Cellular Neuroscience Practical November 2007 **Dr Jane Saffell**

Part I: Background

Effect of FGF2 and FCS on survival, proliferation and morphology of primary cerebellar cells and a cell line in culture

Introduction

Growth factors can have many different effects on cells, such as the stimulation of proliferation, survival, morphological changes or even apoptosis. Using simple, well-controlled *in vitro* cellular assays we can investigate the effects on cells of specific growth factors and the intracellular signalling pathways underlying these responses. We have talked about the importance of growth factors *in vivo* in the nervous system both during development and for the neuronal plasticity underlying learning and memory. It is important that researchers understand the mechanisms underlying these activities. In this project we will compare the effects of a specific growth factor (FGF2) and foetal calf serum (FCS) on cells in two different assays. The first is a quantitative colorimetric assay to monitor the effects of FGF2 and FCS on cell number over three days, cell number being dependent on survival and proliferation of the cells under the given conditions. We will also investigate the intracellular signalling pathways required for responses observed using well-characterised pharmacological inhibitors of three key signalling intermediates: PI-3 kinase, Erk MAP kinase and p38 MAP kinase. For convenience, we will use the 3T3 fibroblast cell line for this assay, but the assay would be equally suitable for nervous system cells such as purified astrocytes or oligodendrocytes. The second approach is a qualitative assay to monitor the effect of FGF2 on primary neurons, astrocytes and oligodendrocytes dissociated from rat cerebellum, using fluorescence immunocytochemistry to identify the specific cell types, and assess their relative number and morphology.

Aims

To design and execute your own experiments to:

- Determine the effect of FGF2 and FCS on cell number (proliferation and/or survival) of the NIH 3T3 fibroblast cell line.
- Determine whether effects stimulated by FGF2 and FCS are mediated via the signalling intermediates PI-3 kinase, Erk MAP kinase and p38 MAP kinase.
- Determine the effect of FGF2 on the morphology and relative numbers of neurons, oligodendrocytes and astrocytes present in primary dissociated cerebellar cells cultured for three days.

Assessment

This practical is worth 13% of the total mark for this course. (Practical 13% + Article 7% = 20% coursework total). It will be assessed from two pieces of work:

1. (10%) Research plan

This should set out the specific aims of YOUR experiment clearly and logically, and should not be longer than 3 sides of A4, at least 1.5 line spacing and 12 point text. What is the purpose of YOUR experiments? What concentrations of growth factors and inhibitors did you use? What controls have you included? What composition of 'basal medium' did you choose? How are the conditions you have chosen spread between your plates and slides (diagrams will help to illustrate this)? Include all pertinent details. (N.B. Write it as a PLAN, not a record of what you did, i.e. what you want to find out, what you plan to do, why and how, rather than what you did. If your actual experiment deviated from your plan, for example as a result of circumstances or a last-minute change of mind, give brief details of the differences and the reason for them).

2. (90%) Practical report in the form of a research paper, with Abstract, Results, Discussion, References

Abstract (5%) should be a summary of the WHOLE paper and include: background, rationale, approach, main findings, conclusions/significance of the findings. **(Word limit: 250 words)**

Introduction (5%) should be succinct, sufficient only to introduce the reader to the context and purpose of the work, and provide brief background information on factors and pathways to be tested. End with a short paragraph of a few sentences setting out for the reader what they can expect from reading on, i.e. how you approached the investigation, and your main findings. **(Word limit: 500 words)**

Results (60%)
You need a **commentary** for your results, not simply a string of figures with figure legends. I would recommend breaking up the results into sections with sub-headings. In order that the results section makes sense you need to make clear at the beginning of each section after the heading the nature of the experiment described (e.g. "…we carried out a quantitative colorimetric cell number assay…"), what it was trying to find out (e.g. "…to determine the effect of a range of FGF concentrations on 3T3 fibroblast proliferation over three days…"), and key elements of its set-up ("….cells were plated at a density of 12,000 per well in basal medium comprising…..in the presence and absence of……"). In the same way, it is helpful to include a sentence at the end of each section making clear what you are concluding before you move on the next section. The results section must include quantitative data presented in graphic form, qualitative data in the form of photographs, (both with clear figure legends) and must stand alone as a 'story'. This is the heart of your paper and should be built around the figures but with clear logical progression as you unfold the story in the commentary. For quantitative data, make full consideration of statistical significance of differences or changes you report. Data points should be expressed as mean +/- SEM (n). This will be covered in next week's lectures.

Discussion (20%)
What were your main findings? What can you conclude from your data? What further experiments or different approaches could be taken to elucidate the mechanisms you are investigating? Comment on the experimental design and possible improvements, particularly if your findings are difficult to interpret or you had problems. Highlight the implications of your results in the light of the published findings of others. **(Word limit: 800 words).**

(Methods: Do not write a methods section for your paper as the information is given in the handout)

The Research Plan and Practical Report must be handed in to the Student Office on or before Monday 14 January 2007.

Experimental Design

You will design your own experiments for this practical, in groups of three, during two days of guided, interactive tutorials on Thursday 29 and Friday 30 November. Your group must decide during this time what it is that it wants to discover: the effect of a range of FGF and FCS concentrations? More limited concentrations but over 1, 2 and 3 days? A range of inhibitor concentrations? The effect of combining inhibitors? etc. There are countless possibilities, but you cannot do everything, so the group will need to make decisions about what it is will and will not investigate, and design feasible and well-controlled experiments during the tutorials.

Practical Overview

Day	3T3 MTS assay	Primary cerebellar slides
Day 1 Mon 3 Dec	Plate cells in 96-well plates and incubate overnight (1 'Day 0 control' plate and up to 3 test plates)	
Day 2 Tues 4 Dec	Add growth factors & inhibitors etc to 96-well test plates and incubate for up to 3 days Add MTS to 'Day 0 control' plate you set up on Day 1	
Day 3 Wed 5 Dec	[Optional] Add MTS to test plate to determine cell number after 1 day in culture (if set up)	
Day 4 Thurs 6 Dec	[Optional] Add MTS to test plate to determine cell number after 2 days in culture (if set up)	
Day 5 Fri 7 Dec	Add MTS to test plate(s) to determine cell number after 3 days in culture.	Dissociate cerebellar cells and plate in 8-chamber Lab-Tek slides in different growth media +/- FGF
Day 6 Mon 10 Dec		Fix and stain cultures

Fluorescence Microscopy

Tues 11 Dec	9.30 – 1	Group Xa
Tues 11 Dec	2 – 5.30	Group Xb
Thurs 13 Dec	9.30 – 1.00	Group Ya
Thurs 13 Dec	2 - 5.30	Group Yb
Fri 14 Dec	9.30 – 5.30	Optional

Background information – What is available for the practical?

Cells available
NIH3T3 fibroblast cells (Monday)
Post-natal day four (PND4) rat cerebellum (Friday)

Assays
Colorimetric MTS 96-well plate assay (this is an **end-point assay**, i.e. the cells cannot continue to grow once the MTS assay has been done).
Immunocytochemistry for GAP-43, GFAP and O4 (see below)
Light and fluorescence microscopy

Plates and slides
4 x 96-well plates for 3T3 experiment
3 x 8-chamber Lab-Tek slides for cerebellar cells

Growth promoting supplements available
Fibroblast Growth Factor 2 (FGF2)	100 µg/ml stock
Foetal calf serum (FCS)	Neat

Signalling inhibitors available
PD98059	Inhibits Erk MAPK	Stock 20 mM
LY294002	Inhibits AKT/PKB	Stock 50 mM
SB203580	Inhibits p38 MAPK	Stock 50 mM

Culture Media available
DMEM
(Neat FCS is also available)

Antibodies available
All antibodies will be supplied to you as **x1 ready-to-use solutions**. You simply need to tell the demonstrator which antibody you require and what volume.
Primary:
Anti-GAP43 rabbit polyclonal (neurons)
Anti-GFAP monoclonal mouse IgG (astrocytes)
Anti-O4 monoclonal mouse IgM (oligodendrocytes). **Note: unlike all the other antibodies, this one only works on LIVE cells. The rest all work on fixed cells.**
Secondary:
Goat anti-mouse IgG conjugated to Alexa-488 (green)
Goat anti-rabbit IgG conjugated to Alexa-555 (red)
Goat anti-mouse IgM conjugated to Alexa-488 (green)

All other standard solutions (such as trypsin solution), PBS, etc and equipment, pipettes, etc necessary for the practical will also be provided of course.