed by the APPSwe clone and finally the lowest increase was in the $APP_{Swe,Ind}$ clone. At all other time points, however, there was no significant difference in pCREB levels between the clones.

ERK PHOSPHORYLATION BEFORE AND AFTER STIMULATION

UNSTIMULATED, 30 MINUTES, 1 HOUR, 2 HOURS, 3 HOURS STIMULATION

Upon stimulation, pERK levels were increased in all clones (Fig. 5), and phosphorylative activation was sustained for all four time points. The difference in pERK levels between the clones was not significant.

TOTAL PROTEIN EXPRESSION

Total protein expression in the transfected PC12 cells was determined by β-tubulin levels. Western blot results showed an irregularity in the amount of protein loading between the samples (Fig. 5). All western blot statistical analyses of pCREB and pERK were normalized to these levels.

DISCUSSION

The goal of studying the consequences of low pathogenic levels of the Aβ protein is to enable intervention in the early stages of AD while intracellular Aβ is present, prior to plaque formation. The hope is to cure behavioural abnormalities and reverse the decline of cognitive function. The motivation prompting the use of the three different stably transfected genes was to correlate increasing levels of the Aβ protein to the amount of dysregulation in ERK-CREB signalling and the subsequent effects on CRE-regulated gene expression.

The three stably transfected cell lines used in this study expressed homogenous levels of APP protein. Constant expression was achieved by three methods: first, the same promoter was used for all three plasmid constructs; second, a selectable marker of transgene expression, EGFP, was co-translated with the inserted gene; third, the cells were selected using FACS. Stimulation of the cells by intracellular Ca2+ and cAMP production activated ERK-CREB signalling. The level of pathway activation was influenced by the amount of Aβ protein present, which increased across the APP_{Wt}, APP_{Swe} and APP_{Swe}, Ind cell lines.

ERK phosphorylation levels increased upon stimulation and were sustained during all four time points in all four cell lines. *In vivo*, this phosphorylation is greatest in $APP_{Swe,Ind}$ compared to wild-type animals (unpublished results) suggesting the Aβ protein induces constitutive signalling; however, these results were not replicated *in vitro*. Arvanitis *et al.* observed a peak CRE-regulated gene expression 30 minutes after PC12 cell stimulation in APP transfected cells (15). In the current study, the first investigative time point for ERK phosphorylation was 30 minutes. Therefore, investigating an earlier time point could yield more conclusive results since ERK phosphorylation is upstream of CRE activation.

There was a statistically significant ($p<0.05$) increase in pCREB levels at 5 minutes, with moderate levels of the Aβ protein, as expressed by the APP_{Swe} clone (Fig. 4). The intermediate time point of 15 minutes demonstrated that high levels of the Aβ protein, expressed by the $APP_{Swe,Ind}$ clone, inhibited CREB phosphorylation. At 30 minutes, CREB phosphorylation levels progressively decreased with increasing levels of Aβ protein (Fig. 4). The APP clones differed in their amount of CREB phosphorylation; the wild-type clone demonstrated the largest increase, followed by the Swe clone and then the $APP_{Swe,Ind}$ clone, (Figs. 4 and 5). These results corroborate the theory that APP has an endogenous role in the cell by favouring CRE-regulated gene expression, while the overexpression of Aβ has negative consequences on CREB phosphorylation and subsequently CRE-regulated gene expression.

Taglitatela et al. (18) have suggested a possible explanation for increased ERK phosphorylation, a kinase of CREB, and decreased CREB phosphorylation in the presence of Aβ. Since ERK is upstream of CREB, it is theorized that there are phosphatases activated simultaneously or after ERK which could act to dephosphorylate and rescue CREB activation. One such phosphate being investigated is the Ca2+/calmodulin dependent phosphatase calcineurin (PP2B). Supporting this idea, calcineurin showed increased activity with the application of exogenous Aβ oligomers (Fig. 6) (16), suggesting a potential explanation for the results shown here. After 15 minutes of stimulation, increased PP2B activity induced by the high level of Aβ protein could have rescued CREB from activation, elucidating why pCREB levels were lowest in the $APP_{Swe,Ind}$ clone. Accordingly, PP2B may not have been activated at 5 minutes, allowing moderate levels of the Aβ protein to exert positive effects on CREB phosphorylation. The time course of PP2B activation upon stimulation and its interaction with intracellular Aβ will have to be investigated to verify this hypothesis.

Another interesting feature of calcineurin which implicates the phosphatase in the pathogenesis of AD is its activation of the proapoptotic protein BAD (17). As apoptosis is a well-characterized feature of the later stages of AD, controlling the dephosphorylative abilities of PP2B through inhibition could help manage some of the negative consequences arising from the downregulation of CREB phosphorylation and CRE-regulated gene expression seen in AD.

An alternative explanation for high pERK and low pCREB levels has been suggested by Echeverria et al. (11). Depending on the duration of stimulation, ERK could have varying consequences on its downstream substrates. p90RSK, a CREB kinase and an ERK substrate, could be hypophosphorylated either due to the simultaneous stimulation of rescue phosphatases or by the duration of ERK activity disrupting its kinase activity. This would result in a decreased level of CREB phosphorylation (18) and CRE-regulated gene expression. Further investigations are required to confirm this hypothesis.

The work described here has several limitations. First, the level of CRE-regulated gene expression was not examined and so the final product of the ERK-CREB activation observed is still under question. Secondly, a quantitative analysis of Aβ by western blot detection was unsuccessful. A likely explanation for this failure could be the low loading level of the samples. Future investigations should perform an enzyme-linked-immunosorbent-assay (ELISA) which would not only determine the level of Aβ protein, but would also distinguish the isoforms $Aβ_{40}$ and $Aβ_{42}$. Nonetheless, it may be assumed that the results from the ICC of the PC12 cells represent the differential intracellular accumulation of Aβ between the APP_{Wt} and $APP_{Swe,Ind}$ clones; however, APPSwe staining has yet to be completed. In the normal human neuron, the percentage of $Aβ_{40}$ to total Aβ produced is 90% and $Aβ_{42}$ only 10% (19). In contrast, the APPSwe mutation causes a shift in the ratio to 20% $Aβ_{42}$, the APPInd mutation creates a 50/50 ratio $Aβ_{40}/Aβ_{42}$, and the $APP_{Swe,Ind}$ mutation results in almost solely $Aβ_{42}$ production (20). These investigations support the presence of increasing $Aβ_{42}$ levels within the produced cell lines. Finally, the continuing study of AD requires both an animal model which mimics the phenotype as well as a replicating cellular model. The types of cells used in vitro differ in the amplitude, magnitude and importance of their respective signaling pathways (21), hence it is difficult to produce a reliable cell line. While the rat PC12 cell line is neuronal in nature, it is not human. The McSA1 antibody used in these experiments is specific to human Aβ. Consequently, there is limited influence in the results from endogenously produced rat Aβ. Due to these collective limitations, the experiments are being repeated to confirm the reliability of the observed results.

The findings from this study can lead to a variety of future research endeavours. One possible direction will be to examine CRE-regulated genes implicated in synaptic plasticity using reverse transcriptase–polymerase chain reaction to compare gene expression levels between samples. The possibility of localizing the expressed Aβ protein within the cell to specific vesicles would also be of interest. Furthermore, empirical investigations to determine the form of Aβ protein accumulated by the cells should also be conducted, for example, by using the oligomeric recognizing antibody Nu1. Therapeutic testing could also be done. Therapeutic testing could be done as well, for instance by using the calcineurin inhibitor FK506 to compare its effects on varying levels of endogenously produced iAβ.

To conclude, the stably transfected cell lines of APP_{Wt}, APP_{Swe} and $APP_{Swe,Ind}$ in PC12 cells demonstrate that increased levels of the Aβ protein have a downregulating effect on the phospho-

rylation of the CREB protein, validating part of the hypothesis. While not replicated in these preliminary *in vitro* experiments, ERK phosphorylation is also disrupted in the presence of increased Aβ *in vivo*. This study, with its intended continuation, will help to further our understanding of the AD pathology and possible therapeutic remedies.

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