In Vitro Investigations of the ACTH4-9 Analogue, Org2766

Ralph F. Murry
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Neuroscience and Neurobiology Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1994 Ralph F. Murry
IN VITRO INVESTIGATIONS OF THE
ACTH$_{4-9}$ ANALOGUE, ORG2766

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE NEUROSCIENCE GRADUATE PROGRAM
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

NEUROSCIENCE GRADUATE PROGRAM

BY
RALPH F. MURRY

MAYWOOD, ILLINOIS
JANUARY, 1994
ACKNOWLEDGMENTS

The efforts of a number of people across departments and institutions were needed for the successful completion of this work. In particular I express my thanks to Drs. Jerry McLane, Samuel Bowen, Robert Wurster, Mary Druse-Manteuffel and Talat Khan whose various efforts will remain appreciated. The willingness to suspend disbelief should not be under-valued.

"Can be discriminate, or, is be merely erudite?"

Sri Ramakrishna
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................ iii

LIST OF FIGURES ........................................ viii

LIST OF TABLES ........................................ x

LIST OF EQUATIONS ........................................ xi

LIST OF ABBREVIATIONS ................................ xiv

Chapter

I. INTRODUCTION ........................................ 1

II. LITERATURE REVIEW ................................ 3

Non-hormonal Activities of ACTH and Fragments ........ 3

Source of ACTH and α-MSH .................................. 3
Early Evidence for ACTH Effects on CNS Tissue .......... 3
ACTH Behavioral Effects ...................................... 4
Minimal ACTH Sequence for Behavioral Activity ......... 4
ACTH Fragments Affect CNS Protein Synthesis .......... 6
ACTH and Fragments Affect Cyclic Nucleotide Metabolism in CNS 8
Neurotrophic Properties of ACTH ....................... 9

Neurotrophic and Behavioral Effects of Org2766 ....... 10

Developmental Effects of Org2766 ......................... 10
Org2766 and PNS Mechanical Injury ....................... 11
Org2766 and PNS Chemical Injury: Rat & Human ....... 14
Org2766 and CNS Injury .................................. 18
Org2766 Affects Learning/Memory in Intact Animals .... 22
Org2766 and Aging ........................................ 26
Clinical Effects of Org2766 ............................... 27
Org2766: Mechanistic Studies ......................... 29
Conclusions ............................................. 34

Measurement of Finite Closed Curves .................... 35

Introduction ............................................... 35
Morphometric Approach .................................. 35
Fractal Geometric Approach ............................. 37
A Fourier Analytic Approach ............................ 40
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III. MATERIALS &amp; METHODS</td>
<td>44</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>44</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>45</td>
</tr>
<tr>
<td>Overview</td>
<td>45</td>
</tr>
<tr>
<td>Protocol</td>
<td>45</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>48</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>48</td>
</tr>
<tr>
<td>Overview</td>
<td>48</td>
</tr>
<tr>
<td>Protocol</td>
<td>49</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>51</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>51</td>
</tr>
<tr>
<td>Overview</td>
<td>51</td>
</tr>
<tr>
<td>Protocol:</td>
<td>52</td>
</tr>
<tr>
<td>Micro-incubation Environment</td>
<td>52</td>
</tr>
<tr>
<td>Experimental Treatment</td>
<td>54</td>
</tr>
<tr>
<td>Image Acquisition</td>
<td>55</td>
</tr>
<tr>
<td>Image Processing</td>
<td>56</td>
</tr>
<tr>
<td>Image Analysis</td>
<td>57</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>60</td>
</tr>
<tr>
<td>i) Fourier Analysis of ϕ(t)</td>
<td>60</td>
</tr>
<tr>
<td>ii) Area, Perimeter and Form Factor</td>
<td>61</td>
</tr>
<tr>
<td>iii) Fractal Dimension</td>
<td>62</td>
</tr>
<tr>
<td>IV. ORG2766 AMELIORATES THE IN VITRO CYTOTOXIC EFFECTS OF A VARIETY OF COMPOUNDS</td>
<td>64</td>
</tr>
<tr>
<td>Overview</td>
<td>64</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>64</td>
</tr>
<tr>
<td>i) 10⁻⁷ M Colchicine</td>
<td>64</td>
</tr>
<tr>
<td>ii) 10⁻⁵ M Colchicine</td>
<td>66</td>
</tr>
<tr>
<td>iii) 10⁻⁷ M Vincristine sulfate</td>
<td>67</td>
</tr>
<tr>
<td>iv) 10⁻⁵ M Vincristine sulfate</td>
<td>67</td>
</tr>
<tr>
<td>v) 10⁻⁷ M Cytochalasin D</td>
<td>67</td>
</tr>
<tr>
<td>vi) 10⁻⁵ M Cytochalasin D</td>
<td>68</td>
</tr>
<tr>
<td>vii) 10⁻⁷ M A23187</td>
<td>68</td>
</tr>
<tr>
<td>viii) 10⁻⁵ M A23187</td>
<td>68</td>
</tr>
<tr>
<td>ix) Org2766</td>
<td>69</td>
</tr>
<tr>
<td>V. ORG2766 STIMULATES PROTEIN SYNTHESIS IN NEUROBLASTOMA CELLS IN VITRO</td>
<td>71</td>
</tr>
<tr>
<td>Overview</td>
<td>71</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>71</td>
</tr>
</tbody>
</table>
VI. ORG2766 SPEEDS MORPHOLOGICAL RECOVERY FROM COLCHICINE INSULT

Experiment 1
Overview
Results and Discussion
  Mean % Change in Area
  Mean % Change in Perimeter
  Mean % Change in Form Factor
  Mean % Change in Fractal Dimension
dS/dt via Fourier Magnitudes

Experiment 2
Overview
Results and Discussion
  Mean % Change in Area
  Mean % Change in Perimeter
  Mean % Change in Form Factor
  Mean % Change in Fractal Dimension
dS/dt via Fourier Magnitudes

VII. SUMMARY & CONCLUSIONS

Summary
Conclusions

Appendix

A1 Fourier magnitudes of the first 300 harmonics at t=0
A2 Fourier magnitudes of the first 300 harmonics at t=5
A3 Fourier magnitudes of the first 300 harmonics at t=10
A4 Fourier magnitudes of the first 50 harmonics at t=0
A5 Fourier magnitudes of the first 50 harmonics at t=5
A6 Fourier magnitudes of the first 50 harmonics at t=10
B Reduction in per cent error (e) with increasing number of harmonics
C1 Change in cell shape as a function of time
C2 Change in cell shape as a function of time
C4 Change in cell shape as a function of time
C5 Change in cell shape as a function of time
<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6</td>
<td>Single time-lapse image of each of the five cells of Group AP3</td>
</tr>
<tr>
<td>C7</td>
<td>Single time-lapse image of each of the five cells of control group at P3</td>
</tr>
<tr>
<td>C8</td>
<td>Single time-lapse image of each of the five cells of control group at P1</td>
</tr>
<tr>
<td>C9</td>
<td>Single time-lapse image of each of the five cells of control group at P2</td>
</tr>
<tr>
<td>C10</td>
<td>Single time-lapse image of each of the five cells of Group AP1</td>
</tr>
<tr>
<td>C11</td>
<td>Single time-lapse image of each of the five cells of Group AP2</td>
</tr>
<tr>
<td>C12</td>
<td>Single time-lapse image of each of the five cells of Group GP2</td>
</tr>
<tr>
<td>REFERENCE LIST</td>
<td></td>
</tr>
<tr>
<td>VITA</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4.1</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-7} M colchicine and graded doses of Org2766</td>
</tr>
<tr>
<td>4.2</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-5} M colchicine and graded doses of Org2766</td>
</tr>
<tr>
<td>4.3</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-7} M vincristine sulfate and graded doses of Org2766</td>
</tr>
<tr>
<td>4.4</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-5} M vincristine sulfate and graded doses of Org2766</td>
</tr>
<tr>
<td>4.5</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-7} M cytochalasin D and graded doses of Org2766</td>
</tr>
<tr>
<td>4.6</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-5} M cytochalasin D and graded doses of Org2766</td>
</tr>
<tr>
<td>4.7</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-7} M A23187 and graded doses of Org2766</td>
</tr>
<tr>
<td>4.8</td>
<td>Per cent change in LDH activity vs. Control in the presence of 10^{-5} M A23187 and graded doses of Org2766</td>
</tr>
<tr>
<td>4.9</td>
<td>Per cent change in LDH activity vs. Control in the presence of graded doses of Org2766</td>
</tr>
<tr>
<td>4.10</td>
<td>Cell number following ten days of incubation in the presence of control media or 10^{-8} M Org2766</td>
</tr>
<tr>
<td>5.1</td>
<td>Per cent change in 35S-Met incorporation vs. Control in the presence of graded doses of Org2766</td>
</tr>
<tr>
<td>6.1</td>
<td>Average % change in area of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media</td>
</tr>
<tr>
<td>6.2</td>
<td>Average % change in perimeter of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media</td>
</tr>
<tr>
<td>6.3</td>
<td>Average % change in form factor of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media</td>
</tr>
<tr>
<td>6.4</td>
<td>Average % change in the fractal dimension (D_f) of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>6.5</td>
<td>Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media</td>
</tr>
<tr>
<td>6.6</td>
<td>Average % change in area of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.7</td>
<td>Average % change in perimeter of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.8</td>
<td>Average % change in form factor of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.9</td>
<td>Average % change in the fractal dimension ($D_f$) of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.10</td>
<td>Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.11</td>
<td>Net % change in area of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
<tr>
<td>6.12</td>
<td>Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after colchicine wash-out</td>
</tr>
</tbody>
</table>
## LIST OF EQUATIONS

<table>
<thead>
<tr>
<th>Equation</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 $FF = 4\pi \text{Area} / \text{Perimeter}^2$</td>
<td>36</td>
</tr>
<tr>
<td>2.2 $N(\varepsilon) \propto \varepsilon^\delta$</td>
<td>38</td>
</tr>
<tr>
<td>2.3 $L(\varepsilon) \propto \varepsilon^{1+\delta}$</td>
<td>38</td>
</tr>
<tr>
<td>2.4 $N(\varepsilon) \propto \varepsilon^{-D}$</td>
<td>38</td>
</tr>
<tr>
<td>2.5 $L(\varepsilon) \propto \varepsilon^{1-D}$</td>
<td>39</td>
</tr>
<tr>
<td>2.6 $f(x) = \exp(-</td>
<td>x</td>
</tr>
<tr>
<td>2.7 $\exp(-i2\pi vx)$</td>
<td>42</td>
</tr>
<tr>
<td>2.8 $F(\nu) = 2\left[1 + (2\pi \nu)^2\right]$</td>
<td>42</td>
</tr>
<tr>
<td>2.9 $F(x) = \sin[\exp(-</td>
<td>x</td>
</tr>
<tr>
<td>2.10 $F(\nu) = \frac{1}{N} \sum_{\tau=0}^{N-1} f(\tau) \exp(-i2\pi \nu \tau/N)$</td>
<td>42</td>
</tr>
<tr>
<td>2.11 $[\phi(l)]<em>N = \sum</em>{n=0}^{N} A_n \cos\left(\frac{2\pi nl}{L}\right) + B_n \sin\left(\frac{2\pi nl}{L}\right)$</td>
<td>43</td>
</tr>
<tr>
<td>2.12 $A_n = -\frac{1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \cos\left(\frac{2\pi nk}{L}\right)$</td>
<td>43</td>
</tr>
<tr>
<td>2.13 $B_n = -\frac{1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \sin\left(\frac{2\pi nk}{L}\right)$</td>
<td>43</td>
</tr>
<tr>
<td>2.14 $M_n = \sqrt{(A_n)^2 + (B_n)^2}$</td>
<td>43</td>
</tr>
<tr>
<td>3.1 $LDH\text{activity}[U/L] = \frac{\Delta A \times \text{min}^{-1} \times TV \times 1000}{6.22 \times SV \times LP \times 10^4}$</td>
<td>48</td>
</tr>
</tbody>
</table>
3.2 \[ z = \mu g \text{ protein per tube} = \frac{\text{O.D.}_{\text{sample}} - \text{O.D.}_{\text{blank}}}{\text{O.D.} / \mu g \text{ protein}} \]

3.3 \( \mu g \text{ protein per ml original sample} = z \cdot \text{dilution} \cdot (1 \text{ ml/vol used}) \)

3.4 \( \varepsilon = \frac{1}{L} \int_{0}^{L} \left| \phi(l) - \phi(l)_{\text{series}} \right|^2 \, dl \)

3.5 \( \phi(l)_{\text{series}} = A_0 + \sum_{n=1}^{299} A_n \cos \left( \frac{n\pi l}{L} \right) + B_n \sin \left( \frac{n\pi l}{L} \right) \)

3.6 \( \phi(l) = \arctan \left( \frac{\Delta y}{\Delta x} \right) \)

3.7 \( \Delta y = 0.5(y_p - y_{p-1}) \)

3.8 \( \Delta x = 0.5(x_p - x_{p-1}) \)

3.9 \( [\phi(l)]_N = \sum_{n=0}^{N} A_n \cos \left( \frac{2\pi nl}{L} \right) + B_n \sin \left( \frac{2\pi nl}{L} \right) \)

3.10 \( A_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \cos \left( \frac{2\pi nl_k}{L} \right) \)

3.11 \( B_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \sin \left( \frac{2\pi nl_k}{L} \right) \)

3.12 \( M_n = \sqrt{(A_n)^2 + (B_n)^2} \)

3.13 \( \frac{dS}{dt} = \sum_{n=1}^{300} \Delta M_n \)

3.14 \( \Delta A = \sum_{t=0}^{9} |A_{t+1} - A_t| \)

3.15 \( \Delta P = \sum_{t=0}^{9} |P_{t+1} - P_t| \)
<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.16</td>
<td>$\Delta FF = \sum_{t=0}^{9}</td>
</tr>
<tr>
<td>3.17</td>
<td>$\Delta D_t = \sum_{t=0}^{9}</td>
</tr>
<tr>
<td>3.18</td>
<td>$D_t = 1 + d$</td>
</tr>
<tr>
<td>3.19</td>
<td>$d = \frac{d \log_b (l(\varepsilon))}{d \log_b (\varepsilon)}$</td>
</tr>
<tr>
<td>3.20</td>
<td>$\varepsilon_i = 2^L \left( \frac{L}{N} \right)$</td>
</tr>
<tr>
<td>3.21</td>
<td>$d_i = \frac{-\log_b \left[ \frac{l(i+1)}{l(i)} \right]}{\log_b (2.0)}$</td>
</tr>
<tr>
<td>3.22</td>
<td>$d = \left[ \frac{dd(3) + dd(4)}{2} \right]$</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

α-MSH . . . . . alpha-melanocyte stimulating hormone
β-MSH . . . . . beta-melanocyte stimulating hormone
°C . . . . . . . degrees Centigrade
µCi . . . . . . microcurie
µg . . . . . . microgram
µl . . . . . . microliter
µM . . . . . . micromole
µm² . . . . . square micrometer
[³H] . . . . . . tritiated
1-D . . . . . one-dimensional
2-D . . . . . two-dimensional
2-DG . . . . . 2-deoxy-glucose
³²P . . . . . phosphorous-32
6-OHDA . . . 6-hydroxy-dopamine
A/D . . . . . analog to digital
ACTH . . . . . adrenocorticotropic hormone
ANOVA . . . . analysis of variance
Arg . . . . . arginine
ASCII . . . . American standard code for information interchange
ATCC . . . . American Type Culture Collection
ATP . . . . . adenosine tri-phosphate
ATPases . . . adenosine tri-phosphatases
B-50 . . . . . . growth associated protein-43
BID . . . . . . twice daily
BSA . . . . . . bovine serum albumin
Ca++ . . . . . . divalent calcium
cAMP . . . . . . adenosine 3',5'-cyclic monophosphate
CCTV . . . . . . closed circuit television
CD . . . . . . cytochalasin D
cGMP . . . . . . guanosine 3',5'-cyclic monophosphate
cm² . . . . . . square centimeter
CNS . . . . . . central nervous system
CO₂ . . . . . . carbon dioxide
cpm . . . . . . counts per minute
CPU . . . . . . central processing unit
CRF . . . . . . corticotropin releasing factor
CS . . . . . . conditioned stimulus
CSF . . . . . . cerebral spinal fluid
CuSO₄ . . . . . copper sulfate
d.c. . . . . . . direct current
DA . . . . . . dopamine
dB . . . . . . decibel
Dₙ . . . . . . fractal dimension
DMSO . . . . . dimethyl-sulfoxide
DNA . . . . . . deoxyribonucleic acid
DRG . . . . . . dorsal root ganglion
dS/dt . . . . . . shape as a time function
DSM III . . . . Diagnostic and Statistical Manual - III
DSM III-R . . . Diagnostic and Statistical Manual III-Revised

$D_T$ . . . . . . . . . . topological dimension

EBSS . . . . . . Earle's Balanced Salt Solution

$ED_{50}$ . . . . . . effective dose in 50% of sample

EDL . . . . . . . . extensor digitorum longus

EMG . . . . . . . . electro-myogram

F1 . . . . . . . . growth associated protein-43

FBS . . . . . . . . fetal bovine serum

FF . . . . . . . . form factor

g . . . . . . . . gravity

GAP-43 . . . . growth-associated-protein-43

GDP . . . . . . . guanosine di-phosphate

GFAP . . . . . . . glial-fibrillary-acidic-protein

Glu . . . . . . . . glutamate

Gly . . . . . . . . glycine

GTP . . . . . . . . guanosine triphosphate

h . . . . . . . . hour

$H_2O$ . . . . . . water

His . . . . . . . . histidine

HSD . . . . . . . Honest Significant Difference

HVA . . . . . . . homovanillic acid

HVAC . . . . . . 3,4-dihydroxyphenylacetic acid

Hz . . . . . . . . hertz

i.c.v. . . . . . . . . intracerebral-ventricular

I.D. . . . . . . inside diameter

i.m. . . . . . . intramuscular
i.p. ........ intraperitoneal
I.U. ........ International Unit
i.v. ........ intravenous
IgM .......... immunoglobulin
kB .......... kilobyte
$K_d$ ........ dissociation constant
kDa .......... kilodalton
kg .......... kilogram
L .......... liter
L2 .......... second lumbar vertebrae
LDH .......... lactate dehydrogenase
Lys .......... lysine
M .......... molar
MAP .......... microtubule associated protein
MEM .......... minimum essential medium
Met .......... methionine
mg .......... milligram
min .......... minute
ml .......... milliliter
mM .......... millimolar
mm .......... millimeter
mmol ......... millimole
MNCV .......... motor nerve conduction velocity
mo .......... month
mos .......... months
mRNA .......... messenger ribonucleic acid
NAD . . . . nicotinamide adenine dinucleotide
NADH . . . . reduced nicotinamide adenine dinucleotide
NaHCO₃ . . . . sodium bicarbonate
NaOH . . . . sodium hydroxide
NF . . . . . . neurofilament
nM . . . . . . nanomole
nm . . . . . . nanometer
NMJ . . . . . . neuromuscular junction
O.D. . . . . . . outside diameter
O₂ . . . . . . molecular oxygen
p.o. . . . . . per os
P/S . . . . . . penicillin/streptomycin
PC . . . . . . Personal Computer
PE . . . . . . polyethylene
Phe . . . . . . phenylalanine
PNS . . . . . . peripheral nervous system
POMC . . . . . pro-opio-melanocortin
RNA . . . . ribonucleic acid
s.c. . . . . . sub-cutaneously
SAR . . . . . . structure-activity-relationship
SC . . . . . . spinal cord
SDAT . . . . . . Senile Dementia Alzheimer's Type
SDS-PAGE . . sodium dodecyl sulfate - polyacrylamide gel electrophoresis
sec . . . . . . second
SEM . . . . . . standard error of the mean
SNCV . . . . . sensory nerve conduction velocity

xviii
T10 . . . . . . tenth thoracic vertebrae
TCA . . . . . . trichloroacetic acid
TIFF-G . . . . Tagged-Image-File-Format-Greyscale
Trp . . . . . . tryptophan
TTL . . . . . . transistor-transistor logic
UCS . . . . . . unconditioned stimulus
V . . . . . . . volt
VCR . . . . . . video cassette recorder
VCR . . . . . . vincristine sulfate
vs. . . . . . versus
W . . . . . . . watt
Zn$_3$(PO$_4$)$_2$ . . zinc phosphate
CHAPTER 1

INTRODUCTION

Central and peripheral nervous tissues embody and direct the motor, sensory and cognitive experience of all mammals. Damage to these tissues results in functional impairment which is only ameliorated upon repair of the damaged area or a shift in function to an undamaged area. Central nervous system (CNS) tissue is less adaptable to injury than peripheral nervous system (PNS) tissue, especially in the adult animal. While endogenous repair and remodeling mechanisms are crucial to a favorable prognosis following nervous tissue injury, exogenous repair aids can, depending on injury type and location, greatly improve functional outcome. Such exogenous interventions include the use of drugs, surgery, stimulation by electrical fields, acupuncture, diet and physical therapy.

Studies in recent times have shown that adrenocorticotropic hormone (ACTH) has significant neurotrophic properties when administered to rats with experimentally crushed sciatic nerve e.g. (Strand & Kung, 1980). These effects are enhanced in adrenalectomized animals. Structure-activity-relationship (SAR) studies reveal that the neurotrophic properties of the ACTH peptide reside within the first 13 amino acids e.g. (Lüneburg & Flohr, 1988). This same series of studies demonstrated neurotrophic effects of residues 4-7. A degradation resistant tri-substituted ACTH\textsubscript{4-9} analogue, Org2766, retains the neurotrophic properties of the parent peptide, but is devoid of corticotropic activity.

The present work describes the development of a cell culture model in which to investigate the effects of and mechanisms by which Org2766 protects and/or speeds recovery from damage sustained as a result of chemical insult. The cultures were derived from an immortalized murine neuroblastoma tumor cell line.
Three general approaches were taken. In the first, the cultures were exposed to either vincristine sulfate, colchicine (microtubule depolymerizing agents), cytochalasin D (microfilament depolymerizing agent) or A23187 (Ca\(^{++}\) ionophore) in the presence or absence of graded doses of Org2766. Cell media supernatant was then sampled at 8, 24 and 48 hrs and assayed for lactate dehydrogenase (LDH) activity. Extracellular LDH levels rise concomitant with damage to the cell membrane (or cell death); therefore, LDH levels were taken to correlate positively with toxic damage. The neurotrophic effects of Org2766 in this experiment result in reduced LDH activity relative to control conditions. A positive finding in the first experiment prompted a study to determine whether Org2766 could modulate the rate of \textit{de novo} protein synthesis. Cultures were pulsed with \(^{35}\)S-methionine in the presence or absence of graded doses of Org2766 for 60 min. Protein synthetic rates were then calculated from the combined results of total protein determined by the method of Lowry and a scintillation count to measure incorporation of the labeled methionine. Org2766 was found to stimulate protein synthesis in a dose-dependent fashion with \(10^{-8}\) M representing an optimal dose. The final approach utilized time-lapse video microscopy to measure morphological recovery of the cultured cells following colchicine insult under various Org2766 treatment conditions. Cells recovered more rapidly when treated with Org2766 irrespective of whether the peptide was administered before, during or after colchicine treatment.

At present, neither the \textit{in vivo} nor \textit{in vitro} neuroprotective mechanism(s) of action of Org2766 are known. The studies described here do show that the peptide can exert trophic effects directly on nerve-like cells in culture. This suggests that a primary \textit{in vivo} neurotrophic action may be directed at neurons, but does not rule out concomitant effects mediated by glial cells.
CHAPTER II
LITERATURE REVIEW

Non-hormonal Activities of ACTH and Fragments

Source of ACTH and α-MSH

Adrenocorticotropin [ACTH] and α-melanocyte stimulating hormone [α-MSH] are pituitary released peptides, with the latter consisting of the first 13 amino acids of the 39 amino acid ACTH molecule. ACTH is derived from the parent pro-opio-melanocortin peptide (Mains et al., 1977). In addition to their known corticotropic and melanotropic activities, both have been shown to exert tropic and trophic effects on mammalian PNS and CNS tissues, a topic of this review.

Early Evidence for ACTH Effects on CNS Tissue

As early as the 1950's, ACTH was shown to promote axonal regeneration in the spinal cord of the cat and dog (Windle & Chambers, 1950) and was thought to mimic the effects of Piromen (Windle et al., 1952b), a potent pyrogenic bacterial polysaccharide, which some claimed could enhance CNS regeneration. Piromen stimulates release of large quantities of pituitary hormones, including ACTH (Chambers et al., 1949). The resultant high levels of circulating adrenal corticoids were thought to reduce glial scarring (Oppenheimer & Reister, 1954), thus enhancing CNS axon regeneration (Windle et al., 1952a). More recent studies have failed to confirm these early findings regarding the stimulation of CNS regeneration by ACTH or Piromen (e.g. McMasters, 1962). Note that these unconfirmed early studies, however, were performed on animals with intact adrenals. Despite their shortcomings, these studies provided a rationale for performing additional studies on the effects of ACTH and derivatives on a number of behavioral and biochemical indices.
**ACTH Behavioral Effects**

In a seminal study, adenohypophysectomized male rats were trained in a one-way active avoidance shuttlebox task (De Wied, 1964). Those animals receiving sufficient ACTH replacement performed conditioned avoidance response behaviors at a level indistinguishable from sham-operated rats. Similarly, ACTH improved escape latency in a noxious stimuli runway task in the same set of experiments. This is one of the first published works demonstrating the involvement of ACTH in learning and behavior since the work of Mirsky (Mirsky et al., 1953). The behavioral effects of ACTH are not dependent on the adrenal hormones as ACTH has similar effects in adrenalectomized animals when measuring extinction of conditioned avoidance behaviors (Miller & Ogawa, 1962).

Structure-activity-relation (SAR) studies are crucial in the determination of the minimal peptide sequence necessary for induction of the behavioral effects of ACTH. \( \text{ACTH}_{1-16} \) is sufficient to produce corticotrophic activity of the \( \text{ACTH}_{1-39} \) molecule. Increasing peptide length from 17 to 23 residues only increases the potency of the molecule. Since the behavioral effects are not mediated via adrenal hormones, it was thought the behavioral effects might require less than the entire 39 residue sequence.

**Minimal ACTH Sequence for Behavioral Activity**

A series of experiments were performed, using male rats, to assess the minimal peptide sequence necessary to facilitate acquisition and extinction performance in a variety of behavioral paradigms as reviewed by De Wied (Greven & De Wied, 1973). All peptides were administered subcutaneously (s.c.). Dosing quantities and schedules were experiment-dependent.

Avoidance acquisition was tested in both a one-way shuttlebox (De Wied, 1964) (active avoidance) and a one-trial (passive) avoidance paradigm (Ader et al., 1972). The animals in the shuttlebox experiment were hypophysectomized and allowed to recover from the surgery prior to
testing. The peptides were administered on alternate days in the active avoidance experiment and as a single injection 1 h before the passive avoidance test.

Extinction trials were begun in the shuttlebox experiment after each rat had achieved a criterion of 80% or better avoidance responses for 3 consecutive days. Extinction trials were performed identically to the acquisition phase minus the footshock unconditioned stimulus (UCS) and the visible light conditioned stimulus (CS) was terminated after 5 sec in the absence of an avoidance response. Trials to extinction were also measured following training to criterion in an active avoidance pole-jump experiment in which the rat was presented with a CS (light) and then received a shock via the grid floor if the avoidance response (jumping onto and grasping a vertical pole) did not occur within 5 sec (De Wied, 1966). The pole-jump extinction trials were identical to the acquisition trials minus the footshock UCS. Only a single peptide injection 1 h prior to beginning of extinction was used in the pole-jump experiment.

Drug treatment conditions for the shuttlebox experiment were ACTH₁₋₃₉, ACTH₁₋₂₄, α-MSH (residues 1-13 of ACTH), ACTH₁₋₁₀, β-MSH (ACTH₁₋₁₈ plus the 3 amino acids preceding position 1), ACTH₄₋₁₀, sham-operated placebo and hypophysectomized placebo. All peptide conditions resulted in similar avoidance acquisition scores relative to sham-operated placebo conditions, with all scores roughly three times higher than hypophysectomized control animals.

In the experiment measuring extinction of shuttlebox avoidance behavior the peptides studied were ACTH₁₋₃₉ (1.5 I.U.), α-MSH (6 µg), ACTH₁₋₁₀ (10 µg), β-MSH (6 µg) and ACTH₄₋₁₀ (20 µg), all doses s.c. Avoidance behaviors took roughly twice as many trials to extinction in the peptide vs. saline condition (more trials to extinction is interpreted as an adaptive behavior in this paradigm).

The dosing and peptide regimen for the extinction of pole-jumping response experiment were ACTH₁₋₃₉ (3 I.U.), ACTH₁₋₂₄ (10 µg), α-MSH (6 µg), ACTH₁₋₁₀ (10 µg), ACTH₁₁₋₂₄ (10 µg), β-MSH (6 µg), ACTH₄₋₁₀ (20 µg) and saline control. Only ACTH₁₁₋₂₄ was ineffective in delaying extinction vs. control condition. Only four ACTH fragments were tested in the passive avoidance
test, 30 µg each of ACTH₁₋₁₀, ACTH₁₁₋₂₄, ACTH₂₅₋₃₉ and ACTH₄₋₁₀, as well as a saline control. Fragments 1-10 and 4-10 resulted in longer latencies relative to control, while 11-24 and 25-39 did not.

In the experiments described above only those peptides sharing a common core of residues 4-10 were effective in altering behavior relative to control. This relationship between peptide length and behavioral (biological) activity was studied in a more systematic fashion to determine the minimum sequence retaining the ability to enhance performance on the extinction of a pole-jumping avoidance response and on the response latency of a passive avoidance task (Greven & De Wied, 1967). The sequences looked at were ACTH₁₋₁₀⇒ACTH₇₋₁₀ with the shortening taking place one residue at a time from the amino-terminus. ACTH₄₋₁₀ was shown to be the shortest fragment that retains behavioral effects on these tasks with a potency similar to that of the parent molecule. A follow-up experiment by the same group shortened ACTH₄₋₁₀ one residue at a time from the carboxy-terminus. ACTH₄₋₇ was shown to be the shortest fragment with behavioral effects. Slightly higher doses, however, were required to equal the effects of ACTH₄₋₁₀.

The studies outlined above led researchers to search for ACTH analogues more efficacious with greater potency. One such analogue with a potency three magnitudes greater than that of ACTH₄₋₁₀ is a tri-substituted ACTH₄₋₉ analogue (later manufactured by Organon, International [Org2766]). The sequences of the two peptides are as follows:

\[
\text{ACTH}_{4-10}: \text{Met-Glu-His-Phe-Arg-Trp-Gly} \\
\text{Org2766}: \text{Met(O₂)}-\text{Glu-His-Phe-D-Lys-Phe}
\]

The potent behavioral effects of ACTH fragments led workers to more directly test for the involvement of CNS structures in the activity of these peptides.

**ACTH Fragments Affect CNS Protein Synthesis**

Male, hypophysectomized rats were treated with 20 µg s.c. of either ACTH₁₋₁₀₋₇-L-Phe, ACTH₁₋₁₀₋₇-D-Phe or ACTH₁₁₋₂₄ as a long-lasting zinc-phosphate preparation or saline every
other day for 12 days beginning 5 days post-surgery (Schotman et al., 1972). Radioactive incorporation experiments were performed following the last peptide treatment by injection of 20 µCi of [2-14C]-uridine or 20 µCi of [4,5-3H]-leucine into the diencephalon to measure RNA and protein synthesis, respectively. Animals were sacrificed either 70 min after uridine injection or 5 min after leucine injection. ACTH₁₋₁₀₋₇-L-Phe had no effect on rapid uridine incorporation; whereas, a 28% increase in rapidly labeled protein was observed. A 28% decrease in protein synthesis was observed when treated with ACTH₁₋₁₀₋₇-D-Phe. This is an interesting result in light of the finding that these two peptides exert opposite effects in an extinction of avoidance behavior paradigm (Bohus & De Wied, 1966); specifically, increased protein synthesis positively correlates with an inhibitory effect and the D-isomer peptide, which decreases protein synthesis, correlates with a facilitation of extinction of avoidance behavior. Consistent with this finding, ACTH₁₁₋₂₄ which does not have behavioral activity, had no effect on protein or RNA synthesis. Neither of the ACTH₁₋₁₀ analogues modulated RNA metabolism. These results are consistent with the idea that the behavioral effects of ACTH-like analogues are mediated via a translational vs. a transcriptional mechanism. The labeling kinetics of nuclear vs. cytoplasmic protein were also determined in this study. The labeled protein in the nuclear fraction was associated with ribosomes attached to the outer nuclear membrane suggesting that the newly synthesized protein in the nuclear and soluble cytoplasmic fraction are the same.

A follow-up study (Reith et al., 1974) looked at the in vitro effects of ACTH₁₋₁₀₋₇-L-Phe on brainstem slices obtained from hypophysectomized male rats. Drug protocols were similar to those described previously. First, hypophysectomy resulted in a 30% decline in [U-14C]-Leu incorporation vs. sham-operated controls. Peptide treatment reversed this decline at sub-nanomolar doses with a 39% increase seen at 10⁻⁵ M. These results are further evidence for a direct action of ACTH-like peptides on brain structures. A similar in vitro study compared ACTH₁₋₂₄, ACTH₁₁₋₂₄, ACTH₁₋₁₀₋₇-L-Phe and ACTH₁₋₁₀₋₇-D-Phe vs. control on labeled leucine incorporation into rat brainstem slices obtained from hypophysectomized rats. Consistent with
previous findings ACTH$_{1-24}$ and ACTH$_{1-10}$-7-L-Phe stimulated leucine incorporation while
ACTH$_{1-10}$-7-D-Phe decreased and ACTH$_{11-24}$ had no effect on protein synthesis (Reith et al.,
1975).

The administration of ACTH or β-MSH, i.p., increases protein synthesis in mouse
cerebrum, cerebellum, pons and medulla by 20 to 100% at 6, 12 and 24, but not 3, hrs post-
administration (Rudman et al., 1974). No effects on protein synthesis were observed in
thalamus, hypothalamus, liver, kidney, striated muscle or spleen. Uptake of [U-$^{14}$C]-leucine into
brain stem slices taken from hypophysectomized versus sham-operated controls demonstrate a
30% decrease in labeled protein (Reith et al., 1974). This decline could be due to the loss of
endogenous ACTH and ACTH-derived peptides.

**ACTH and Fragments Affect Cyclic Nucleotide Metabolism in CNS**

Early reports showed no effect of ACTH on adenylate cyclase activity in cell-free
membrane preparations (Burkhard & Gey, 1968; Von Hungen & Roberts, 1973) or on cAMP
accumulation in rat cerebral cortex slices (Form & Krishna, 1971). However, intrathecal
injections of μg amounts of ACTH or β-MSH increased cAMP, but not cGMP, levels in rabbit
cerebrospinal fluid (CSF) 30-120 min post-injection (Rudman, 1976). Obviously, the source of
the cAMP remains obscure in the *in vivo* study. In a follow-up study they showed that ACTH$_{1-24}$,
β-MSH and α-MSH stimulate the *in vitro* production and accumulation of cAMP in the choroid
plexus of the lateral, 3rd and 4th ventricles, pineal gland, sub-commissural organ and the area
postrema (Rudman, 1978). Rats chronically treated with α-MSH show elevated levels of cAMP
in occipital cortex of both intact and hypophysectomized animals (Christensen et al., 1976).
Chronic α-MSH had no effect on cGMP in rat CNS of intact animals, but did raise cGMP in the
thalamus of hypophysectomized rats (Spirtes et al., 1978).

ACTH$_{1-24}$ stimulates adenylate cyclase activity at 1 and 10 μM, but inhibits the enzyme at
higher concentrations in a CNS sub-cortical broken cell preparation. In the same study, cAMP
levels were shown to peak between 5 and 10 min post-ACTH$_{1-24}$ in rat striatal slices with levels
gradually descending towards control levels after 10 min (Wiegant & Gispen, 1975). No effects on cAMP concentrations were observed in other brain regions. These results cannot be taken as evidence that the cAMP second messenger system(s) mediate all or any of the neurotrophic properties of N-terminal ACTH fragments, but only suggest a possible role for cAMP.

Interestingly, the cAMP-sensitive phosphoprotein GAP-43 (also known as B-50 and F1), which at the cellular level stains most heavily in synaptic regions (Gispen et al., 1985), but is ubiquitous at the whole-brain level and is important in synaptic development and maintenance, is indirectly sensitive to ACTH fragments through the adenylate cyclase pathway (Matus et al., 1976). Those ACTH fragments which are effective in stimulating phosphorylation of GAP-43 also induce excessive grooming behavior in the rat (Gispen & Isaacson, 1980). Similarly, ACTH₁₋₂₄ stimulated the incorporation of ³²P into rat brain synaptosomal plasma membranes in a biphasic fashion. Millimolar concentrations of ACTH₁₋₂₄ decreased ³²P incorporation while µM amounts had no effect and nM concentrations inhibited phosphorylation (Zwiers et al., 1976). The net phosphorylation measured in the previous study is the combined result of phosphorylation by protein kinases, dephosphorylation by protein phosphatases and the depletion of labeled ATP by various ATPases.

**Neurotrophic Properties of ACTH**

ACTH has been shown to exert neurotrophic effects on dissociated neurons derived from chick embryo cerebral hemispheres. These neurons grow well in serum-supplemented, but not serum-free medium. In the absence of serum the cells develop vacuoles, retract their neurites and begin to fragment. ACTH prevents these degenerative morphological signs, increases protein and RNA synthesis and enhances 2-deoxyglucose uptake at ACTH concentrations ranging from 10⁻⁹ to 10⁻⁷ M, with 10⁻⁸ M being optimal for all effects (Daval et al., 1983). Neurons in the serum-free condition with no ACTH died within one to two days. There is no ready reason as to why ACTH increases RNA synthesis in this preparation and not those described previously.
As stated earlier, the failure of ACTH to induce neurotrophic effects in animal studies may have been related to the fact that ACTH causes release of steroid hormones produced in the adrenal glands. The steroids may have countered the neurotrophic properties of ACTH. To test this hypothesis, the sciatic nerve was unilaterally crushed in adrenalectomized and sham-operated control rats. ACTH treated, adrenalectomized rats recovered sensation and functional movement sooner than saline controls (Strand & Kung, 1980). Axonal regrowth proceeds at a faster pace and the number of large endplates and the frequency of preterminal branching increases. No effect was seen in either intact or denervated muscle.

Low-dose (10^{-8} M) \text{ACTH}_{4-10} and \text{ACTH}_{1-24} increase the density of the neural network, stimulates formation of neuronal aggregates and enhances neurite fasciculation of embryonic rat cerebral cells \textit{in vitro} (Richter-Landsberg \textit{et al.}, 1987). The same study also showed a 30% increase in acetylcholinesterase activity, with no changes seen in the acid-insoluble protein fraction.

**Neurotrophic and Behavioral Effects of Org2766**

**Developmental Effects of Org2766**

Both spontaneous activity and cold-stress-induced motor activity are increased three to five-fold in 11 to 13 day old rats following daily treatment with 10 \(\mu\)g/kg Org2766 i.p. (Acker \textit{et al.}, 1985). These levels of activity are not observed in the saline controls until day 14 or 15. Such findings are consistent with an acceleration of developmental events as regards motor behaviors and, presumably, central and peripheral motor systems. Consistent with the above, 10 \(\mu\)g/kg 24 h^{-1} of Org2766 increases twitch and tetanic tensions in the \textit{extensor digitorum longus} (EDL) muscle-peroneal nerve \textit{in situ} preparation of 15 day old rats (Acker \textit{et al.}, 1986). This study, however, failed to confirm the increase in spontaneous and cold-evoked behavior seen earlier by this group (Acker \textit{et al.}, 1985).

Even more direct evidence for an effect of Org2766 on developing motor systems is provided by an experiment showing an increased number of posijunctional folds and a greater
endplate area following 0.01 µg/kg/day of sub-cutaneously administered peptide from birth to day 14, at which time scanning electron microscopy was used to examine the neuromuscular junction (NMJ) (Frischer et al., 1985). These results are interpreted as an acceleration of maturation of the NMJ. Interestingly, nicotine had a similar effect. Whether the observed effects are primarily a result of protein synthetic regulation, nerve/muscle electrochemical stimulation or some other mechanism, is not certain.

Org2766, administered using the same regimen as in the Acker 1985 study, increased end-plate perimeter and nerve terminal branching during the first seven postnatal days at a dose of 0.01 µg/kg/day (Frischer & Strand, 1988). Only terminal branching remained elevated relative to saline control by day 14. No differences in any of the measured parameters was observed by day 21. These effects are very dose-dependent as 10 µg/kg inhibits nerve terminal sprouting at 7 and 14 days. It is difficult, however, to reconcile the inhibition seen at 10 µg/kg as the 1985 Acker et al. study noted an increase in spontaneous and cold-stress-induced motor activity at the same dose. Differential CNS effects are likely responsible. The results are of particular interest as the peptide effects correlate with the critical period for NMJ maturation. The effects stop at the timepoint corresponding with cessation of polyneuronal innervation of individual muscle fibres.

These results are consistent with a role for Org2766 in regulation of the development of the NMJ and perhaps in CNS structures.

**Org2766 and PNS Mechanical Injury**

Following unilateral crush of the sciatic nerve, female rats were tested for return of sensorimotor function using a foot-flick paradigm in which a 47°C air stream was applied to the sole of the foot and latency to retraction was measured. Three doses of sub-cutaneously injected Org2766 were used, 0.1, 1.0 and 10.0 µg, in either a long-acting zinc phosphate (Zn₃(PO₄)₂)-preparation or the unconjugated peptide every other day for the 18 day duration of the experiment (Bijlsma et al., 1983). The peptide significantly decreased latency to recovery at
1 and 10, but not 0.1 µg. Accelerated return of sensorimotor function was observed in 4, 8, 12 and 52 week old animals, but to a lesser extent in the one year rats. Restriction of peptide treatment to either the first or last 9 days inhibited the effect, suggesting that sensitivity to Org2766 is maintained throughout the entire recovery period. Of interest, the three carboxy-terminal residues of Org2766 (Phe\textsuperscript{7}-D-Lys\textsuperscript{8}-Phe\textsuperscript{9}) had no effect on recovery. The tripeptide is the major metabolic product of Org2766 (Witter \textit{et al.}, 1975), suggesting the neurotrophic properties do not reside exclusively in the carboxyl portion of the peptide. This is mentioned here as DeWied had noted a facilitating effect of Org2766 on passive avoidance behavior resides in the (low dose) amino terminal residues, while an inhibition of passive avoidance learning is induced by the tripeptide carboxy portion (Fekete & DeWied, 1982b).

In the crushed rat sciatic nerve, Org2766 increases the number of outgrowing neurites at 2, 3 and 4 days post-injury following a 1 µg s.c. injection immediately after and again 48 hrs after the crush (Verhaagen \textit{et al.}, 1987a). The increase in neurite number could be seen 0, 3 and 7 mm distal to the injury site at day 4 suggesting the increase was not solely due to sprouting occurring at earlier times vs. control. In the same study a single dose of Org2766 was administered immediately post-crush at either 0, 0.01, 1 or 100 µg and the number of neurites was measured at the distal border of the nerve on day 3. Both 1 and 100 µg doses were effective in increasing neurite number.

The dosing protocol was also shown to be an important factor, again using the number of outgrowing neurites as the dependent variable. The results showed 8 µg of Org2766 to be more effective if administered in two 4 µg injections 48 hrs apart vs. eight 1 µg injection separated by 12 hrs. The Verhaagen results suggest that it is the number of neurites, not the growth rate of new sprouts, which is affected. The dosing regimen study demonstrates that higher peak blood levels of Org2766 may be more effective than a more intermittent dosing schedule resulting in lower peak blood levels. These studies further indicate the importance of testing a range of
doses over a variety of administration protocols in order to determine an optimal dose of Org2766.

Route of peptide administration is also a key factor as regards the efficacy of Org2766. Using a recovery of sensorimotor function paradigm similar to that of Bijlsma and co-workers (Bijlsma et al., 1983), young adult female rats were administered Org2766 under the following protocols: two daily s.c. injections of 10 µg; 20-40 µg/day via subcutaneously implanted osmotic mini-pumps; 40 µg/day delivered by s.c. biodegradable micro-spheres; 1.5-20 mg/day via peptide dissolved in drinking water or by gavage (Dekker et al., 1987). Direct s.c. injections, mini-pumps and micro-spheres were equally effective in speeding recovery from sciatic crush. Neither administration via drinking water nor by gavage were effective.

Confirming the increase in neurite number following peripheral nerve crush in the presence of Org2766, rats were treated with 1 µg of peptide immediately after sciatic crush and again at 48 hrs (Verhaagen et al., 1987b). Using antibodies against α-MSH which selectively cross-reacts with the 150 kDa neurofilament protein, investigators were able to more accurately count neurites. Light microscopic, even electron microscopic, counting of neurites is difficult due to their small diameter (<1 µm) and the degenerative debris. Previous results showing an increase in neurite number were confirmed in this experiment.

Building on these results, the sciatic crush paradigm was performed using a similar dose regime and neurite counting and recovery of sensorimotor function assays employed 13 days after crush injury (Tonnaer et al., 1992). The independent variable was modified to include two types of nerve crush: 1) the standard crush with parallel-grooved forceps; 2) a crush with forceps having cross-hatched jaws. A positive peptide effect was only seen when the injury was made with parallel-grooved forceps. The endoneural tubes (epineurium) and Schwann cells appeared to receive less damage in this condition. A slightly larger axon diameter was also observed in the parallel-grooved condition. These results may be suggestive of a direct action of
Org2766 on Schwann cells as a primary mechanism of action because efficacy appears to correlate positively with Schwann cell survival.

Van der Zee et al. were able to replicate the return of sensorimotor function results in 6-7 week, 5 mo and 20 mo old rats (Van Der Zee et al., 1991) using 7.5 µg/kg 48 h⁻¹ s.c. Org2766. This is an important result as they demonstrate that Org2766 can also stimulate regenerative processes in aged animals. A direct comparison with α-MSH showed Org2766 to be as potent and as efficacious in accelerating recovery from peripheral nerve damage.

Laryngeal abductor reinnervation surgery was performed on ten cats, half of which received 25 µg/kg/48 hrs for 30 days prior to surgery. Both EMG and video-laryngoscopic recordings showed a trend towards earlier and more complete recovery in the Org2766 group. Histological evaluation showed no difference in myelin thickness and axon diameter. Axon number, however, was 26% higher in the peptide group (Mahieu et al., 1993).

Org2766 can also improve electrophysiological aspects of regenerating rat sciatic nerve (De Koning & Gispen, 1987). Functional recovery of sensory and motor parameters were accelerated by peptide, but both saline and peptide groups demonstrated complete recovery by 4 weeks. However, compared to the contra-lateral uncrushed sciatic, motor nerve conduction velocity (MNCV) measured by the M-reflex, and sensory nerve conduction velocity (SNCV) measured via the H-reflex, fully recovered in the presence of peptide by 90 and 120 days, respectively. The saline controls displayed a 20-40% deficit in both MNCV and SNCV as late as 214 days following crush. These results suggest functional measures may lack sensitivity in detecting recovery differences and that electrophysiological parameters should also be followed.

**Org2766 and PNS Chemical Injury: Rat & Human**

Cisplatin [cis-diaminedichloroplatinum (II)] has proven an effective drug in the treatment of ovarian, testicular and bladder carcinomas (Gerhenson et al., 1981). There exists a strong dose-response relationship between Cisplatin administration and clinical efficacy (Dembo, 1987) with some reports suggesting that larger doses of Cisplatin would be more effective (Ozols et
The development of a largely sensory peripheral neuropathy, however, limits Cisplatin to a dosage below the optimum (Legha & Dimery, 1985).

A preclinical model using the rat has shown a time and dose-dependent onset of peripheral neuropathy by measuring the H and M-reflexes (De Koning et al., 1987). Female 13 week old rats were treated with 1 mg/kg i.p. Cisplatin twice a week to a cumulative dosage of 19 mg/kg body weight. Furosemide (Lasix) was co-administered to prevent nephrotoxicity. After 47 days, corresponding to a cumulative Cisplatin dose of 13 mg/kg body weight, the SNCV began to slow in the Cisplatin/saline controls. By day 67 (19 mg/kg body weight) SNCV had slowed to 76% of saline controls. No slowing of SNCV was observed in Cisplatin/Org2766 animals. No change in MNCV was seen in either of the Cisplatin groups.

The De Koning et al. study also tested for interference of Org2766 with the anti-tumour activity of Cisplatin. Female Lou/M Wsl inbred rats were injected with IgM-immunocytoma cells on the left flank. Tumour growth was assessed twice weekly with vernier calipers and expressed as the mean of three perpendicular measurements. Cisplatin treatment began when tumour mean diameter reached 1 to 1.5 cm. Animals were then divided into three groups: Cisplatin plus saline; Cisplatin plus 10 µg Org2766 four times weekly; Cisplatin alone. No differences were found between groups indicating Org2766 does not block the anti-tumour activity of Cisplatin.

These findings were confirmed and extended demonstrating the neurotrophic actions of Org2766 in the presence of Cisplatin. In addition to preventing the neuropathy it was demonstrated that Org2766 blocks further deterioration and accelerates recovery of an already established Cisplatin-induced neuropathy in 12-13 week old rats (Van Der Hoop et al., 1988). Administration of the peptide during an initial Cisplatin cycle results in improved resistance to neurotoxicity during a second Cisplatin treatment cycle. The non-interference of Org2766 with the anti-tumour properties of Cisplatin were again demonstrated in this study.
In older rats, aged 7 mos, while Org2766 prevents onset of the peripheral neuropathy when administered concomitantly with Cisplatin, no effects are observed when the peptide is given to an animal with an existing neuropathy (Hamers et al., 1993a). The authors speculate that regenerative mechanisms are already maximally stimulated following 10 weeks of Cisplatin treatment in the older animals. However, others have shown that Org2766 can improve an existing neuropathy in streptozocin-induced diabetic rats at 10, but not 1 μg/rat every 48 hrs (Bravenboer et al., 1993).

Org2766 does not, however, influence the toxicity of Cisplatin in vitro on embryonic chick brain cell cultures (Bruinink & Birchler, 1993). The same study did show that Org2766 increased lysosomal activity, as assessed by neutral red uptake, and stimulated production of glial-fibrillary-acidic-protein (GFAP).

Clinically, Org2766 has proven effective in preventing onset of peripheral sensory neuropathy in women undergoing Cisplatin treatment for ovarian cancer (Van Der Hoop et al., 1990). The analogue was administered s.c. (note the importance of route of administration as discussed previously) at a dose of .25 mg (low dose) or 1 mg (high dose) per square meter of body surface area immediately before Cisplatin administration and again 24 hrs later. The chemotherapeutic cycle was repeated every three weeks (with the same Org2766 regimen) for six complete cycles.

The threshold of vibration perception, measured at the fingertips, was the main measure of neurotoxicity. The threshold value for vibration perception increased more than two-fold after four drug cycles in the placebo group. No change was observed in the high dose peptide group. At the end of six cycles of chemotherapy, threshold values had increased by a factor of eight in the placebo group, but only two-fold in the peptide group. Fewer neurologic signs were noted in peptide vs. placebo conditions as well. Protective effects were noted, but less prominent, in the low dose peptide group. No negative side effects were observed in either peptide condition.
A continued deterioration in vibration perception threshold values and clinical symptomology occurred in the patients described in the above study was observed after the last chemotherapeutic cycle. These changes were independent of treatment condition (peptide administration ceased with the last Cisplatin cycle). After 4 months of worsening of symptoms a gradual but incomplete recovery was observed at 12 and 24 months (Hovestadt et al., 1992). The deterioration, however, was less severe in peptide patients. Such results indicate peptide treatment should continue following cessation of Cisplatin administration. The data further suggest that Org2766 is effective in stimulating recovery long after the initial sprouting period.

Taxol has proven an effective anti-neoplastic agent of clinical importance (Rowinsky et al., 1990) via its ability to greatly enhance the stability of microtubule polymers (Schiff et al., 1979; Horwitz et al., 1986). The major current clinical indication of taxol is in treatment of ovarian cancer refractory to Cisplatin (McGuire et al., 1989). A largely peripheral sensory neuropathy, similar to that of Cisplatin, limits dose and treatment length. Using the rat/SNCV model, Org2766 completely blocks the deficit in SNCV observed in low (1.2 mg/kg 5 times weekly for 7 weeks) and high dose (9 mg/kg once a week for 6 weeks) taxol administration (Hamers et al., 1993b).

A double-blind, placebo-controlled trial showed that the peripheral neuropathy in patients undergoing vinblastine-vincristine combination therapy for Hodgkin's and non-Hodgkin's lymphoma could be greatly ameliorated by concomitant treatment with 2 mg s.c. Org2766 on the first and tenth day of a chemotherapeutic cycle (Van Kooten et al., 1992). Improvements were noted in vibration and temperature sense, a reduction in autonomic complaints and fewer motor and sensory disturbances following eight drug cycles.

Using electroreception in the catfish, Ictalurus nebulosus, as a model for vincristine-induced peripheral neuropathy, it was shown that Org2766 administered 2 days, but not 1 day, before local vincristine application, prevents the onset of the phase lag in electroreceptor organ afferent activity (Neuman et al., 1993).
Acrylamide, a synthetic resin, is a cumulative toxin that results in peripheral neuropathies in both man and other animals by an, as yet, unknown mechanism (Spencer & Schaumburg, 1974). Similar to Cisplatin, the neuropathy is of the dying-back type with the sensory fibres much more susceptible. Org2766 did not prevent the initial loss of motor coordination (due to the loss of sensory feedback) as assayed by the landing foot spread and footprint length analysis paradigms. The peptide did reverse the SNCV deficit when given concurrently with acrylamide, but had no effect when administration did not begin until after the last acrylamide dose. These results suggest a need for early peptide treatment of chemically induced peripheral neuropathies. While recovery of normal motor coordination, as measured, was unaffected by peptide, the animals were more resistant to a second acrylamide challenge, possibly indicative of a more complete recovery relative to saline controls.

In all, Org2766 has proven effective in preventing/ameliorating damage induced by chemical challenge of more than one type.

**Org2766 and CNS Injury**

Septal lesions in the rat results in hyperemotionality as indexed by a large number of behavioral parameters (Brady & Nauta, 1953) and facilitates acquisition and performance of shuttlebox active avoidance behaviour (Poplawsky, 1978). The hyperemotionality decreases progressively with time while the enhanced performance on the active avoidance task appears permanent.

Adult female rats received bilateral electrolytic lesions of the septal area after having been rated for emotionality on five rating scales: 1) reaction to object presentation; 2) response to tap on back; 3) resistance to capture; 4) resistance to handling; 5) vocalization to capture and handling (Isaacson, 1983). The peptide group received 1 μg Org2766 s.c. on the day of surgery and post-surgical days 1, 2 and 3. All animals were again rated for emotionality on days 3 through 12 in a blinded fashion. The two-way shuttlebox behavioral testing was conducted on day 35.
Mean emotionality was roughly half that of the saline control group in peptide treated animals. Both peptide and control rats made correct avoidance responses 82% of the time. However, the Org2766 group made significantly fewer inter-trial crossings. Inter-trial crossings are generally thought to reflect anxiety or some related hyperemotional state. Therefore, only those behaviors which were of an emotional nature were affected. The facilitation of the avoidance response was unchanged by the peptide. This study did not differentiate between whether the functional recovery was due to partial repair of the lesion area or to stimulation of compensatory mechanisms, though it is likely the latter due to the relatively short duration of the behavioral testing.

Adult male rats received bilateral electrolytic lesions of the parafascicular area of the posteromedial thalamus (Nyakas et al., 1985). Peptide groups were given s.c. injections of either 10 or 50 µg of Org2766 every 48 hrs beginning on post-operative day 3 and ending on day 17. On days 7, 13 and 19 the lesion and sham-lesion groups were tested for open-field behavior and T-maze reversal learning. In the open-field test 50 µg Org2766 decreased ambulation at days 13 and 19 relative to saline control in the sham-lesion group. No significant effects were noted in the 10 µg group. For the T-maze reversal learning task rats were trained to go (either) to the left or right arm of a T-maze on post-operative day 21. Animals with parafascicular lesions make a greater number of errors during the (day 22) reversal phase. Both 10 and 50 µg of chronically administered Org2766 reverse this deficit with 50 µg returning error numbers to near sham-lesion/saline levels. This reversal was not seen with a single (acute) peptide injection 1 hr prior to testing.

Functional recovery from aspirative cortical, cortico-hippocampal or sham lesions was followed in adult hooded rats (Hannigan & Isaacson, 1985). Animals were given either 1 µg s.c. Org2766 or saline daily for one week post-surgery. Training and testing in a food search task began on the day following the last peptide or saline injection. Rats with cortical lesions only, in general, were profoundly impaired in food search performance and were not aided by peptide
administration. Animals with cortical plus hippocampal damage were hyperactive, but learned the task quickly. Note that behavioral impairments observed in rats with cortical lesions are not seen in rats with both (overlying) cortical and hippocampal removal e.g. (Schmajuk et al., 1983). The deficits in the hippocampectomized animals were of the type most commonly thought to relate to attentional vs. memory processes. Such attentional processes are thought to be sensitive to emotive states.

Evidence has been reviewed above which is consistent with a role for Org2766 in regulation of emotionality. Org2766 dramatically improved performance using attentional measures in the study being discussed presently. Sham-operated rats treated with peptide showed a decrement in performance also indicative of impaired ability to attend to ongoing goal-oriented type behaviors relative to saline animals (this is contrary to the human behavioral data to be reviewed later). The authors conjecture emotional reactivity in the peptide-treated sham animals was lowered below an optimal level. Similarly, reduced emotionality may have resulted in improved performance on those tasks where high emotionality was thought to inhibit performance. However, it is not possible, using the present literature, to differentiate between effects of Org2766 on rat emotionality and possible effects on the reward value, if any, of the food. It is clear that Org2766 attenuates error in goal-directed behavior of rats in a food search task. The peptide can also result in a decrement in performance of control animals on those types of behaviors postulated to be significantly influenced by affective state of the animal.

Org2766 can also stimulate recovery following damage to the vestibular system. Frogs (Rana temporaria) underwent unilateral labyrinthectomy. Two groups of functional deficits are generally noted with such lesions: postural changes, such as curvature of the longitudinal body axis, head deviation across the long axis and changes in extensor/flexor tone; secondly, impaired dynamic vestibular reflexes. In general, the postural symptoms are greatly ameliorated through compensatory mechanisms while the dynamic deficits do not recover as well (Precht & Dieringer, 1985). Either .25 nM/kg/day or 1 nM/kg/day of Org2766 or saline was injected into the
dorsal lymph sac each day for 24 days following unilateral labyrinthectomy. Half-compensation times are operationally defined as the time over which the initial head deviation is reduced to 50% of that on the first day post-surgery. The half-compensation times serve as the dependent variable. Similar to the rat sciatic crush model, those frogs treated with either peptide dose demonstrated a dramatic acceleration of recovery. The compensatory mechanism is not known though the authors speculate a stimulation of collateral sprouts from remaining inputs in the deafferented vestibular nuclei.

A series of experiments was performed to determine the effects of Org2766 on functional and biochemical parameters following 6-hydroxydopamine (6-OHDA) lesions of the nucleus accumbens, a key area of the ventral striatal motor system in mammals. Functional recovery from lesion-induced motor hypoactivity is accelerated by daily s.c. or intra-accumbal injections with 10 µg/kg or 10 ng per left and right nucleus accumbens, respectively (Wolterink et al., 1990b). That is, the spontaneous recovery period of 3 weeks is shortened to 7 days by peptide administration. Following behavioral testing, the in vitro levels of dopamine (DA), homovanillic acid (HVA), a DA metabolite indicative of DA turnover, 3,4-dihydroxyphenylacetic acid (HVAC), another DA metabolite, and [3H]-DA uptake were found to be reduced to 30-40% of control levels in both control and peptide-treated animals (at least following 6 days of peptide treatment with measurements performed on the seventh day). A Scatchard analysis of [3H]-haloperidol binding of placebo or peptide treated animals on day 7 of sham operated animals suggested a single type of DA binding site. A similar Scatchard plot was found with placebo-treated 6-OHDA lesion animals. A curvilinear Scatchard plot was found for 6-OHDA/Org2766 animals suggesting induction of a second type of DA binding site by day 7. An enhanced behavioral response following apomorphine challenge in peptide-treated animals suggests induction of functional DA (denervation) supersensitivity by Org2766.

Further experiments revealed intra-accumbal, s.c. and per os (p.o.) Org2766 routes of administration were all effective as regards functional recovery from 6-OHDA lesions of the
nucleus accumbens (Wolterink & Van Ree, 1990) at doses ranging from 0.01 to 100 ng/kg for intra-accumbal injections, 0.01 to 10 µg/kg for s.c. administration and 1 to 10,000 µg/kg p.o. in a dose-dependent fashion. It is not known why p.o. doses are effective in this model. The greatly larger non-intra-accumbal doses needed to effect functional change indicate that Org2766 likely exerts its effects locally on accumbens tissue. If peptide treatment is delayed until the fourth post-operative day no beneficial effect is observed indicating the need for timely Org2766 administration in cases of CNS damage. A single injection of biodegradable micro-spheres releasing ~8-9 ng/kg/day Org2766 was also effective in acceleration of functional recovery from 6-OHDA lesions of the nucleus accumbens in rat (Wolterink et al., 1990a).

Org2766 is not effective in facilitating recovery from all types of CNS damage. Young adult rats received aspirative lesions of the medial or lateral occipital cortex. Lateral lesions have been found to have more severe functional consequences vs. medially placed lesions (Dean, 1990). A third sham-lesion group served as the control. Half of the animals were administered 25 µg of Org2766 s.c. beginning on the day of surgery and continuing daily until post-operative day 9 (Schmidt et al., 1992). All animals had been trained to criterion on a black vs. white stimulus card used as an indicator of escape platform location in a Morris water maze. Retention testing began 5 weeks post-operatively. A group was also included which was given peptide during the retention testing only. Neither immediate post-surgical nor concurrent peptide administration improved performance in this discrimination task. A group receiving peptide for the full seven week period would have been desirable in this experiment.

The authors speculate the failure of Org2766 to promote functional recovery from medial or lateral occipital lesions is because the nature of the impairment is neither memory nor attentional, but may best be characterized as sensory/perceptual. Since control animals do not recover from occipital lesions this may be indicative that the peptide only affects recovery processes that would have occurred in its absence.
Org2766 Affects Learning/Memory in Intact Animals

Low dose (50, 100, 200 ng s.c.) and high dose (500, 1000 ng s.c.) Org2766 have differential effects on a one-trial learning passive avoidance task whether administered pre- or post-learning (Fekete & De Wied, 1982a). Irrespective of administration schedule, low doses facilitated while high doses attenuated retention of a passive avoidance response. Both high and low doses of the peptide decelerated the rate of extinction of a pole-jumping active avoidance behaviour in the same series of experiments.

As described in an earlier section, the differential effects on the passive avoidance task can be attributed to different portions of the tri-substituted ACTH₄₋₉ molecule. It is not known why these differential effects are limited to the passive avoidance task though it is possibly due to involvement of only partially overlapping brain areas between the two behaviors.

Spontaneous motor activity was measured in normal and cold-stressed 13 day old rats pre-treated with 0.1 µg/kg/day Org2766 or saline from birth to the day before testing (Saint-Côme et al., 1982). Motor behaviour was quantified and categorized according to the following scheme: horizontal movements consisting of head waving, forelimb and hindlimb movements, wriggling, rolling, sniffing, pivoting, walking, running and grooming; ambulatory movements which were a subset of horizontal movements including crawling, walking and running; the number of stop and go movements. No peptide effects were found in non-stressed animals. When animals were placed on a cold metal plate (0°C) centered in the activity chamber, both peptide and control animals display an elevated level of motor activity after escaping the cold-stress. However, Org2766 pups maintained the high motor activity for the duration of the 5 min test period, while controls were only highly active for the first 2 min.

Because Org2766 is known to accelerate development of neural circuitry, perhaps the prolonged high activity under cold-stress is a function of this early maturation which becomes evident under stress. Depressed function and high metabolic rates such as that seen in developing and regenerating systems are the prerequisites for neuropeptide effects on
neuromuscular performance as reviewed in previous sections. The authors speculate that stress is an additional state in which the effects of Org2766 become evident.

The tendency of ambulatory rat neonates to return to their mothers when separated was used to study the influence of Org2766 on learning (Acker et al., 1985). The young animals were treated with 0.01 µg/kg/day or 10 µg/kg/day s.c. Org2766 or saline from days 1 to 8. The injections were changed to an i.p. route from day 9 onward including the onset of the learning acquisition phase. The learning task consisted of a T-maze with the mother placed in either the left or right goal box. A successful response was tallied when the pup went first to the goal box containing the mother. Pups treated with 0.01 µg/kg peptide, only, achieved the goal in half the time vs. controls and made less than half as many errors in doing so. During the extinction phase 24 hrs later low dose animals took significantly more trials to extinguish while maintaining faster trial times with fewer errors (i.e. peptide pups went to the arm to which they were trained to find their mother). Normal developing rats are known to run faster to the left than the right. This differential is due to an asymmetry in dopaminergic nigrostriatal (Zimmerberg et al., 1974) and cholinergic (Zimmerberg et al., 1974) function seen in developing animals. Abolition of the turning preference during the extinction phase in the .01 µg/kg group is interpreted by the authors as an acceleration of maturation of the brain systems involved in the asymmetry.

Housing adult rats singly and/or under intense light levels markedly decreases motor activity while group housing under low light conditions elevates motor activity. Subcutaneously administered Org2766 (ED₅₀: 0.01-0.03 µg/kg) 50 min prior to testing normalizes motor activity under both conditions (Wolterink & Van Ree, 1987). The normalization is blocked by systemic naltrexone suggesting the direct or indirect involvement of opioid systems. Perhaps these effects on motor activity are indicative of the previously described effects of Org2766 on the regulation of emotionality.

A followup by the same group showed that intra-amygdaloid injection of Org2766 produced the same normalizing effect (Wolterink & Van Ree, 1989) which, again, is blocked by naltrexone.
Intra-accumbal injections were not effective. Antibodies to endorphins also blocked the 
behavioral effects of Org2766, more directly indicating endogenous opioid systems are involved 
in the normalizing effect.

An odor discrimination paradigm in the rat quantifies the difficulty the animals have in 
rapidly reversing behavioral responses to previously learned odors. The difficulty positively 
correlates with an increase in the storage (depth) of olfactory information. Org2766 enhances 
this storage when administered at either 5 or 10 µg/kg immediately before or after the learning 
phase (Roman et al., 1989). Treated animals also demonstrated superior retention when re-
tested 24 hrs after meeting acquisition criteria.

The learning/memory literature, in toto, is vast and will not be reviewed here. While 
various dichotomies have been used to classify learning/memory sub-types, one particular 
scheme views learning/memory as either procedural (acquiring and performing skilled behaviors) 
or declarative (storage and retrieval of single facts and associations) (Schacter, 1985). 
Declarative learning/memory is thought to be probed in the odor discrimination paradigm.

Org2766 also has been tested for behavioral effects on humans in a non-clinical setting. 
Adult male volunteers, in a double-blind placebo study, were administered 40 mg Org2766 p.o. 
or placebo (Fehm-Wolfsdorf et al., 1981). In a two-stimulus reaction time paradigm the peptide 
was found to facilitate attention directed to one set of stimuli, but impaired shifts of attention 
between attentional sets. Similarly, in a constant foreperiod reaction time paradigm 40 mg 
Org2766 quickens reaction time (Rockstroh et al., 1981).

In a related double-blind study adult male volunteers received 40 mg Org2766 or placebo 
in a paradigm designed to test for distractibility (Rockstroh et al., 1983). This experimental 
design is a modification of the constant foreperiod reaction time test. An acoustic stimulus S1 
(pure sine tone of 1200 Hz, 65 dB) is presented for 6 sec and is immediately followed by tone S2 
(600 Hz, 65 dB), which is to be interrupted by the subject as quickly as possible by pressing a 
button. Having reached criterion performance level the test is repeated, but with 50% of the
trials a distracting background pulse is pseudo-randomly interspersed during the entire S1-S2 interval. All experimental sessions were scheduled at 1430 and 1630 hrs when endogenous ACTH secretion is low. No statistically significant differences were found between groups, but response latencies of the peptide subjects, with and without distraction, were ~10% longer suggesting an impairment of switching between attentional sets.

**Org2766 and Aging**

The rat hippocampus, which is rich in corticosterone receptors, undergoes reduced morphological correlates of aging following long-term adrenalectomy. Adrenalectomized animals not only have greatly reduced circulating levels of steroid hormones, but also display elevated serum concentrations of ACTH. Perhaps the elevated ACTH levels are responsible for the retardant effects of adrenalectomy on aging morphological correlates rather than reduced circulating steroids. To address this question, middle-aged rats were either adrenalectomized and chronically maintained with corticosteroids or left intact and chronically treated with s.c. Org2766 for 9 to 10 mos (Landfield et al., 1981). Both the peptide group and the animals maintained on steroids showed increased neuronal density, nuclear roundness, decreased gliosis and a lowered brain aging index, which is a composite of the listed variables, relative to aged controls. Of interest, several adrenalectomized animals exhibited markedly fewer brain aging signs vs. peptide animals suggesting steroids may interfere with peptide effects.

Aged rats demonstrate an attenuated glucocorticoid feedback response which results in chronically elevated circulating steroids (Sapolsky et al., 1984). The Type-I corticosterone-preferring receptor, located in highest densities in hippocampal and septal areas (Reul & de Kloet, 1985), is involved in the coordination and synchronization of such daily activities as exploration, food-seeking and sleep-related events (Bohus et al., 1982). The Type-II glucocorticoid receptor regulates glucocorticoid feedback repression of corticotropin-releasing-factor (CRF) and POMC pituitary release (Dallman et al., 1987).
To assess the effects of Org2766 on Type-I and Type-II glucocorticoid receptors, three and thirty month old rats were treated for two weeks with Org2766 via a 10 µg s.c. injection on day 1 and a s.c. implanted mini-pump releasing 0.5 µg/hr or saline control for 13 days (Reul et al., 1988). All animals were adrenalectomized on day 13 to deplete the receptors of endogenous corticosterone and were sacrificed 24 hrs later. In the aged control group, a 52 and 28% decline in Type-I and Type-II receptors, respectively, was noted in both hippocampus and septal areas. Org2766 induced a modest 8% increase in Type-I receptors in young rats, but a 68% increase in old rats. No changes were observed in Type-II receptors in young or aged animals. Neither were any alterations in Kᵰ seen for Type-I or Type-II receptors for corticosterone.

The effects of chronically administered Org2766 were investigated as regards the age-related degeneration of serotonergic fibres and on gliosis in the rat hippocampus and caudate-putamen. Eleven month old rats were s.c. injected with either 1 or 100 µg/kg Org2766 three times a week for 6-7 mos (Van Luijtelaar et al., 1992). The peptide had no effect on the incidence of age-related degeneration of serotonergic fibres in the hippocampus or caudate-putamen. Neither did Org2766 demonstrate neurotrophic properties when administered to young rats with hippocampal lesions implanted with fetal serotonergic cells. Staining for glial fibrillary acidic protein (GFAP) revealed no changes in gliosis in peptide vs. control.

Clinical Effects of Org2766

Patients with Senile Dementia Alzheimer's Type (SDAT) as defined by the Diagnostic and Statistical Manual III (DSM III) were studied in a double-blind, placebo-controlled study (Kragh-Sorensen et al., 1986). Subjects were excluded with confounding medical or psychiatric conditions. Those patients on psychoactive medication were also excluded. Only those individuals with mild to moderate dementia were used. Subjects received placebo, 5, 20, 40 or 80 mg Org2766 p.o. per day for 28 days. Org2766 was found to have a small but significant therapeutic effect as assessed by the Sandoz Clinical Assessment Geriatric Scale. However, only 25% of the subjects displayed improvement and no dose-response relation was observed.
These results suggest the need for studies of a much longer time-course. The degenerative processes resulting in the clinical dementia likely develop over many years and it is not reasonable to expect a neurotrophic agent to show maximal effects, if any, following a 28 day treatment period.

A recent study looked at larger doses (40, 60 and 80 mg/BID p.o. for 12 weeks) of Org2766 in a double-blind study similar to the study outlined above (Miller et al., 1993). High-dose peptide resulted in significantly faster reaction times on behavioral tasks and a clinically defined improvement in alertness relative to control. No effects were seen on the Global Deterioration Scale designed to assess overall cognitive and affective function. This study shows that high oral doses of Org2766 are well tolerated, but longer treatment periods may yet be indicated.

In a double-blind, placebo controlled study, patients diagnosed with either panic disorder, generalized anxiety disorder or social phobia were treated for six weeks with 80 mg Org2766 p.o. (Den Boer et al., 1992). A small reduction on the Hamilton Anxiety Scale was observed in the panic disorder group with no change in the number of attacks (as in the den Boer (1989) study). No anxiolytic effects were seen in the generalized anxiety or social phobia subjects.

Adult male and female subjects diagnosed with panic disorder (DSM III-R) with and without agoraphobia were treated with 40 mg/BID Org2766 in a double-blind, placebo controlled study (Den Boer et al., 1989). While the peptide had no effect on the number of panic attacks, it did lessen the anxiety associated with the disorder much as in the previous study.

Autism is a child-onset persistent psychiatric disorder characterized by deficits in social interaction including stereotyped behavior patterns, abnormal cognitive, language and communicative function (Cohen & Donnellan, 1987). Daily treatment with 40 mg/day Org2766 p.o. in a double-blind, placebo-controlled, cross-over study resulted in improved eye contact, disconnection of verbal initiative (e.g. putting a question or changing a topic of conversation) from stereotypes (e.g. repetitive asking of a question) and connection of verbal initiative to non-
verbal gestures (e.g. pointing or showing). Additionally, psychiatrists and psychologists not associated with the experiment noted substantial improvement on clinical scales. Parents reported a decrease in social withdrawal behaviors at home. A second controlled cross-over trial resulted in lessened social withdrawal and improved play behaviour (Buitelaar et al., 1992).

**Org2766: Mechanistic Studies**

A seminal study investigated the effects of substitutions in the ACTH$_{4-9}$ molecule on the extinction of an active avoidance behaviour in intact rats (Witter et al., 1975). The previously described introduction of 4-Met-sulfoxide, 8-D-Lys and 9-Phe resulted in a 1000-fold increase in behavioral potency with a concomitant 1000-fold decline in melanotropic activity. Since there exists a strong positive correlation between behavioral activity and the *in vitro* half-life of substituted ACTH$_{4-9}$ it is believed that resistance to bio-transformation explains, in part, the increased potency of the analogue.

Uptake of [³H]-Org2766 into specific brain regions of the rat were studied via i.c.v. injections of the labeled peptide (Verhoef et al., 1977). Intra-cisternal (cisterna magna) or peripheral administration of the peptide resulted in inadequate brain labeling likely due to CSF flow dynamics and degradation, respectively. All major brain regions were then assayed for peptide using quantitative autoradiography. Highest uptake occurred in the septal area, thalamus and hypothalamus with equal labeling observed in most other brain regions including hippocampus and basal ganglia. The lack of high uptake in hippocampus and basal ganglia is somewhat surprising given the involvement of these areas in peptide effects in previously reviewed studies. The answer may, in part, be that high uptake may not necessarily correlate strongly with functional effects. Although there is a positive correlation between peptide uptake and distance from the ventricular system, this does not explain all of the variance as other major unlabeled structures reside adjacent to the ventricles, yet do not label heavily (e.g. caudate-putamen). [³H]-Phe was used as a control and showed more even labeling vs. Org2766. This suggests that the uptake and/or binding sites for labeled Org2766 are distinct from those which
result in labeling with the single residue. This study is suggestive only as preferential uptake does not necessarily indicate site(s) of action. Uneven labeling may largely be due to differences in accessibility and uptake capacity for the peptide. Likely the most important factor in peptide activity is its availability in optimal amounts at appropriate sites. Other labeling may be irrelevant or antagonistic as regards a specific effect.

Related to the above study, local cerebral glucose utilization was studied in rat brain following 100 µg/kg/day i.p. Org2766 using an autoradiographic 2-deoxyglucose (2-DG) technique (McCulloch et al., 1982). Significant increases in glucose utilization measured as 2-DG uptake were observed only in the hippocampus (stratum moleculare lacunosum and parasubiculum show increases in 2-DG uptake of 16 and 17%, respectively), anterior nucleus of the thalamus (23%) and anterior cingulate cortex (30%) of the 49 anatomically distinct brain areas studied. Note that no changes were observed in the septal area, though a non-significant 13% increase was observed in the lateral septal nuclei.

Interestingly, all of the areas showing elevated glucose utilization are classically defined as belonging to the limbic system (a system important in learning/memory, emotive, motivational phenomena). No effects were seen in basal ganglia (motor integrative/output system) or cortical areas involved in visual, auditory or somatosensory processing. This study provides no definitive information as to the primary site(s) of action of Org2766, but is merely suggestive of the functional involvement of these areas in peptide effects.

Of importance, the parasubiculum is the origin of major hippocampal projections to the anterior thalamic nucleus (Carpenter, 1976) while anterior cingulate cortex and anterior thalamic nucleus have extensive reciprocal projections (Domesick, 1969). This hippocampal, thalamic, cingulate circuit could provide the anatomical basis for the motivational hypothesis of ACTH analogue action as proffered by De Wied (De Kloet & De Wied, 1980). In support of this, cingulate cortex interactions with the anteroventral thalamus have been shown to be important in learning and behaviour (Gabriel et al., 1980).
The 150 kDa neurofilament subunit has been shown to contain an immunologically recognized α-MSH segment (Dräger et al., 1983). This is the same protein that has been shown to break down in the early stages of peripheral nerve degeneration (Bignami et al., 1981) coinciding with the onset of neurite regrowth. Degenerating peripheral nerve contains a factor that is active in a MSH bioassay (Edwards et al., 1984) that is not expressed in control nerve. This, as yet, unidentified protein with α-MSH-like growth promoting activity may be a breakdown product of the 150 kDa neurofilament subunit. It is conceivable that Org2766 mimics this endogenous protein since both α-MSH and Org2766 share a common, but modified, primary sequence.

The in vitro binding of [3H]-Org2766 to rat spinal cord sections as assessed via quantitative autoradiography reveals an uneven binding distribution across spinal cord structures which is only partially displaceable by cold peptide (Dekker & Tonnaer, 1989) possibly indicating the involvement of a non-classical binding site or, alternatively, non-specific binding. Labeled peptide was not naloxone-displaceable making the binding site distinct from the opiate receptor. A left-right difference was observed in spinal cord dorsal horn binding 6 days following unilateral sciatic crush at level L2, but not T10. Only the L2 binding sites were displaced by cold peptide with neither L2 nor T10 peptide binding being naloxone displaceable. This differential disappeared at 28 days corresponding with return of sensorimotor function. The relation, if any, between CNS Org2766 binding and regeneration of crushed sciatic nerve is not made clear by this experiment. Perhaps the peptide is binding afferent neurites or glia in the dorsal horn.

A partial answer is provided by a study measuring the biotinylated-Org2766 (b-Org2766) binding to in vitro dorsal root ganglion (DRG) and spinal cord (SC) cultures (Van Huizen et al., 1991). Displaceable b-Org2766 binding occurred on neurofilament containing cells with round somata and fine processes in both culture types. In comparison with nerve growth factor (NGF), Org2766 had little effect on neurite outgrowth in DRG cultures. The authors did report, in the same abstract, preliminary evidence that neurite branching may be altered by Org2766.
Delayed neuronal death of non-dissociated DRG and SC cultures obtained from embryonic (day 15) rats is reported in the presence of Org2766 (Lee et al., 1991). The same abstract reports preliminary evidence that Org2766 may stimulate neurite outgrowth in these cultures, contrary to the findings of Van Huizen and co-workers.

In contrast, others have shown that α-MSH and ACTH$_{1-24}$ stimulate synthesis of GAP-43 and neurofilament dose-dependently at 48 and 96, but not 24 hrs in dissociated fetal rat spinal cord neurons (Van Der Neut et al., 1992); whereas, Org2766 had no effect on these parameters. In DRG cells in vitro in the same study, α-MSH increased neurofilament levels by 22% and GAP-43 by 54% with an inverted-U shaped dose-response curve. Neither ACTH$_{4-10}$ nor Org2766 were effective in changing GAP-43 or neurofilament levels in either of these preparations. Alpha-MSH, but not Org2766, induces expression of c-fos, one of the immediate early gene proteins implicated in a variety of processes related to cell change, in dissociated rat spinal cord neurons at dose ranging from $10^{-13}$ to $10^{-4}$ M (Hol et al., 1993a). The authors chose this particular culture system because, of the two peptides, only α-MSH has a trophic effect on these neurons. This is suggestive that c-fos may be involved in the mediation of the neurotrophic effect of α-MSH. Further studies are needed to ascertain whether those in vitro systems responsive to Org2766 also show an increase in c-fos mRNA. This result is of particular interest, as the same group showed in a later study that both α-MSH and Org2766 induce an increase in cAMP-activity in dissociated rat spinal cord neurons (Hol et al., 1993b).

Large axons of the cerebral commissure of Lymnaea Stagnalis are stimulated to produce a 30% increase in the number of microtubules in vitro in the presence of $10^{-8}$ M Org2766 (Muller et al., 1992), with $10^{-8}$ M producing a maximal effect. Log doses one to three magnitudes greater produced no further increase in the number of microtubules. A dose of $10^{-9}$ M had no effect on microtubule number. This experiment does not reveal whether the mechanism for the effect involves tubulin synthesis, increased microtubule assembly or a stabilization of existing microtubules. However, ultrastructural data shows alterations in glial cell morphology such as an
increase in the number of filament bundles, the presence of irregular circular structures resembling smooth endoplasmic reticulum and less pronounced chromatin condensation along the nuclear envelop suggesting a more general effect of Org2766 on CNS tissue.

Org2766 showed no effect on nerve cell differentiation as indicated by changes in microtubule-associated-protein-5 (MAP-5) and the 68 kD neurofilament (NF 68 kD) subunit in embryonic chick brain cell cultures (Bruinink & Birchler, 1993) indicating that these growth-associated proteins are not affected. This study, however, used 400 µM Org2766; lower doses were not tested. The inability of Org2766 to lessen Cisplatin toxicity in the same in vitro study may indicate that compounds working directly at the DNA level are more resistant to the protective effects of Org2766, though others have shown that the peptide does ameliorate Cisplatin neurotoxicity in an in vivo rat model (De Koning et al., 1987). The Bruinink study did, however, show significant delayed toxicity of Cisplatin on the culture even 192 hrs after Cisplatin wash-out. A significant increase in GFAP, but not MAP-2 was noted at 10^0 and 10^{-1} M, but not at lower doses. These results indicate the importance of dose-response testing when conducting studies using Org2766.

Fast anterograde axonal transport rates in normal rat sciatic nerve is not altered by 7 day treatment with either 1 or 10 µg/kg/day i.p. Org2766 (Crescitelli et al., 1989) following injection of {\[^3\text{H}\]-Leu into lumbar spinal cord. Crest height, indicative of the quantity of transported labeled protein, was decreased 62 and 64%, respectively, at 1 and 10 µg/kg/day. Damaged nerve, unfortunately, was not used with Org2766 in this study. ACTH_{4-10}, however, changes neither rate nor crest height in regenerating sciatic nerve. The crest height is doubled by ACTH_{4-10} in undamaged nerve, though a large variance prevents statistical significance.

Rats receiving intra-accumbal injection of control serum during the first week following 6-OHDA lesions display similar levels of motor activity at 3 weeks post-lesion as sham-lesion with control serum (Wolterink et al., 1990c). Rats with similar lesions treated with antisera to α-MSH or Org2766 fail to recover at 3 weeks. The antisera had no effect on sham-lesioned animals.
Apomorphine challenge in recovering lesion animals treated with control serum results in increased motor activity, but not in animals administered antisera. This result and the absence of elevated [³H]-haloperidol binding in nucleus accumbens of antisera treated animals is indicative of the lack of development of denervation supersensitivity. This evidence is suggestive that Org2766 and other MSH-like analogues may mimic endogenous peptides which mediate CNS recovery processes.

Though GAP-43 is generally thought to be a neuron-specific protein, the mRNA has been found in the sciatic nerve of control rats (Plantinga et al., 1993, 1992) indicating localization in Schwann cells. The message is up-regulated in DRG 18-fold following crush injury. Surprisingly, mRNA message increases dramatically in the distal nerve stump 2 days following crush or transection, remaining elevated for the period corresponding with return to complete sensorimotor function. No changes in message are observed in the proximal portion of injured nerve. In situ hybridization showed heavy staining for GAP-43 mRNA adjacent to Schwann cell nuclei. These results were confirmed by Northern blot analysis.

Transcription of GAP-43 message appears, therefore, to be regulated by Schwann cell interactions with nerve cell axons. Given the previously reviewed evidence for regulation of protein synthesis by ACTH and derivatives, perhaps Org2766 stimulates GAP-43 expression in DRG and in Schwann cells distal to the lesion site as a (partial) mechanism of action in recovery from peripheral nerve mechanical damage. Of interest, there is evidence of developmental regulation of GAP-43 in rat neonatal cortex. GAP-43 also remains at detectable levels in adult rat cortex and co-migrates with B-50, a synaptic membrane protein which is a preferred substrate of protein kinase C in adult brain (Jacobson et al., 1986). In line with the action of Org2766 at the lesion site it has been shown that transected rat sciatic nerve wrapped in Accurel™ tubing impregnated with α-MSH (tubing restricts peptide to lesion site) accelerates functional recovery (Edwards et al., 1986).
Conclusions

Evidence has been reviewed here implicating Org2766 in a variety of developmental, regenerative, behavioral, aging and degenerative phenomena. The mechanisms by which these effects are produced largely remain to be elucidated. The experiments described in this work test for neurotrophic effects of Org2766 on a nerve cell-like immortalized cell line. These in vitro studies have the advantage of controlling for effects on glial cells and/or other systemic actions of the peptide. Disadvantages include the limited generality of any findings as these are transformed cells operating in the absence of the native systemic environment.

Measurement of Finite Closed Curves

Introduction

For the purposes of the present work a finite closed curve may be conceptualized as a line segment of determinable length having the same start and end-points with the further stipulation that it be non-intersecting, though these criteria obviously do not necessarily hold for all closed curves. The curves considered here are embedded in two-dimensional Euclidean space; hence, each point of the curve can be referenced by two descriptors such as two real numbers corresponding with positions on the x and y-co-ordinate axes of a Cartesian plane. The intersection of the perpendiculars to the axes for each co-ordinate x-y pair maps the positions of the points in two-space.

The closed curves of interest here define the 2-D contour of well-differentiated nerve cell-like tumor cells grown in culture at the peripheral attachment points of the cell to the culture dish. The problem arises as how to quantifiably define these shapes as well as the first time derivative of the shape. Traditional approaches within the cell morphometry literature are discussed in light of their relative advantages and disadvantages.

Morphometric Approach

A common approach to quantifying changes in cell morphology is to measure cell area or the change in area over time e.g. (Davies & Cox, 1991). Others have measured neurite length
or change in length as a function of time e.g. (Gupta et al., 1987). The enumeration of cell structures of a particular type such as veil, filopodia e.g. (Burmeister & Goldberg, 1988) or growth cone configuration (Aletta & Greene, 1988) has also been done, as well as the mere listing of the presence or absence of structures of a type e.g. (Yong et al., 1988; Van Der Neut et al., 1988). The dynamic extension/retraction of filopodia (Sheetz et al., 1992) and single axons (Jhaveri et al., 1991) has also been studied. In addition, various morphological qualifying schemes have been employed such as defining a given morphology as to roundness, thinness and branchedness (Luckenbill-Edds & Kleinman, 1988).

Each of the above methods has demonstrated a degree of utility correspondent with individual applications. The size measurements such as area and length, however, fail to encode any shape information. The qualifiers (e.g. branchedness), while providing some intuitive indice relating to shape fall short of specifying shape information in terms that are useful in making comparisons within or between groups of contours.

The form factor (FF) has also been used to quantify the relative complexity of a closed contour. This measure is operationally defined as:

\[
FF = \frac{4 \pi \text{Area}}{\text{Perimeter}^2}
\]

For a circle of any radius, FF is equal to 1. As a shape tends towards the infinitely complex, FF approaches zero in the limit. However, while FF does provide information as to relative complexity, it suffers for its lack of ability to provide a unique measure. That is, there are an infinite number of shapes with identical form factors. The form factor is, in fact, merely a reformulation of size data (area and perimeter); hence, contains no shape information.

The landmark approach to morphological characterization has been well developed (Bookstein, 1991). Landmarks are reproducibly recognizable features of a contour or surface. The shape or changes in shape can then be quantified by changes in location of general and specific features or points in relation to the landmark points. This method is of little use in the
description of the closed curves of concern here as candidate landmark features tend to be transient if existent.

**Fractal Geometric Approach**

The geometry of Euclid serves well in describing and defining many curvilinear structures, especially those forms and surfaces associated with architecture and engineering. The Euclidean framework, however, is often inefficient or ineffective in describing natural entities such as PNS or vascular geometry in the spatial domain or the probabilistic opening/closing of an ion channel in the temporal domain.

When studying the spatial or temporal characteristics of these phenomena a repetitiveness in form and spacing, respectively, can be observed that is resolution independent (Barnsley *et al.*, 1986). A geometry has been developed to describe and analyze such fragmented non-Euclidean structures. This mathematical framework is commonly referred to as *fractal geometry* (Tsonis & Tsonis, 1987).

A brief discussion of *dimension* (*D*) is necessary in order to explain the concept of a fractal and the accompanying *fractal dimension* (*D_f*). In general, there are two basic definitions of dimension: Euclidean dimension (*D_E*) and topological dimension (*D_T*), both of which can assume only integer values. For a given object *D_E* and *D_T* cannot be identical. Cuts, called surfaces, are made in order to divide space into local domains. In the same sense, curvilinears can be used to divide surfaces. A point, not being a continua, cannot be further divided.

Topology, the study of surfaces (Barr, 1964), tells us that since curvilinears can be divided by point-like objects which are not continua (*D_T = 0*), the curvilinear is a continua of dimension one (*D_T = 1*). A surface is a continua of dimension two (*D_T = 2*) since it can be divided by a curvilinear. Everyday (non-relativistic) space is a continua of dimension three (*D_T = 3*) since it can be partitioned by a planar surface of dimension two. In an intuitive sense, *D_T* reveals the number of parameters necessary to describe any point on the surface once on the surface.
$D_E$ is a measure of the minimum number of parameters needed to map the points of an object in entirety. A curved line in two-space, which has a $D_T$ equal to one, has $D_E$ equal to two since both an $x$ and a $y$ value must be given to describe each point. Similarly, a curved planar surface in three-space requires $x$, $y$ and $z$ co-ordinates for its full description ($D_E = 3$). Only two values are needed to describe each point once on the surface ($D_T = 2$). As is clear, in general $D_T = D_E - 1$ with exceptions such as the case of a straight line embedded in one-space where $D_T = D_E$ or the case of a curved line embedded in three-space or any higher dimensional space. Again, intuitively, $D_E$ is an index of the number of parameters needed to describe each point on the surface without already being on the surface.

Consider the case of a curved line embedded in two-space ($D_T = 1$; $D_E = 2$). For this object $1 \leq D_T \leq 2$; i.e. $D_T$ can assume non-integer values. In a physical sense the curve under consideration has a dimension residing between that of a line and a surface so that, in a sense, the line approaches having an area. For a planar surface where $2 \leq D_T \leq 3$, the surface approaches attainment of a non-zero volume metric. With the above as background the discussion proceeds to the concept of fractal.

The following is a modified recapitulation of the classic example of a fractal as related by Mandelbrot (Mandelbrot, 1983). Assume measurement of a straight line segment using a rigid index of length $\varepsilon$. The total length is measured by walking along the segment in steps of length $\varepsilon$ with each new length beginning where the previous step left off. If the number of steps is $N(\varepsilon)$, then $\varepsilon \times N(\varepsilon)$ gives the total length $L(\varepsilon)$. If the procedure is repeated as $\varepsilon$ becomes vanishingly small, $N(\varepsilon)$ tends toward infinity in the limit of small $\varepsilon$, but $L(\varepsilon)$ remains constant. If the logarithm of $N(\varepsilon)$ is then plotted as a function of the logarithm of $\varepsilon$, the function describes a straight line with negative slope, $S$. So, it follows:

$$N(\varepsilon) \propto \varepsilon^\delta \quad (2.2)$$

and;

$$L(\varepsilon) \propto \varepsilon^{1+\delta} \quad (2.3)$$
By letting $\delta = S = -D$:

$$N(\varepsilon) \propto \varepsilon^{-D} \quad (2.4)$$

and:

$$L(\varepsilon) \propto \varepsilon^{1-D} \quad (2.5)$$

The quantity $D$ is variously referred to as the similarity or Hausdorff-Besicovitch dimension (Mandelbrot, 1983). In the case of a straight line, $D = 1$, which also is the value of $D_T$ for a straight line.

In the event of repeating the described procedure using a curve which cannot be described by Euclidean geometry, such as a coastline, $L(\varepsilon)$ tends to infinity in the limit of vanishing $\varepsilon$. The reason for this outcome is that greater detail of the coastline becomes evident with shrinking measuring step, $\varepsilon$. In the case of the coastline, relations (2.4) and (2.5) hold, but $D$ can now assume non-integer values; therefore, $D$ can now be fractional (i.e. fractal dimension terminology arises from this fact). From this Mandelbrot offered the following definition of a fractal (Mandelbrot, 1983): "A fractal is a set for which the Hausdorff-Besicovitch dimension ($D_f$, fractal dimension) strictly exceeds the topological dimension ($D_T$)." As mentioned previously, $D_f$ must also be less than or equal to $D_E$.

The fractal geometric approach has been successful in such applications as predicting the location of an enzymes active site as a function of the value of $D_f$ (Lewis & Rees, 1985) and revealing correlations between the physical behaviour of pharmaceutical granules and their topology (Thibert et al., 1988). Further applications include the use of fractal geometry in the study of the effects of surface morphology on drug dissolution (Farin & Avnir, 1992) and the modeling of discharge patterns following dielectric breakdown (Niemeyer et al., 1984).

The major shortcoming in using a $D_f$ value as an indice of shape is the same as that of the form factor (FF); that is, $D_f$ is not a unique descriptor. This non-uniqueness means that a given closed curve can, in principle and fact, change shape with no concomitant change in $D_f$. If there is a change in $D_f$ following alterations in the contour, $D_f$ does not provide a meaningful indication
of the magnitude of change. It is also important to realize that the scaling $D_f$ detects occurs only over a limited range in the biological structures of interest in this work. Contributions to $L(E)$ by molecular and sub-molecular structures are beyond the resolution of the optical and digital devices used.

Delta analysis is a related method well-suited to the characterization of fine structure or surface roughness (Clark & Meloy, 1985). The delta analysis measures the angular change between two vectors which are stepped around the contour. Smooth surfaces generate low values for the delta value while larger angles are indicative of a curve with a highly irregular shape at small scales. This is the most useful approach reviewed to this point as regards providing true shape information. Similar to FF and $D_h$, however, delta analysis is a non-unique measure and is not necessarily sensitive to changes in shape.

**A Fourier Analytic Approach**

In light of the above discussion, it becomes apparent that what is needed is an analytic method which not only embodies shape information, but is also sensitive to all size changes in shape in a reliable, reproducible and quantifiable manner. Additional requirements include insensitivity to size scale (dilation), relative rotation of the contour in the plane of study and relative position (translation) of the contour within the plane. Such methods are now used in applications to analyze cell and nuclear contours for automated cell-recognition routines e.g. (Diaz et al., 1989; Kuhl & Giardina, 1982; Diaz et al., 1990; Holmquist et al., 1978) and recognition of surface features in quality control routines where a fluid energy mill is used to break solid particles to minimal size in order to maximize surface area for optimal solubilization and bioavailability (Akbarieh & Tawashi, 1987). Each of these methods is derived from the basic technique Fourier used to describe the conduction of heat within an iron ring (Fourier, 1822).

A brief discussion of the application of Fourier's method (by Fourier) to describe the heat distribution as a function of time of an iron ring will be helpful in gaining an intuitive understanding of the method. This intuitive explanation adapted from Bracewell (Bracewell,
An iron ring is placed part way into a fire until a portion of the circumference is red hot. The ring is then withdrawn and buried in a fine insulating sand and the temperature is recorded at multiple points around the circumference as a function of time. The initial heat distribution is irregular, with part of the ring uniformly hot and part uniformly cool with abrupt shifts in temperature in between. As time progresses the temperature around the ring can be described by a $\sin$ function with a uniform increase and decrease in temperature around the circumference. The sinusoidal temperature distribution slowly flattens until the ring is of uniform temperature.

Fourier's idea was that the initial irregular temperature distribution could be decomposed into a number of $\sin$ functions, each with its own amplitude, frequency (of some integer value) and phase (corresponding with position on the circumference). The single-cycle variation is known as the fundamental or first harmonic while the second, third, fourth, etc. cycles are the second, third, fourth, etc. harmonics. The Fourier transform is the mathematical function that gives the maximum temperature (amplitude) and position (phase) for each harmonic. The initial heat distribution which is difficult to describe mathematically has been reduced to a more manageable series of $\sin$ and $\cos$ functions that could be summed (or integrated in some cases) to produce the original heat distribution function.

The Fourier analysis, in the example given, takes an amplitude existing in the spatial domain and transforms it into an amplitude and phase residing in the frequency domain. It is important to note that for a given function if a sufficient number of harmonics is included in the analysis the original function can be described to any desired degree of accuracy.

A more formal presentation of the transform concept follows. Start with some function, such as:

$$f(x) = \exp(-|x|)$$

(2.6)
The discrete Fourier transform of \( f(x) \) consists of multiplying the original function, in this case \((2.6)\), by:

\[
\exp(-i2\pi nx)
\]  

(2.7)

where \( n \) is the transform variable and \( i = \sqrt{-1} \). Now, having multiplied \((2.6)\) by \((2.7)\) the resultant function \( F(n) \) is said to be the Fourier transform of \( f(x) \). The result of this operation is:

\[
F(n) = 2 \left[ 1 + (2\pi n)^2 \right]
\]  

(2.8)

Note that for any given value of \( F(n) \), such as \( F(3) = 0.00561 \), the result 0.00561 depends on the entire range of \( x \), not on any single value of the original function. This dependency is quite different from what happens when an operation converts \((2.6)\) to:

\[
F(x) = \sin\left[ \exp(-|x|) \right]
\]  

(2.9)

The result \((2.9)\) is referred to as a "function of a function" and each output value of \((2.9)\) depends only on the input value, not on the shape of \( f(x) \) as a whole.

The Discrete Fourier transform of \( f(t) \) is given by:

\[
F(n) = \frac{1}{N} \sum_{r=0}^{N-1} f(t) \exp(-i2\pi nt/N)
\]  

(2.10)

Consider the case of the Fourier transform of a space-varying signal, such as the closed finite curves of interest to the present work: A curve can be represented parametrically as a function of arc length by the cumulative vector tangent angle to the curve at each point since the (arbitrary) starting point (Zahn & Roskies, 1972). Call this net tangent angle, \( \phi \), and the associated length function, \( \phi(t) \). The net tangent angle, \( \phi(t) \), in a sense, takes the place of the
time-varying amplitude function discussed earlier; therefore, $\phi(t)$ becomes the independent variable and the analysis proceeds, in general, as follows:

The spatial function, $\phi(t)$, can be expanded in a Fourier series:

$$[\phi(t)]_N = \sum_{n=0}^{N} A_n \cos\left(\frac{2\pi n l}{L}\right) + B_n \sin\left(\frac{2\pi n l}{L}\right)$$  \hspace{1cm} (2.11)

Where:

$$A_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \cos\left(\frac{2\pi l_k}{L}\right)$$ \hspace{1cm} (2.12)

$$B_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \sin\left(\frac{2\pi l_k}{L}\right)$$ \hspace{1cm} (2.13)

The Fourier coefficients $A_n$ and $B_n$ index the contribution of the $\sin$ and $\cos$ functions at each harmonic. The harmonic magnitude, $M_n$, is then defined as:

$$M_n = \sqrt{(A_n)^2 + (B_n)^2}$$ \hspace{1cm} (2.14)

The detection of changes in shape, in the present work, is made via comparison of the $M_n$'s at each harmonic (n) as the contour changes over time. Intuitively, the value of $M_1$ is an indication of the circular contribution to the contour shape (any finite closed contour is going to have a very significant value at $M_1$ due to the $2\pi$ periodicity). Similarly, $M_2$ quantifies the elliptical contribution, $M_3$, the triangular component, etc. The finest shape features are contained in the higher frequency terms (Bookstein et al., 1982).

The strength of this method lies in the fact that the Fourier transform of the $\phi(t)$ function retains shape information, is sensitive to small changes in shape if a sufficient number of harmonics are computed and is invariant to dilation, rotation, translation and reflection.
CHAPTER III
MATERIALS & METHODS

Cell Culture

The cells used in all experiments described here were murine neuroblastoma cells, designated Neuro-2a. These cells, obtained from American Type Culture Collection (ATCC; Rockville, MD), were first isolated from a spontaneous mouse CNS tumor in 1940 and maintained via serial transplantation until 1969 (Van Kooten et al., 1992), at which time they were sub-cloned to produce the present strain. The cell-line was received from ATCC following Passage #172. The Neuro-2a line exhibits a variety of morphologies with most being neuron-like and well-differentiated. These cells express microtubular protein in high concentration as well as levels of tyrosine hydroxylase similar to that seen in midbrain structures.

The line was further sub-cloned for the morphological study (Experiment 3) to yield a cell-line with maximum arborization. The sub-clone is designated Neuro-2a-C8 in the present work.

No more than 20 passages were performed during any experiment before fresh cells were retrieved from liquid nitrogen storage. Stock cells were grown in 25 cm² Falcon tissue culture flasks at 37 °C in a humidified, 10% CO₂ / balance air atmosphere. Culture media consisted of Eagle Minimum Essential Medium (MEM; Sigma) with non-essential amino acids, Earle's Balanced Salt Solution (EBSS) with L-glutamine (L-Gln). Sodium bicarbonate (NaHCO₃) is added to adjust media pH to 7.4. The MEM, purchased as a powder, was solubilized with double-distilled, de-ionized water. The media solution was then filter-sterilized to ensure freedom from contamination. Penicillin (100 IU/ml) and streptomycin (100 µg/ml) (P/S) were added to decrease the probability of bacterial infection.

44
MEM is designed to closely approximate the protein composition of cultured mammalian cells. The MEM was supplemented with 10% fetal bovine serum (FBS; Gibco BRL) for the growing of stock cells. The FBS used in all experiments was from the same lot number to minimize variability. FBS concentration was reduced to 0.5% for individual experiments, as indicated. The 0.5% FBS concentration was empirically determined to maximize cell differentiation.

As cells grew to confluence in the parent flask or were needed for experimental protocols, removal from the parent container proceeded as follows: Media was aspirated and the culture was washed twice with 5 ml of serum-free MEM. Approximately 2 ml of 0.25% trypsin (Sigma) was added to flask until cells began to detach from the culture flask, at which time ~10 ml of serum-supplemented MEM was used to suspend the cells (proteins in the serum inactivate trypsin). The cell suspension was centrifuged at 500 x g for 5 min. The supernatant was then aspirated and the pellet resuspended in fresh MEM and seeded at the appropriate, experiment-dependent, concentration.

Experiment 1

Overview

The first experiment was designed to screen for any neurotrophic properties of Org2766 in the presence of a variety of compounds that are shown to be neurotoxic to Neuro-2a cells at the doses used. The optimal dose(s) of Org2766 at which any protective effect was observed was also determined.

Protocol

Neuro-2a cells were plated at a density of 5x10^4 cells per 35 mm tissue culture dish (Falcon) at a volume of 1 ml in MEM plus P/S supplemented with 0.5% FBS to maximize morphological differentiation. A cell cytometer was used to determine cell densities under
phase-contrast optics (Nikon). The cultures were then grown in the incubation conditions outlined above for 48 hrs.

At the end of the 48 hr differentiation period groups of dishes (n=5) were treated with one of the following drug protocols: 1) a control condition containing MEM+0.5% FBS (control media) with no drugs added; 2) $10^{-5}$ and $10^{-7}$ M of vincristine sulfate, a vinca alkaloid derived from *Cantharanthus roseus*, which binds to or crystallizes microtubule protein preventing polymerization (Creasey, 1975); 3) $10^{-5}$ and $10^{-7}$ M of colchicine (Sigma), an alkaloid derived from *Colchicum autumnale* which results in the depolymerization of microtubules and also exerts many effects on DNA (deoxy-ribonucleic acid) and RNA (ribonucleic acid) metabolism (Creasey, 1975); 4) cytochalasin D (Sigma), a microfilament depolymerizing agent; 5) the Ca++-ionophore, A23187 (Sigma). Each of these groups also received a log integer dose of Org2766 (Organon, Intl.) ranging from $10^{-13}$ to $10^{-6}$ M or zero peptide.

Aliquots of Org2766 (M.W. 854) were lyophilized to ensure dosing accuracy as follows. Org2766 was diluted in double-distilled water at a ratio of 0.854 mg peptide per 5 ml water. This yielded a stock solution of $2 \times 10^{-4}$ M. Fifty µl of stock was then put into 1.5 ml cryo-vials (Corning). Lyophilization of the aliquots was performed in a rotary vacuum lyophilizer (Savant). Addition of 10 ml MEM to the lyophilized aliquots yields a $10^{-6}$ M solution. Serial dilutions were then performed to yield the desired peptide concentrations. No data was available on the stability of Org2766 in the lyophilized form, but the *in vitro* half-life exceeds 11 days.

Cytochalasin D and A23187 were solubilized in dimethylsulfoxide (DMSO) to produce 50mM stock solutions. This stock could then be diluted in MEM/Org2766 to produce the $10^{-7}$ or $10^{-5}$ M concentrations, as needed. Vincristine and colchicine solutions, both of which are water soluble, were made on an as needed basis. The drug solutions were syringe-filter sterilized using 0.22 µm membrane filters (Corning) immediately prior to use.

At the end of the 48 hr differentiation period the media was aspirated off and the appropriate media/drug combination added at a volume of 2 ml. Serial samples, 100 µl in
volume, were then taken at 8, 24 and 48 hrs. The samples were placed in microcentrifuge tubes and spun at 500 x g for 5 min. Ninety µl of supernatant was then drawn off and placed in borosilicate glass test tubes (Baxter S/P) and placed on ice in preparation for the spectrophotometric assay for lactate dehydrogenase (LDH) activity.

LDH is an enzyme which catalyzes the oxidation of lactate to pyruvate with the concomitant reduction of nicotinamide adenine dinucleotide (NAD) to NADH (Lajtha et al., 1981). The NADH can be quantified via an increase in absorbance at 340 nm. The rate of increase of NADH in the sample positively correlates with LDH activity. Since the stock medium contains no LDH, the source of any LDH detectable in the supernatant must be intracellular. There were detectable LDH background levels under control condition. Large increases in extracellular LDH, however, can be used as an index of cell membrane compromise or failure which is indicative of cytotoxicity. Elevations in serum LDH activity have been shown to correlate with a variety of injury and disease states including various liver diseases, pernicious anemia (Zimmerman & Henry, 1979; Erickson & Morales, 1961) and myocardial infarction (Kachmar & Moss, 1976). The change in LDH activity in the presence and absence of the cytotoxins and/or Org2766 served as the dependent variable in this experiment.

An LDH reagent kit (Sigma) containing 50 mmol/L lactate and 7 mmol/L NAD was reconstituted with deionized water. Four-hundred-eighty µl of reagent substrate at ambient temperature was placed in a 1.5 ml plastic spectrophotometer cuvet. Seventy µl of sample was added to the substrate and the cuvet was inverted five times and placed in a Beckman DU-64 spectrophotometer. Five samples could be measured each run, with the sixth position in the spectrophotometer rack occupied by a standard containing 0.55 ml reagent alone. A Beckman programmable spectrophotometric kinetic software module directed absorbance readings to be made every 60 sec for a 10 min period at 340 nm. The rate of change in absorbance was computed using only the last 5 min of the data. The reason for using only the latter 5 min was that in most samples no change in absorbance was observed for the first 1 to 2 min. The second
5 min period showed a much more stable and reproducible absorbance time derivative. LDH activity was computed as follows:

\[
LDH_{activity}[U/L] = \frac{\Delta A \times \text{min}^{-1} \times TV \times 1000 \times 10^4}{6.22 \times SV \times LP}
\]  

(3.1)

Where: \(\Delta A\) = Change in absorbance per minute at 340 nm.

TV = Total reaction mixture volume [0.5 ml].

SV = Sample volume [70 µl].

6.22 = Millimolar absorptivity of NADH at 340 nm.

LP = Lightpath length.

1000 = Converts units per ml to units per liter.

**Data Analysis**

Changes in LDH activity were expressed as the mean±SEM (standard error of the mean) per cent change from the zero peptide control condition. Statistically significant differences were assessed via a 1-way ANOVA with a Tukey HSD follow-up (\(\alpha = 0.05\)) at 8, 24 and 48 hrs.

**Experiment 2**

**Overview**

A positive neurotrophic response in Experiment 1 prompted an investigation to determine whether the observed reduction in LDH activity was merely a function of a decrease in the rate at which LDH was being synthesized. To this end cell cultures pre-treated for 24 hrs in the presence or absence of the same range of Org2766 dosages used in Experiment 1 were pulsed for 60 min with 16 µCi \(^{35}\)S-Met. Total protein was determined via the method of Lowry and differences in \textit{de novo} synthesis were calculated as a function of scintillation counts per µg of protein.
**Protocol**

Cells were plated at a density of 5\times10^5 cells per 35 mm tissue culture dish in MEM plus 0.5% FBS with P/S at a volume of 2 ml. The incubation environment was identical to that of Experiment 1. At the end of the 48 hr differentiation period the culture medium was replaced with medium containing Org2766 at log integer concentrations ranging from $10^{-13} \rightarrow 10^{-6}$ M, plus a control condition with no peptide added ($n = 7$).

Twenty-four hrs later the cultures were washed three times with 1 ml of non-serum supplemented Met-free media taking care not to disrupt the attached cells. The cultures were then pulsed for 60 min with 16 µCi/ml $^{35}$S-Met (TRAN $^{35}$S-LABEL, Sp. Act. $> 1000$ Ci/mmol, ICN Biomedical, Inc.) in Met-free media with zero serum. The labeled amino acid is guaranteed to contain $\geq 70\%$ $^{35}$S-Met and $\leq 15\%$ $^{35}$S-Cys. Methionine incorporation into protein was stopped by addition of 100% trichloroacetic acid (TCA) to a final concentration of 10% (110 µl in this case). The TCA causes the protein to precipitate and aggregate. A Teflon scraper was used to loosen cell tissue from the culture dish and the solution was transferred to a glass test tube. The dish was then washed with 5% TCA and this solution was added to the test tube. The samples were put on ice for ~15 min to allow completion of protein precipitation.

The samples were spun at low speed and the radioactive supernatant aspirated. The pellets were washed by resuspending in 1.7 ml of 5% TCA. The washing steps were repeated three more times. Following the final centrifugation the pellet was resuspended in 0.5 ml H$_2$O. One-half ml 1M NaOH was added and the solution mixed well in 1.5 ml polypropylene micro-centrifuge tubes. Samples were then heated in a boiling water bath for ~2 min at which time the solution would be clear indicating all the soluble components had gone into solution. Two hundred µl of the sample was then used for the Lowry protein determination assay (Lowry et al., 1951) (to determine total protein) while a second 500 µl was used for the scintillation count (to determine the quantity of radiolabel).
The Lowry protein assay reagents used were as follows:

\[ A = 2\% \text{ Na}_2\text{CO}_3 \text{ in } 0.1 \text{ N NaOH.} \]

\[ B = 1\% \text{ CuSO}_4 \cdot 5\text{H}_2\text{O.} \]

\[ C = 2\% \text{ Na,K-tartrate.} \]

\[ D = 50 \text{ ml } A + 0.5 \text{ ml } B + 0.5 \text{ ml } C. \]

\[ E = 2\text{N phenol stock reagent diluted 1:1 with H}_2\text{O.} \]

All samples were mixed in 12 x 75 mm borosilicate glass test tubes (Baxter S/P). The standard blank tubes contained 200 µl of 0.1 N NaOH. The protein standards used were 12.5, 25, 50, 75 and 100 µg/ml bovine serum albumin (BSA). The standard samples were mixed with H₂O to a final volume of 200 µl. For the experimental samples, 200 µl was added to each tube. To each sample, 1 ml of reagent D was added followed by vortex mixing and the tubes were left to stand for 10 min at room temperature. One hundred µl of reagent E was then added followed immediately by vortex mixing. The absorbance was read 30 min later at 750 nm using a glass cuvette in a Gilford 250 spectrophotometer.

To calculate µg protein per ml of original sample the following conversion formulae were used:

\[ z = \mu g \text{ protein per tube} = \frac{O.D._{\text{sample}} - O.D._{\text{blank}}}{O.D./\mu g \text{ protein}} \]  \hspace{1cm} (3.2)

\[ \mu g \text{ protein per ml original sample} = z \times \text{dilution} \times (1 \text{ ml/vol used}) \] \hspace{1cm} (3.3)

Equation (3.3) gives the total protein per ml of the original sample. Another step was needed to calculate the specific activity of the ³⁵S-Met label as counts per min (cpm) per µg protein. The ³⁵S-Met is incorporated into newly synthesized protein and therefore can be used as an indication of protein synthetic rate. Ten ml of neutralizing scintillation fluid was added to 0.5 ml of sample. The experimental sample was replaced with 0.5 N NaOH to compute
background disintegration counts to be entered into the final cpm as a correction factor. Two runs were made on a Beckman LS3801 scintillation counter and the average of the two values was used as the final value. The data were then expressed as cpm/µg protein. This number, therefore, positively correlates with de novo protein synthesis and serves an index of synthetic rate.

Data Analysis

The indicator of de novo protein synthesis, cpm/µg protein, was expressed as the mean ± SEM for each group (n=7). A 1-way ANOVA with a Duncan Gap-Order follow-up was used as the test for significant group differences (α = 0.05). The Duncan Gap Order test is a slightly less stringent follow-up test vs. the Tukey HSD resulting in several more conditions reaching significance. The means were then expressed as ± per cent change from control.

Experiment 3

Overview

The third experiment utilized time-lapse video microscopy to study recovery from the effects of 12 hrs of treatment with 10⁻⁷ M colchicine in the presence and absence of 10⁻⁸ M Org2766. This dose of peptide was shown to be optimal for the induction of neurotrophic effects as indicated by the first two experiments. Video-microscopic images of individual cells were captured every 5 min for a 45 min period at 8, 24 and 48 hrs following colchicine wash-out under a variety of peptide treatment conditions including combinations of pre-, post- and co-treatment. A control group received colchicine but no peptide treatment. The rate of change in shape of the digitized cell contour was then calculated using area, perimeter, form factor, fractal dimensional and Fourier analysis of the tangent angle length function measures. Comparisons of rate of shape change were then made between peptide groups and control.
Protocol:

Micro-incubation Environment

The Neuro-2a-C8 sub-clone was chosen for use in this study due to the complex arbors that this cell line tends to produce. The initial differentiation period culture conditions were identical to those of the first two experiments with the exception that a different brand of culture dishes were used. A description of the hardware used in maintaining the cell cultures at proper temperature, gas and pH conditions will be followed by a description of the time-lapse video recording, digitization and image processing hardware and software. Experimental treatment conditions will be described in the last section of the protocol.

The cells were grown in 35 mm (Nunc, Denmark) tissue culture dishes. This brand of dish was chosen due to its superior optical properties relative to those of other manufacturers. A stock dish cover was modified to allow gassing and the changing of media conditions as follows: The lip of the dish was ground off and a 9x15 mm opening milled in the center. A plastic coverslip was then cemented in place over the opening with epoxy resin. The plastic coverslip was used as it is thinner, yielding greater passage of light vs. the plastic in the dish cover.

Two 14-gauge tubing adapters (Becton-Dickinson) were cemented into holes drilled on opposite sides of the periphery of the dish. Polyethylene (PE) tubing, .060" O.D., was then fed through the opening in one tubing adapter extending to the bottom of the dish. The medium could be changed through this tube using a 3 ml syringe. A gas line was attached to the other stub adapter through which a 10% CO₂/balance air mixture was fed in from a regulated supply via a .066" I.D. PE tube. The gas first passed through a sealed glass 20 ml bottle so that flow could be monitored by counting the number of bubbles per unit time passing through the purified water in the bottle. A rate corresponding to ~0.5 ml min⁻¹ was found to be sufficient to maintain pH and O₂ tension and keep the evaporation in the dish below 3% of the total volume following 60 hrs of continuous service.
A 0.015" chromel-constantan thermocouple (Omega Engineering) fed through and was cemented into another hole with epoxy resin and extended to just above the surface of the dish bottom. The thermocouple leads led to a digital temperature bridge (Omega) whose output fed a voltage inverter constructed for the purpose of making the thermocouple output compatible with the direct-current proportional feedback controller (also constructed for this experiment) that regulated the temperature of the microincubator described below. An inert coating (Thermcoat SL, Omega) applied to the portion of the thermocouple immersed in media prevented shortcircuiting of the thermocouple and leaching of ions into the media.

The microincubator, machined out of brass, was 106 mm in diameter which fit snugly into the microscope stage opening. A 38 mm diameter hole in the center of the microincubator allowed for placement of the culture dish directly over the microscope optics. A 20x25 mm opening was machined into a thin brass plate attached to the bottom of the microincubator. This made for a sufficiently large opening to view the culture and yet provided sufficient surface contact to maximize temperature homogeneity within the dish.

The body of the microincubator was hollowed and a Nikrome heating element attached to electrodes extended to the outer surface of the body for connection to the feedback controller. A thin coating of high temperature silicone sealant (General Electric) in the incubator cavity prevented electrical shortcircuit between the heating element and body of the incubator. The cavity was filled with mineral oil which transferred heat efficiently from the element to the incubator body.

The temperature gradient across the dish was shown to be less than 1.5 °C with the cover removed in bench studies. The gradient is likely less than this under experimental conditions where the system is closed. Measuring the temperature with thermocouple placement at the periphery ensures that the temperature at the lowest point near the dish center, where the cell of interest resides, will be ≤ 37 °C. This system maintained temperature at 37± 0.1 °C with normal variations in ambient temperature.
Several number 1 glass coverslips were placed over the center opening of the incubator and a d.c. current was fed through a 2-watt power resistor placed on the coverslips to collapse the temperature differential between the culture dish and room temperature. This prevented the buildup of condensation on the inside of the cover of the culture dish which otherwise interferes with light transmission.

The dish cover was attached to the culture immediately prior to placement in the microincubator. A thin strip of Parafilm was used to make a seal tight enough to allow for maintenance of the proper gas environment and to ensure low evaporative rates, yet did not allow positive pressure to build. A three-way neoprene valve on the gas feed between the flow monitor bottle and the culture dish allowed the gas seal to be broken so that the media could be withdrawn or added without building pressure differentials in the system.

**Experimental Treatment**

Cultures were plated at a density of $5 \times 10^3$ cells per dish and differentiated for 24 hrs. The peptide and colchicine treatment protocols are summarized in Table 3.1.

Each culture could then be rapidly transferred to the laboratory where the time-lapse video microscopy hardware was located. The standard dish cover was replaced with the special top described in the previous section and the culture was placed in the microincubator. The standard medium was withdrawn and replaced with 2 ml of pre-warmed and gassed MEM containing 0.5% FBS and $10^{-7}$ M colchicine following three washes spaced 5 min apart with MEM alone. The current flow needed to maintain the culture at 37 °C was determined in pilot studies prior to experimental testing. The proportional feedback controller, therefore, could be manually overridden to bring the culture medium up to temperature following fluid replacement without temperature overshoot.

An additional experiment (n=10) looked at the effects of $10^{-8}$ M Org2766 and 0.5% FBS in MEM vs. control media containing no peptide. Neither group in this experiment received
peptide treatment during the 24 hr differentiation period. The time-lapse recording protocol was identical to that described above.

**TABLE 3.1**

<table>
<thead>
<tr>
<th>COLCHICINE &amp; ORG2766 TREATMENT PROTOCOLS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>P1</strong></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>Org2766b</td>
</tr>
<tr>
<td>B</td>
<td>Org2766</td>
</tr>
<tr>
<td>C</td>
<td>Org2766</td>
</tr>
<tr>
<td>D</td>
<td>Org2766</td>
</tr>
<tr>
<td>E</td>
<td>Org2766</td>
</tr>
<tr>
<td>F</td>
<td>Org2766</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
</tr>
</tbody>
</table>

*P1 = 24 hr period prior to addition of colchicine.*

*P2 = 12 hr colchicine treatment period.*

*P3 = 48 hr period following colchicine washout.*

*b 10⁻⁶ M

c n=5 per group.

**Image Acquisition**

The microincubator and cell culture were placed on the stage of an inverted Nikon Diaphot-TMD phase-contrast microscope with a 12V-50W halogen lamp. A Javelin black and white solid-state video camera was connected to the video port on the microscope via a closed-circuit television (CCTV) adapter with 2X magnification. A 20X microscope objective was used (Nikon) providing a total magnification of 400X.

Custom software (Ross Naheedy, LUMC Medical Informatics) directed a 5V TTL pulse from an XT-type computer every 5 min for 10 sec to: a) a solid-state relay which switched the microscope lamp on; b) a Sony TS-9600 Superior Performance 3/4" video cassette recorder (VCR), switching it into Play/Record mode. Before the video signal from the camera reached the
VCR it passed through a VTG-33 time/date generator (For-A Co. Ltd.) which superimposed the current time and date on the final recorded image.

Following completion of acquisition of the images to KSP-60 high-resolution 3/4" video tape (Sony), the next step was to digitize the images for processing and analysis. A DT-2861 analogue to digital (A/D) framegrabber (Data Translation) installed in a 386-based AT-type computer converted the images from analogue form on the video tape to a 512x512 pixel 8-bit image (256 levels of grey). With the magnifications used for these experiments, each pixel was 440 nm along the x-axis and 352 nm along the y-axis. The correction for the nonsquare pixels was made in a later processing step. The image was stored in a buffer on the DT-2861 in a format specific to Data Translation A/D boards. A conversion program provided by DT was used to change the DT-format to Target-Image-File-Format-Greyscale (TIFF-G), which is a format recognized by the image processing programs used in the later stages of image manipulation. The 8-bit image files (1800 in toto, 262 kB per image) were then stored on a Colorado Memory Systems cassette tape.

**Image Processing**

All image processing was performed on 486-based AT-type computers. The central processing units (CPU) in these machines have built-in mathematics co-processors, greatly speeding run time of the arithmetic-intensive processing routines.

The image files were restored to the PC hard drive from the cassette backup tape. Sequential processing subroutines reduced the initial 8-bit greyscale image containing a number of cells and background noise to a 1-bit black and white image containing a single contour representing the perimeter of the cell of interest. Repeated runs on images demonstrated a high degree of repeatability in producing the final contour (See Appendix D, 145-147).

The images were first high-pass filtered using PhotoFinish, an image editing program (Z-Soft Corp.). This software is proprietary so that the convolution kernel values are not available, though the kernel is likely 3x3 pixels. High-pass filtering accentuated greyscale gradients
thereby enhancing edges. Visual detection of the cell boundary in the x-y plane was made easier following the filter routine (See Appendix D).

The image was then manually traced with a mouse controlled one pixel wide line of greyscale value equal to zero within PhotoFinish. A deepest black pixel has a greyscale value of zero, while a deepest white pixel has a value of 255. At this point of the processing the image had a range of greyscale values with the histogram peak centered in the mid-ranges. Because the manual tracing of the cell contour was performed with a greyscale value equal to zero, a histogram equalization was then done to convert all pixels with values \( \geq 1 \) to a value of 255. This processing step produced an image with the contour of interest in black (greyscale equals zero) plus any pixels that had a value of zero prior to the histogram equalization remaining black. All other pixels then became white (greyscale equals 255). The black pixels which were not part of the contour of interest were then erased within the editing program. As a final step, the 8-bit images were converted to 1-bit images greatly saving on storage space for the files.

A custom FORTRAN boundary tracking routine (DAKKRO Corp., Denver, CO) running in batch mode using a floodfill based algorithm detects the contour boundary and outputs the ordered x-y coordinate pairs to an ASCII (American Standard Code for Information Interchange) text file. The enclosed area, perimeter and form factor of the closed contour was then outputted to another ASCII file. File naming was done in such a way that the date, time and experimental condition was encoded in a unique manner for each image, contour coordinate file and contour geometry file. A calibration routine multiplied the x-coordinate by 1.0 and the y-coordinate by 0.8 to adjust for the nonsquare pixels outputted by the framegrabber prior to running of the tracking program.

**Image Analysis**

Custom software written in Borland C++ (Samuel Bowen, Argonne National Laboratory) and running in batch mode computed the tangent angle length function \( \phi(t) \) and then calculated the first 300 Fourier coefficients on \( \phi(t) \). Three hundred harmonics was determined sufficient to
adequately describe the contour for several reasons: The smallest polygons (closed contours) contained ~500 points, while the average was in excess of 1,000 points. Therefore, the 300th harmonic describes details as small as ~3 pixels (1000 / 300) for the longer contours while not dangerously over-sampling for the contours of length 500. The algorithm to calculate % error of the Fourier series in reconstructing \( \phi(l) \) as a function of the number of harmonics computed is given below. Three hundred harmonics gives a % error of less than 3% for the most complex contours in this investigation (see Appendix B for an example, p. 124). In addition, in the output of the analysis section, the higher harmonics contribute to a relatively small portion of the variance so that even when looking at detail in the ~2 pixel range, the digitization error contributes only modestly to the result. Since each pixel was adjusted to 440 nm per side, a three pixel resolution translates to the ability to resolve structures as small as 1.5 \( \mu \)m.

The algorithm to compute % error \( \varepsilon \) is:

\[
\varepsilon = \frac{1}{L} \int_0^L \left| \phi(l) - \phi(l)_{\text{series}} \right|^2 \, dl
\]  

(3.4)

Where:

\[
\phi(l)_{\text{series}} = A_0 + \sum_{n=1}^{299} A_n \cos \left( \frac{n \pi l}{L} \right) + B_n \sin \left( \frac{n \pi l}{L} \right)
\]  

(3.5)

Where: \( A_0 = \) The contour mean radius.

Therefore, as \( \phi(l)_{\text{series}} \) more closely approximates the original (input) function \( \phi(l) \) by increasing the number of calculated harmonics \( n \), the % error \( \varepsilon \) decreases.

The tangent angle length function \( \phi(l) \), intuitively, is the net change in angle of the tangent to the polygon as the curve is circumnavigated from start to endpoint. The value of the function at each point was calculated as:

\[
\phi(l) = \arctan \left( \frac{\Delta y}{\Delta x} \right)
\]  

(3.6)
Where:
\[
\Delta y = 0.5(y_p - y_{p-1})
\]  \hspace{1cm} (3.7)
\[
\Delta x = 0.5(x_p - x_{p-1})
\]  \hspace{1cm} (3.8)

The output values from arctan were defined within the software for all cases where either \(\Delta y\) or \(\Delta x\) is equal to zero due to the problem of dividing by or into zero.

The Fourier expansion of \(\phi(t)\) was accomplished by the following function:

\[
[\phi(t)]_N = \sum_{n=0}^{N} A_n \cos\left(\frac{2\pi nl}{L}\right) + B_n \sin\left(\frac{2\pi nl}{L}\right)
\]  \hspace{1cm} (3.9)

Where:
\[
A_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \cos\left(\frac{2\pi nk}{L}\right)
\]  \hspace{1cm} (3.10)
\[
B_n = \frac{-1}{n\pi} \sum_{k=1}^{m} \Delta \phi_k \sin\left(\frac{2\pi nk}{L}\right)
\]  \hspace{1cm} (3.11)

\(A_n\) and \(B_n\) are the Fourier coefficients and, as such, quantify the \(\cos\) and \(\sin\) contributions at each harmonic. The coefficients were then combined into a single magnitude by:

\[
M_n = \sqrt{(A_n)^2 + (B_n)^2}
\]  \hspace{1cm} (3.12)

The discussion of the intuitive meaning of \(M_n\), first outlined in Chapter II, is repeated here. The detection of changes in shape, in the present work, was made via comparison of the \(M_n\)’s at each harmonic (n) as the contour changes over time. Intuitively, the value of \(M_1\) is an indication of the circular contribution to the contour shape (any finite closed contour is going to have a very significant value at \(M_1\) due to the \(2\pi\) periodicity). Similarly, \(M_2\) quantifies the elliptical contribution, \(M_3\), the triangular component, etc. The finest shape features are contained in the higher frequency terms (Bookstein et al., 1982).

The 300 \(M_n\) values for each contour were outputted to ASCII files for analysis as described in the next section.
Data Analysis:

i) Fourier Analysis of \( \phi(t) \)

The ten ASCII files representing the sequential time-lapse recordings for a given contour were imported into SigmaStat (Jandel Scientific) where the data was transformed in such a manner that the 3,000 \( M_n \) values were reduced to a single number representing the first time derivative of the shape; that is, how rapidly the contour is changing shape as a function of time \( (dS/dt) \). First, a custom transform computed the sum of the absolute value of the differences between pairs of \( M_n \) values for all 300 harmonics; that is, \(|t_{\text{time}2}-t_{\text{time}1}| + |t_{\text{time}3}-t_{\text{time}2}| + \ldots + |t_{\text{time}10}-t_{\text{time}9}| \). These 300 values were then summed using the weighting scheme in Table 3.2.

<table>
<thead>
<tr>
<th>Harmonic Range</th>
<th>Weight via ( \varepsilon )^a</th>
<th>Correction Factor^b</th>
<th>Final Weight(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ( \rightarrow ) 10</td>
<td>6.0</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>11 ( \rightarrow ) 20</td>
<td>5.0</td>
<td>1.15</td>
<td>5.5</td>
</tr>
<tr>
<td>21 ( \rightarrow ) 30</td>
<td>4.0</td>
<td>1.4</td>
<td>5.0</td>
</tr>
<tr>
<td>31 ( \rightarrow ) 40</td>
<td>3.0</td>
<td>1.7</td>
<td>4.5</td>
</tr>
<tr>
<td>41 ( \rightarrow ) 50</td>
<td>2.0</td>
<td>1.9</td>
<td>4.0</td>
</tr>
<tr>
<td>51 ( \rightarrow ) 60</td>
<td>1.8</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>61 ( \rightarrow ) 70</td>
<td>1.7</td>
<td>2.3</td>
<td>3.8</td>
</tr>
<tr>
<td>71 ( \rightarrow ) 80</td>
<td>1.5</td>
<td>2.5</td>
<td>3.7</td>
</tr>
<tr>
<td>81 ( \rightarrow ) 90</td>
<td>1.3</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>91 ( \rightarrow ) 100</td>
<td>1.2</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>101 ( \rightarrow ) 150</td>
<td>1.0</td>
<td>3.3</td>
<td>3.0</td>
</tr>
<tr>
<td>151 ( \rightarrow ) 200</td>
<td>0.5</td>
<td>3.75</td>
<td>2.0</td>
</tr>
<tr>
<td>201 ( \rightarrow ) 250</td>
<td>0.25</td>
<td>4.25</td>
<td>1.5</td>
</tr>
<tr>
<td>251 ( \rightarrow ) 300</td>
<td>0.25</td>
<td>5.0</td>
<td>1.25</td>
</tr>
</tbody>
</table>

\(^a\) defined in Eqn. 3.1. Value is proportional to % reduction in \( \varepsilon \) by increase in harmonic \#.

\(^b\) determined from harmonic contribution to \( \sum_{n=1}^{300} M_n \). Since the higher harmonics have smaller absolute values, weighting is increased by a factor inversely proportional to \( M_n \).

\(^c\) (interpolated) final weight = contribution to % reduction in \( \varepsilon \) times the correction factor.
Therefore, the change in shape over time may be expressed as:

\[
\frac{dS}{dt} = \sum_{n=1}^{300} \Delta M_n
\]  

(3.13)

Where: \( M_n \) is weighted according to Table 3.2.

A Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test was used for testing significant group differences \((\alpha = 0.05)\) at each time-point (i.e. 8, 24 and 48 hrs). This non-parametric test was used as many of the data sets proved to not be normally distributed.

ii) Area, Perimeter and Form Factor

Area, perimeter and form factor data were imported to SigmaStat, a statistics software package, where a custom transform converted the pixel data to \((\mu m^2)\) and \((\mu m)\), respectively. The change in each of these parameters was assessed as a function of time similar to that of the Fourier magnitude data. That is, the total change in area, perimeter and form factor was calculated by the output of the summation \((|time_2-time_1| + |time_3-time_2| \ldots + |time_{10}-time_9|)\).

The output of the summation yielded a single value for each cell characterizing the net change in the parameter of interest. This operation was repeated for each cell in each condition at 8, 24 and 48 hrs.

The calculations for net change in area \((\Delta A)\), perimeter \((\Delta P)\) and form factor \((\Delta FF)\) are summarized as follows:

\[
\Delta A = \sum_{t=0}^{9} |A_{t+1} - A_t|
\]  

(3.14)

\[
\Delta P = \sum_{t=0}^{9} |P_{t+1} - P_t|
\]  

(3.15)
\[ \Delta FF = \sum_{t=0}^{9} |FF_{t+1} - FF_t| \]  

(3.16)

Where: \( t \) = time period 0→9, representing steps of 5 min per period.

A Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test was used for testing for significant group differences \((\alpha =.05)\) at each time-point (i.e. 8, 24 and 48 hrs).

**iii) Fractal Dimension**

A more complete discussion of fractal dimension is contained in Chapter II. Suffice it here to say that the fractal dimension is an index of the scaling properties of an object. That is, in the case of a closed polygon, how does perimeter change as a function of resolution. Resolution can be changed by varying the length of the measuring standard. Therefore, if using every point of a polygon to calculate perimeter yields a given value; what is the length when using every \( nth \) point? The slope of the log-log plot of \( N(\varepsilon) \) [abscissa], the number of points used to describe the polygon, vs. \( L(\varepsilon) \) [ordinate], the perimeter length given steps of length \( \varepsilon \), gives the fractal dimension \( D_f \) of the contour.

The optimal minimum number of points \([N(\varepsilon)]\) to describe the polygon is determined empirically. When \( N(\varepsilon) \) becomes so small that resolving power no longer can detect major structural features of the contour the measure becomes misleading. In the case of the polygons investigated in this work the perimeter is calculated using every 8th, 16th and 32nd point. Again, intuitively, the slope of the log-log plot of \( N(\varepsilon) \) vs. \( L(\varepsilon) \) equals \( D_f \). Only every 8th, 16th and 32nd point was used here as it was empirically determined that these contours only scale as fractals across this small range.

\( D_f \) was calculated using custom software (Samuel Bowen, Argonne National Laboratory).

The algorithm used to compute the total change in \( D_f \) was:
\[ \Delta D_t = \sum_{t=0}^{9} |D_{t+1} - D_{tt}| \]  

(3.17)  

where:

\[ D_t = 1 + d \]  

(3.18)  

where:

\[ d = -\frac{d \log_b(l(\varepsilon))}{d \log_b(\varepsilon)} \]  

(3.19)  

where:

\[ \varepsilon_i = 2^i \left( \frac{L}{N} \right) \]  

(3.20)  

therefore:

\[ d_i = -\frac{-\log_b \left[ \frac{l(i+1)}{l(i)} \right]}{\log_b(2.0)} \]  

(3.21)  

therefore:

\[ d = \left[ \frac{dd(3) + dd(4)}{2} \right] \]  

(3.22)  

where:

\( \varepsilon \) = average stepsize  
\( \varepsilon_i \) = average stepsize using every (2)th point  
\( i = \{3,4\} \)  
\( L \) = length using every point  
\( N \) = total # of points in contour

A Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test was used for testing for significant group differences (\( \alpha = 0.05 \)) at 8, 24 and 48 hrs.
CHAPTER IV

ORG2766 AMELIORATES THE IN VITRO CYTOTOXIC EFFECTS OF A VARIETY OF COMPOUNDS

Experiment 1

Overview

Cultured Neuro-2a cells treated with either colchicine, vincristine sulfate, cytochalasin D or the Ca++-ionophore A23187 show a marked increase in LDH activity at 8, 24 and 48 hrs after addition of cytotoxic to the culture media. See Table 4.1 (p. 97) for raw data. A rise in LDH activity is interpreted as an increase in cytotoxicity. Co-treatment with Org2766 reduces LDH activity relative to control in a dose and time-dependent fashion. The experimental results are integrated and discussed in accordance with the findings of the protein synthesis experiment (Chapter V).

Results and Discussion

i) 10^-7 M Colchicine

The per cent change in LDH activity vs. Control (10^-7 M colchicine alone) in the presence of graded doses of Org2766 is shown in Fig. 4.1 (p. 87). The neurotrophic response is best described by a U-function at 8, 24 and 48 hrs where a negative per cent change corresponds with an increase in the neurotrophic effect of Org2766. The interpretation of these results, however, cannot stand independent of the results of the protein synthesis experiment (Fig. 5.1, p. 101).

Figure 5.1 shows that Org2766 stimulates protein synthesis in a dose-dependent fashion at integer log doses ranging from 10^-13 → 10^-6 M. The peak dose for this effect was found at 10^-8 M with the strength of the effect falling off to either side. Org2766 increases radiolabel into most
bands on SDS-PAGE gel by about 40% at $10^{-8}$ M (Murry et al., 1993), so, the effect seems general and does not appear limited to certain proteins. The reduction in LDH activity is not, therefore, due to a decrease in synthesis of LDH.

The peak increase in protein synthesis found at $10^{-8}$ M would maximally raise the LDH activity index at $10^{-8}$ M with the effect dropping off to either side. An increase in LDH synthesis of ~25% would change the U-shaped neurotrophic function shown in Fig. 4.1 to an inverted-U function. An inverted-U function is consistent with that found by other investigators for the neurotrophic action of ACTH analogues as discussed in Chapter II. Little in vitro work demonstrating positive neurotrophic effects has been published. However, Org2766 does increase the number of microtubules in large CNS axons of the snail Lymnaea stagnalis, with the maximal effect found at $10^{-6}$ M (Muller et al., 1992). Combining this result with the Murry and co-workers finding that $10^{-8}$ M Org2766 increased radiolabel into most bands on 1-D SDS-PAGE gels, provides support for the argument that the U-shaped curves noted in Fig. 4.1 (and following figures) would be changed to an inverted-U shaped function if protein synthetic effects are taken into account. This argument is assumed in all following cytotoxin and dose conditions.

Since the protein synthesis experiment was only performed at 24 hrs it is not known if the peptide has differential effects on de novo protein synthesis at 8 and 48 hrs. As will be seen as the discussion progresses, neurotrophic activity, at least under some conditions, progressively declines from 8 to 48 hrs. The degradation rate of LDH in the extracellular space of these in vitro studies is not known. The assumption made here is that Org2766 stimulates protein synthesis at a fairly constant rate and that the degradation of LDH lags behind synthesis resulting in a net accumulation of LDH as a function of time. Therefore, the smaller neurotrophic effects observed at 24 and 48 hrs under some conditions may well be due to the enhanced protein synthesis resulting in a net accumulation of LDH.
ii) \(10^{-5} \text{ M Colchicine}\)

In this condition the dose of colchicine was increased by two magnitudes. The same general pattern is seen here (Fig. 4.2, p. 88) as in the \(10^{-7} \text{ M}\) experiment. That is, the strongest neurotrophic effects are observed at 8 hrs with decreasing neurotrophic activity at 24 and 48 hrs.

The effect is enhanced with the greater toxic challenge presented in this condition. This is an interesting finding as work reviewed in Chapter II suggests the neurotrophic activity of ACTH analogues is enhanced under developmental (Frischer & Strand, 1988; Strand et al., 1989), regenerative (Dekker et al., 1987) and degenerative (Wolterink et al., 1990a; Wolterink & Van Ree, 1990; Wolterink et al., 1990b) conditions. The higher dose of colchicine does induce greater damage to the cultures as evidenced by increases in LDH activity of 48, 23 and 6% at \(10^{-5} \text{ M}\) colchicine vs. 8, 14 and 17% at \(10^{-7} \text{ M}\) colchicine when comparing colchicine with no peptide to neither colchicine nor peptide conditions (see captions of Figs. 4.1 & 4.2). This is evidence that Org2766 exerts greater neurotrophic effects under conditions of higher toxic challenge.

The difference in LDH activity between cytotoxin alone and zero cytotoxin controls for all compounds tested in these and the following experiments (see Table 4.2, p. 99) tends, in general, to decrease as time progresses. This may indicate that LDH synthetic rate is greater than the degradative rate so that LDH is accumulating in the supernatant due to synthesis in the zero cytotoxin condition. A similar increase in LDH levels in the peptide conditions is, therefore, also possible due to an imbalance in synthetic and degradative rates favoring the former. In the presence of peptide, increased protein synthesis could account for the reduced neurotrophic effects (as assessed by LDH activity) seen under some conditions at 24 and 48 hrs. It is suggested here that the phenomenon is fairly general, though possible differences in cytotoxin influences on protein synthesis are not tested in this work.
iii) 10^{-7} M Vincristine sulfate

Vincristine displayed a pattern similar to that observed in the colchicine experiments Fig. 4.3, p. 89). A U-shaped neurotrophic function is generally observed with smaller effects seen at 24 and 48 vs. 8 hrs. Again, if LDH is accumulating in the sampled supernatant as time progresses due to peptide induced protein synthesis, the neurotrophic activity at 24 and 48 hrs as assessed by LDH assay might be equal to or greater than that seen at earlier times. This would be consistent with the previously discussed evidence that peptide induced neurotrophic activity increases proportionally with the degree of challenge.

iv) 10^{-6} M Vincristine sulfate

The higher VCR dosage displays a generally similar pattern to that seen at 10^{-7} M (Fig. 4.4, p. 90). LDH activity, however, is lower at 48 hrs in the 10^{-5} M VCR condition. This could be due to the fact that the higher VCR dose is more toxic as indicated by a 10% increase in LDH activity in the VCR alone condition vs. the zero VCR condition (see Captions of Figs. 4.3 & 4.4, p. 89 & 90). Again, a greater toxic challenge correlates with a greater neurotrophic response.

v) 10^{-7} M Cytochalasin D

In the presence of 10^{-7} M of the microfilament depolymerizing agent cytochalasin D (CD), the strongest neurotrophic response is seen at 48 hrs (Fig. 4.5, p. 91) at approximately 10^{-6} M Org2766 (see previous argument regarding adjustment of the shape of the function). Interpretation of these and the previous results is made difficult by what is possibly an interaction between not only the dose of Org2766 and peptide synthesis, but there also may be an interaction between cytotoxin, cytotoxin dose and protein synthesis; that is, the shape and amplitude of the function (e.g. Fig. 4.5) varies somewhat as a function of cytotoxin and cytotoxin dose.
vi) \(10^{-5} \text{ M Cytochalasin D}\)

The neurotrophic response is approximately twice as strong in the high-dose CD condition at 8 and 24, but not 48, hrs (Fig. 4.6, p. 92) vs. the lower dose CD group (Fig. 4.5). Again, this is interpreted as an enhanced neurotrophic response in the face of a greater toxic challenge. A possible explanation for the observation that the neurotrophic response is not as strong in the high-dose CD group at 48 hrs is that the cultures have been so damaged by the cytotoxin that the neurotrophic effect of the peptide is negated.

vii) \(10^{-7} \text{ M A23187}\)

The steepness of the curve is greater than that seen for any of the other cytotoxins (Fig. 4.7, p. 93). The same U-shaped function is observed at 8, 24 and 48 hrs. The previous argument is assumed that taking account of protein synthetic effects would change the function into an inverted-U shape.

A23187 differs from the other cytotoxins used in these experiments in that it does not work directly on cytoskeletal components. However, given the importance of intracellular Ca\(^{++}\) concentrations in the regulation of cytoskeletal dynamics e.g. (Brundage \textit{et al.}, 1991; Sweet \textit{et al.}, 1988), A23187 is expected to exert profound indirect effects on the cytoskeleton.

viii) \(10^{-5} \text{ M A23187}\)

The shape of the neurotrophic function in the presence of high-dose A23187 (Fig. 4.8, p. 94) is similar to that observed with low-dose Ca\(^{++}\)-ionophore. It is of interest that both curves are so steep relative to that seen with the other cytotoxins studied. This may be suggestive that the effects of the interaction between A23187 and Org2766 on protein synthesis may be markedly different from those of the other cytotoxins. Such a line of reasoning is in line with a great deal of experimental evidence demonstrating that Ca\(^{++}\) is a regulator of many intracellular processes.
ix) Org2766

In the absence of cytotoxin, Org2766 shows a strong neurotrophic effect (Fig. 4.9 & Table 4.3, pp. 95 & 100) that does not appear time-dependent and is only weakly dose-dependent. That is, all doses of peptide show an approximately equal response except $10^{-13}$ M, which is the lowest dose used in these experiments. This low dose of peptide also showed the weakest effects in the cytotoxin experiments.

It is of interest that the peptide should lower LOH activity in the absence of cytotoxin in light of the demonstrated up-regulation of protein synthesis. The fact that the dose-response curve is essentially flat (Fig. 4.9) when the evidence indicates that protein synthesis is maximal at $10^{-8}$ M Org2766 suggests that peak neurotrophic effects are optimal at $10^{-8}$ M of the peptide.

The above argument could be confirmed or refuted by immuno-staining of the SDS-PAGE gel (see (Murry et al., 1993)) with antibody to LOH to determine more precisely how LOH synthesis varies as a function of treatment with Org2766; or, lysing the cells under each Org2766 condition to determine the maximal LDH response as a function of peptide dose.

The extracellular LOH detected by the assay in the control condition (zero peptide) of this experiment is the normal background LOH. LOH is not thought to be transported to the extracellular space via normal transport processes though some low-level background leakage is expected. The source of the LDH, therefore, is largely a result of cell membrane compromise. The cytotoxins used in these experiments are thought to induce an increase in the normal background LDH through induction of cell death or non-lethal cell membrane compromise.

Ten nanomolar Org2766 results in a 15% increase in cell number vs. control following ten days of incubation (Fig. 4.10, p. 96). This is likely due to an increase in survival rate vs. proliferation rate as the Org2766/LDH experiment (Fig. 4.9) would be expected to show an increase in LDH activity if the peptide were only increasing proliferation rate. This assumes a linear relation between cell number and LDH release. Taken together, the Org2766/LDH experiment and cell proliferation study are consistent with the argument that Org2766 enhances
cell survival at $10^{-8}$ M, a dose which shows the weakest neurotrophic effects in the presence of cytotoxin as measured by LDH activity (see Figs. 4.1 → 4.8). These results strengthen the argument that if protein synthetic effects are accounted for, the Org2766/cytotoxin experiments would show an optimal neurotrophic effect at $10^{-8}$ M Org2766.
Org2766 stimulates protein synthesis in Neuro-2a cells in vitro in a dose-dependent fashion. The function shape is described by an inverted-U. See Table 5.1 (p. 102) for raw data. This result is consistent with findings discussed in Chapter II outlining the enhancement of protein synthesis in vivo (Schotman et al., 1972; Rudman et al., 1974) and in vitro (Reith et al., 1974; Reith et al., 1975) by ACTH analogues. Other studies, however, fail to note an effect of Org2766 on synthesis of neurofilament or GAP-43, which are generally taken to be indices of neuron growth, in cultured rat spinal cord or DRG neurons (Van Der Neut et al., 1992).

Results and Discussion

Fig. 5.1 (p. 101) shows the results obtained from pulsing Neuro-2a cells for 60 min with $^{35}$S-Met in the presence of graded doses of Org2766 ($10^{-13} \rightarrow 10^{-6}$ M) following 24 hrs of peptide pre-treatment. Statistically significant increases in protein synthesis are observed at all doses from $10^{-11}$ to $10^{-8}$ M, with $10^{-8}$ M being an optimal dose (77% increase) for the effect.

The inverted-U function obtained in the present work is consistent with that found in other aspects of the neurotrophic effects of ACTH and analogues such as the enhancement of DNA, RNA and protein synthesis by ACTH$_{1-24}$ in embryonic chick cerebral hemispheres in vitro (Daval et al., 1983). An inverted-U function was also obtained when using Org2766 to enhance performance on a passive avoidance task (Fekete & De Wied, 1982); speeding recovery from sciatic nerve crush (De Koning et al., 1986); and, in increasing the number of neurite sprouts following crush of rat sciatic nerve (Verhaagen et al., 1987).
This experiment does not differentiate between transcriptional and translational effects of the peptide. However, Bohus and De Wied showed that neither ACTH_{1-10}^-7-L-Phe nor ACTH_{1-10}^-7-D-Phe altered RNA metabolism (Bohus & De Wied, 1966). The L-isomer increased while the D-isomer decreased protein synthesis in the same study. Such a finding is consistent with a role for ACTH in translational vs. transcriptional mechanisms.

The generality of the inverted-U shaped function across a variety of *in vitro* and *in vivo* phenomena reviewed in Chapter II in conjunction with the present results is interesting. Unfortunately, no direct connection can be made at this point between the protein synthetic effects and the behavioral and neurotrophic effects observed by others. Experiments to address these relations as well as the translation vs. transcription question are suggested in Chapter VII.
Experiment 1

Overview

In the first experiment the mean per cent changes in area, perimeter, form factor, fractal dimension and shape of Neuro-2a-C8 cells were assessed at 8, 24 and 48 hrs after addition of 10^-8 M Org2766 or control media. The algorithms for calculating FF, D_f and dS/dt are described in Chapter III. For both Experiments 1 and 2, when cell area is discussed, the area under consideration was the 2-D interface with the culture dish. No measurements were made in these studies to determine cell height (z-axis) or changes in cell height. See Tables 6.1 and 6.2 (pp. 115 & 116) for raw data summary.

Results and Discussion

Mean % Change in Area

No differences in per cent change in area were noted at 8, 24 or 48 hrs (Fig. 6.1, p. 103). Area, however, being a measure of size only, is not sensitive to a re-distribution of membrane relative to an arbitrary landmark such as the center of cell mass.

Mean % Change in Perimeter

No differences in per cent change in perimeter were observed at any time point (Fig. 6.2, p. 104). The perimeter measure, like area, is not a unique measure as variations or the lack of variation in perimeter provides no information as to whether the cell is changing shape.
Mean % Change in Form Factor

As in the previous two measures, no differences were found in the average per cent change in form factor (Fig. 6.3, p. 105). The same caveat is noted here as above; that is, FF is not a unique measure so provides no absolute measure regarding cell shape. Changes in cell shape can occur with no corresponding change in FF. Such a result, in fact, was noted in numerous instances during data collection.

Mean % Change in Fractal Dimension

No differences in average per cent change in \( D_f \) were found between groups at 8, 24 or 48 hrs (Fig. 6.4, p. 106). The fractal dimension of the cell contours, like the above measures, is not unique. Non-uniqueness make such measures of limited usefulness when assessing changes in contour shape as a function of time.

dS/dt via Fourier Magnitudes

Determination of the rate of change in shape via the Fourier magnitudes (dS/dt; see Chapter III) provides a true measure of cell shape which is unique and readily amenable to statistical analysis. Any change in the tangent angle length function \( \psi(\theta) \) will be reflected in the Fourier series given the calculation of a sufficient number of Fourier coefficients.

No group differences in dS/dt were found at 8, 24 or 48 hours (Fig. 6.5, p. 107). It is concluded that there are no group differences in per cent size change (as assessed by area and perimeter measures) nor shape change (as assessed by dS/dt) induced by 10^{-6} M Org2766 vs. control in the absence of cytotoxic challenge. Such a result is consistent with literature discussed in Chapter II suggesting that Org2766 effects are optimized under developmental, degenerative and regenerative conditions.

Experiment 2

Overview

Morphological recovery from 12 hrs of colchicine treatment under various Org2766 treatment protocols was assessed at 8, 24 and 48 hrs using the same measures as in
Experiment 1. No group differences were found using any of the peptide protocols via \textit{per cent} change in area, perimeter, FF or $D_f$. Significant changes in $dS/dt$ were observed for all peptide protocols vs. control at 8, 24 and 48 hrs indicating acceleration of recovery of remodeling rate by treatment with $10^{-8}$ M Org2766. The effect was noted regardless of whether Org2766 was administered prior to, during, or after colchicine insult. See Appendix A (pp. 118➔123) for a graphical representation of the Fourier magnitudes vs. harmonic number for a particular cell contour as a function of time. Figs. A.1 to A.3 show the first 300 harmonics while Figs. A.4 to A.6 show the first 50 harmonics with higher resolution for the same cell.

\textbf{Results and Discussion}

\textbf{Mean \% Change in Area}

No differences in \textit{per cent} change in area were found at 8, 24 or 48 hrs under any of the peptide protocols vs. control (Fig. 6.6, p. 108).

\textbf{Mean \% Change in Perimeter}

No differences in \textit{per cent} change in perimeter were found at 8, 24 or 48 hrs under any of the peptide protocols vs. control (Fig. 6.7, p. 109).

\textbf{Mean \% Change in Form Factor}

No differences in \textit{per cent} change in form factor were found at 8, 24 or 48 hrs under any of the peptide protocols vs. control (Fig. 6.8, p. 110).

\textbf{Mean \% Change in Fractal Dimension}

No differences in \textit{per cent} change in $D_f$ were found at 8, 24 or 48 hrs under any of the peptide protocols vs. control (Fig. 6.9, p. 111).

\textit{dS/dt via Fourier Magnitudes}

Significant increases in $dS/dt$ between all peptide protocols and control were observed at 8, 24 and 48 hrs (Fig. 6.10, p. 112) using a Kruskal-Wallis 1-way ANOVA on ranks with a
Student-Newman-Keuls post hoc where $p \leq 0.05$. No differences between peptide groups were found.

These results demonstrate that a true shape measure, such as $dS/dt$, is sensitive to changes in cell contour geometry which are not detected by size (area, perimeter), reformulated size (form factor) or non-unique shape (fractal dimension) measures. In addition, since there were no net per cent changes in cell area between peptide groups and control (Fig. 6.11, p. 113) while $dS/dt$ differences were in evidence, lipid membrane must be undergoing a shift in distribution (this is an alternate way of saying that shape is changing). Such an outcome could be accounted for within several schemes. Existing lipid can be added to or subtracted from the outer cell membrane by endo- or exocytotic processes, respectively. Under one scenario, cell membrane pinches off to form bi-layer vesicles which are either transferred to the extracellular (exocytosis) or intracellular (endocytosis) domain resulting in a decrease in cell area. Alternatively, if the vesicles initially are a part of extra- or intracellular stores, these same processes result in an increase in cell area.

Given the results of this experiment, either the addition and/or subtraction of lipid to/from the cell membrane occurs at levels that does not measurably affect the mean per cent change in cell area or perimeter; or, the additive and subtractive processes, whether via endocytosis, exocytosis or enzymatic processes, are in balance. The calculation of net per cent change in cell area (Fig. 6.11) shows no significant difference between peptide groups and control; that is, neither peptide nor control groups significantly differ from zero in terms of this parameter at 8, 24 or 48 hrs. The relatively large standard errors are indicative of the swing between addition and subtraction of lipid from the membrane, resulting in a statistical net flux of zero. Consequently, the endo- and exocytotic processes regulating cell area are in relative balance across groups. The caution made in the initial Overview of Chapter VI applies regarding this point; that is, no measurements of cell height (z-axis) or changes in cell height were made in these experiments.
It is of finite probability that any change in measured cell area was a function of change in cell height. In such a scenario, no endo- or exocytotic processes need be invoked.

The protein synthesis experiment (Chapter V) shows that protein synthesis was increased by 77% in the presence of $10^{-8}$ M Org2766 relative to control. No difference in per cent change in cell area or perimeter was found under challenge (Figs. 6.6 & 6.7) or non-challenge (Figs. 6.1 & 6.2) conditions. Protein synthesis enhancement in the presence of colchicine was assumed but not definite, though it is known that protein synthesis was enhanced under the non-challenge conditions of the zero colchicine time-lapse experiment.

The relationship between cell size, as evidenced by area and perimeter, and total cell protein is not as direct as that between total cell membrane lipid and size parameters. Cell protein can be involved in enzymatic, regulatory and signaling processes in addition to performing structural roles. Only the structural role was (indirectly) measured in the time-lapse experiments. From the significant increases in dS/dt in the peptide groups relative to control in Experiment 2 it can be inferred that the cytoskeleton was in a more dynamic state in the peptide groups. In fact, dS/dt was not significantly different from a control group in which no colchicine was used (Fig. 6.12, p. 114). A brief description of cytoskeletal organization and dynamics is included below to elucidate this point.

One model of microtubule cytoskeletal dynamics in which selected microtubule polymers undergo rapid shortening (catastrophe) and a slower re-polymerization process (rescue) is called the dynamic instability model (Mitchison & Kirschner, 1984; Kirschner & Schulze, 1986). In this model the overall polymer mass remains stable while individual microtubules are either growing or shrinking with the turnover occurring at the plus-end, located towards the cell periphery. The present results are consistent with a fairly steady overall polymer mass in a more dynamically unstable state as assessed by dS/dt. In the dynamic instability model guanosine tri-phosphate (GTP), when bound to the growing end of the polymer, dramatically lowers the off-rate constant of individual subunits (Voter et al., 1991). As GTP spontaneously hydrolyzes to GDP the off-rate
constant increases favoring microtubule depolymerization. A polymer in such a state can grow rapidly and then be stabilized by end interactions, but can also shrink rapidly with only small shifts in initial conditions. It is possible that, as of yet, unidentified capping proteins are required for long-term stabilization of individual microtubules.

A microtubule depolymerization episode can be either complete (catastrophic) or incomplete (tempered) (Sammak et al., 1987). In vitro, microtubule polymers are in dynamic equilibrium with monomeric α- and β-tubulin subunits (Sammak & Borisy, 1988) with a half-life ranging from several to 20 min. Direct observation of fluorescently labeled tubulin in the fibroblast lamellum demonstrates that reorganization of the microtubule network occurs by tempered dynamic instability (Sammak & Borisy, 1988). A large proportion of the microtubule population remains differentially stable and displays no measurable lengthening or shortening at least over a time scale of 15-20 min (Cassimeris et al., 1988).

Intracellular Ca++ concentrations (Wang et al., 1989; Solomon, 1977; Brundage et al., 1991) and temperature are also important regulators of microtubule dynamics. There exists an optimal [Ca++]i for neurite elongation, above and below which growth stops or reverses (Mattson & Kater, 1987). In general, microtubules extend along the long axis of the neurite and tend to be evenly distributed in the plane perpendicular to that axis (Daniels, 1975). Microfilaments, composed of polymerized actin, are generally arranged along the outer portion of the neurite closest to the cell membrane (Fath & Lasek, 1988). Neurofilaments display a localization similar to that of microtubules and are thought to play an important role in the determination of neurite caliber (Hoffman et al., 1988), though these polymers exist in a much more stable state relative to that of microtubules and microfilaments. Functional interactions occur between the cytoskeletal components via microtubule-associated-proteins (MAPs) which are generally regulated by phosphorylation state (Selden & Pollard, 1986). Others have shown that one of the methods by which second messenger systems control the dynamic properties of the cell cytoplasm is by regulation of the phosphorylation state of proteins which link cytoskeletal
components to each other and to other cell organelles (De Camilli et al., 1988). Watson has argued effectively that neuronal function follows form in that the forces sensed by cells can regulate cell shape. The tandem effects of external stimuli and the internal structural forces which maintain cell shape stimulate further changes in protein synthesis/degradation, ribosome formation, DNA/RNA synthesis, $[\text{Ca}^{++}]_{i}$ regulation, cell volume regulation, etc. (Watson, 1991).

The neurites extending from the cell soma of Neuro-2a-C8 cells likely contain microtubules existing in a dynamic state as evidenced by the non-zero $dS/dt$ values. The microtubules in the soma may represent a more stable polymer population similar to those observed by other workers e.g. (Schulze & Kirschner, 1987). See Figs. C1 and C2 (pp. 125, 126) in Appendix C for an example of the changes in cell contour over 5 min intervals for a 25 min total time period. When a large process changes shape (see arrow in Figs. C1 & C2), the basic skeleton of that neurite seems to be preserved in the remodeled state, at least over the observation periods used here. While both cells in Figs. C1 and C2 are morphologically dynamic, the $dS/dt$ values equal 541 and 482 for the experimental and control cell, respectively, indicating that the cell in C1 is changing shape more rapidly. A discussion of which contour features are changing most rapidly is included below. These particular cells were chosen as the $dS/dt$ values closely approximate the respective group means. The grosser features of the cell in Fig. C1 subjectively appear to change more rapidly than the equivalent features in the control cell. The $dS/dt$ values were obtained from the weighting scheme discussed previously in Chapter III, which emphasizes changes in the lower harmonics. In the control cell the finer features subjectively appear to be changing more rapidly relative to the grosser features. These results are consistent with the interpretation that $dS/dt$, using this particular weighting scheme, tends to produce larger values when large-scale structures, described by the lower harmonics, are in a more dynamic state relative to small-scale structures, represented by the higher harmonics.
The \( \frac{dS}{dt} \) data for the above two cells were re-evaluated using either equal weights or a reversed scheme where the higher frequencies were emphasized (see Appendix C, Table C1, p. 136). When using the scheme emphasizing the higher harmonics (scheme C), \( \frac{dS}{dt} \) increased by 7.4% (581) and 17.0% (564) relative to scheme A for the cells in Figs. C1 and C2, respectively (see Table C2, p. 137). Such a result indicates a greater contribution to \( \frac{dS}{dt} \) by the higher harmonics in the control cell. This finding holds for all cells of Group AP3 and the respective control group in general (see Table C3, p. 138). Scheme B, which weights all harmonics equally, produces an intermediate result in terms of per cent change vs. control, as to be expected. Scheme B per cent change vs. control values are intermediate between the values of schemes A and C for all but two P2 groups, which change very little in terms of per cent change vs. control as the weighting scheme is modified (Table C4, pp. 139➔141). This is also consistent with the interpretation that switching progressively from scheme A to B to C makes \( \frac{dS}{dt} \) progressively more sensitive to the high-frequency components of the contours.

A group EP1 cell (Fig. C5, p. 128) shows a reversed effect relative to the case discussed above in that the per cent change vs. control increases as the weighting scheme is advanced from A (5.6%) to B (7.3%) to C (10.0%) (Table C5, p. 142). The group EP1 cell subjectively appears to have a more complex shape vs. the control P1 cell; therefore, the high frequencies contribute relatively more to the shape of the experimental cell. This outcome is welcomed as it provides support to the argument that weighting scheme A is reliably most sensitive to the grosser features of the contours studied in this work.

The net per cent change in \( \frac{dS}{dt} \) vs. control as the weighting scheme is switched from scheme A to C is shown in Table C6 (p. 143). Importantly, all of the P1 groups show a net increase (mean = 8.4%); all of the P2 groups, with the exception of GP2, oscillate about 0% change (mean = 0.6%); all of the P3 groups have a decrease in per cent change vs. control (mean = -4.5%). A single image from each of the five cells of experimental group GP2 are shown in Figure C12 (p. 135). No obvious subjective differences are noted between the cells of
GP2 and (for instance) AP2 (Fig. C11, p. 134). The reason as to why GP2 should differ from the other P2 groups in terms of net per cent change vs. control when switching from scheme A to C is not known. The decrease seen with the P3 groups means that the experimental groups experienced a greater rate of remodeling of the larger-scale structures relative to control at 48 hrs after colchicine wash-out. This is not surprising as control group P3 shows an 11.5% increase in dS/dt when switching from scheme A to C (Table C7, p. 144); whereas, AP3, for example, shows only a 7.0% increase when switching from A to C (Table C7). A single image of each of the five AP3 and control group P3 cells are shown in Figures C6 and C7 (pp. 129, 130), respectively.

The increase in per cent change in dS/dt vs. control seen in the P1 cells (Table C6) indicates an increase in the remodeling rate of the finer structure relative to the control cells. Much of this can be accounted for by the fact that three of the five control cells at P1 have few processes and are very rounded (Fig. C8, p. 131); therefore, any change in the control cells is represented at relatively low frequencies. This is further shown by the fact that dS/dt for control group P1 actually decreases by 2.5% when switching from weighting scheme A to C (see Table C7). The group AP1 experimental cells (see Fig. C10, p. 133) are morphologically more differentiated vs. control, consistent with this interpretation.

The experimental groups at P2, with the exception of GP2, all demonstrate less than or equal to 1% change vs. control when switching from scheme A to C (Table C6). This means that the gross and fine features changed at rates whose ratio of gross to fine change is similar in experimental vs. control group. Since dS/dt values using scheme A are significantly greater than those of control at P2 (Table C4), the gross and fine morphological features are changing at a greater rate vs. control and it is a difference in amount, not in kind, that is occurring. Figures C9 and C11 show a single image of each of the five cells of a control group and an experimental group at 24 hrs after colchicine wash-out (AP2), respectively.
The net within groups per cent change in $dS/dt$ from P1 to P3 when switching from weighting scheme A to C is listed in Table C7. The mean net per cent change in experimental groups is 2.2% and in control groups is 14.0%. This indicates that the high frequency components of the experimental contours, representing the finer structure, recovered more rapidly and/or received less initial damage from the colchicine treatment vs. the control condition. By 48 hrs after colchicine wash-out, however, the control group shows the largest within groups per cent change in $dS/dt$ of any condition when switching from scheme A to C (11.5%) (Table C7). This likely indicates that the experimental cells are less damaged at the earliest time-point measured here (8 hrs after colchicine wash-out); therefore, the control cells show a greater relative improvement from P1 to P3 because they were relatively more damaged by the colchicine than the Org2766 treated cells.

Aside from which features are changing most rapidly, it can be stated that the experimental groups are morphologically in a more dynamic state vs. the control group cells. Equally as important, this difference is a consequence of treatment with $10^{-8}$ M Org2766. A single image of each of the five cells comprising a group for a number of conditions are shown in Figures C4 through C12 (pp. 127➔135).

Taking into account the cited evidence that cytoskeletal dynamics are dependent on $[Ca^{++}]$, the phosphorylation state of MAPs, concentrations of the individual monomers and other factors, it can be reasoned that these or some subset of these parameters were returned to or remained within control ranges when challenged cells were treated with $10^{-8}$ M Org2766 (Fig. 6.10). Newly published work shows the importance of Ca$^{++}$/calmodulin-dependent protein kinase II in the regulation of neurite outgrowth and growth cone motility in Neuro-2a cells, specifically (Goshima et al., 1993). These same parameters, inferentially, remained within normal range when unchallenged cells were exposed to Org2766 (Fig. 6.5, p. 107). Such an interpretation is consistent with the work discussed in Chapter II that ACTH fragments and analogues display
their strongest neurotrophic effects under developmental, degenerative and regenerative conditions.
CHAPTER VII
SUMMARY & CONCLUSIONS

Summary

Cultured Neuro-2a cells treated with either colchicine, vincristine sulfate, cytochalasin D or the Ca++-ionophore A23187 undergo an increase in LDH activity at 8, 24 and 48 hrs after addition of cytotoxin to the culture media. Increases in LDH activity were taken to correlate positively with cytotoxicity. Co-treatment with Org2766 reduced LDH activity relative to control in a dose and time-dependent manner. Peak neurotrophic activity was, in general, seen at roughly 10^{-10} and 10^{-6} M. A U-shaped function would describe the neurotrophic effect in these instances. However, the literature cited in Chapter II suggests the neurotrophic effects of ACTH and ACTH analogues are best described by an inverted-U function. Taking into account the inverted-U shaped effects of Org2766 on 35S-Met incorporation into Neuro-2a cells, it may well be that the peak neurotrophic effect resides at the intermediate dose of 10^{-8} M correlating with peak stimulation of protein synthesis. This is also consistent with in vitro work describing the effect of Org2766 on microtubule number in snail axons (Muller et al., 1992). Consequently, 10^{-8} M was chosen as the best dose to use in the study to determine the effects of Org2766 on recovery from colchicine insult as assessed by changes in cell morphology.

In Experiment 1 of the time-lapse video study no differences in mean per cent change in area, perimeter, form factor, fractal dimension or dS/dt were observed between control and media with 10^{-8} M Org2766. Such a result is consistent with studies discussed in the Literature Review suggesting that the neurotrophic effects of ACTH analogues are maximized under developmental, degenerative and regenerative conditions.
Morphological recovery from 12 hrs of colchicine treatment under various Org2766 treatment protocols was assessed at 8, 24 and 48 hrs using the same measures as in Experiment 1. No group differences were found using any of the peptide protocols via per cent change in area, perimeter, FF or Df. Significant changes in dS/dt were observed for all peptide protocols vs. control at 8, 24 and 48 hrs indicating acceleration of recovery of remodeling rate by treatment with Org2766. The effect was noted regardless of whether Org2766 was administered prior to, during, or after colchicine insult.

Conclusions

Org2766 protects against the cytotoxic effects of a variety of chemical agents, each having a different mechanism of action. The neurotrophic effects of Org2766, therefore, appear to arise from some general mechanism whose exact nature remains to be elucidated. The effect is not likely due to direct pharmacological anatagonism as the LDH experiments were conducted in the presence of four cytotoxic compounds with differing mechanisms of action. Similarly, as reviewed in Chapter II, Org2766 also shows protective effects in the presence of Cisplatin, taxol and acrylamide. The neurotrophic mechanism(s) may be related to the ability of the peptide to stimulate protein synthesis.

The morphological studies indicate that either pre-treatment, co-treatment or post-treatment with Org2766 protects against and/or accelerates recovery from 12 hrs of colchicine treatment as measured by dS/dt, but not by the other measures used. No differential effects on cell contour morphology were observed in the absence of chemical insult. The three general findings regarding significant effects of Org2766 on colchicine insulted cells were: 1) the finer features of the cell contours were more profoundly affected relative to control at 8 hrs after colchicine washout; 2) the gross and fine features both changed at a greater rate vs. control at 24 hrs after colchicine washout, though the ratios of gross to fine change were approximately
equal between peptide and control; 3) the grosser features were most profoundly affected relative to control at 48 hrs after colchicine washout.

Use of the Fourier magnitudes, derived from the Fourier analysis of the tangent angle length function, is shown to be an effective and reliable index of shape for the class of curves studied in this work. This method has potential for development, as the Fourier series contains complete shape information of a quantized curve given the calculation of a sufficient number of harmonics. Other analyses based on a more direct use of the Fourier coefficients per se might be fruitful. The present work has analyzed a large number of complex shapes and attempted to extract rudimentary morphological information from the Fourier magnitudes. An exacting analysis of well-defined simple and complex closed contours would be useful in advancing an understanding of the precise relations between the Fourier magnitudes and individual contour features. The use of dS/dt, as defined and developed in the present work, has shown itself to be sensitive to shape changes for a variety of closed curves where more traditional approaches have failed. As such, dS/dt is proposed to be a useful addition to the collection of tools available to the morphometrician interested in the dynamic behavior of finite closed curves.

The present work demonstrates that Org2766 provides protection against the effects of a variety of cytotoxic chemical agents possessing differing mechanisms of action for single cells in culture. This suggests that the peptide may not directly antagonize the effect of a given cytotoxin, but may activate/enhance intrinsic cellular repair mechanisms. While the cells used here were transformed cells, they retain many neuron-like properties hinting that the in vivo neurotrophic actions of Org2766 may not require systemic mechanisms, but may work directly on the injured tissue.

The majority of recent references pertaining to Org2766 focus on pre-clinical and clinical studies. Little work is being published regarding the underlying cellular (or systemic) mechanism(s) which mediate the neurotrophic properties of this ACTH analogue. A potentially fruitful future area of investigation would be to determine more precisely which proteins are
being affected by Org2766 in terms of increased synthesis and/or regulation of phosphorylation state, the second messenger system(s) involved and effects on cellular electrophysiology. Experiments measuring incorporation of labeled uridine would determine whether Org2766 primarily affects protein synthesis at the transcriptional or translational levels would also be of value.
FIGURES & TABLES
Fig. 4.1 Per cent change in LDH activity vs. Control in the presence of $10^{-7}$ M colchicine and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -8%*, -14%* and -17%*, respectively.
Fig. 4.2 Per cent change in LDH activity vs. Control in the presence of $10^{-5}$ M colchicine and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -48%*, -23%* and -6%, respectively.
Fig. 4.3 Per cent change in LDH activity vs. Control in the presence of $10^{-7} \text{ M}$ vincristine sulfate and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -32%, -30% and -2%, respectively.
Fig. 4.4 Per cent change in LDH activity vs. Control in the presence of $10^{-5}$ M vincristine sulfate and graded doses of Org2766. *Significantly different vs. Control using a t-way ANOVA with a Duncan follow-up where $p \leq 0.05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -39%, -30% and -10%, respectively.
Fig. 4.5 Per cent change in LDH activity vs. Control in the presence of $10^{-7}$ M cytochalasin D and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p < .05$. The colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal 0%, +10% and -23%*, respectively.
Fig. 4.6 Per cent change in LDH activity vs. Control in the presence of $10^{-5}$ M cytochalasin D and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq 0.05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -40%, -50% and -33%, respectively.
Fig. 4.7 Per cent change in LDH activity vs. Control in the presence of $10^{-7}$ M A23187 and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -24%*, -18%* and -2%, respectively.
Fig. 4.8 Per cent change in LDH activity vs. Control in the presence of $10^{-5}$ M A23187 and graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. The 0 colchicine/0 Org2766 condition means relative to cytotoxin alone for 8, 24 and 48 hrs equal -39%*, -41%* and -34%*, respectively.
Fig. 4.9 Per cent change in LDH activity vs. Control in the presence of graded doses of Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where p ≤ .05.
Fig. 4.10. Cell number following ten days of incubation in the presence of control media or $10^{-8}$ M Org2766. Initial plating density equalled $5 \times 10^3$ cells per dish ($n=5$). *Significantly different from control using the Student's $t$-test ($p \leq .05$).
# TABLE 4.1

**EFFECTS OF ORG 2766 ON LACTATE DEHYDROGENASE ACTIVITY IN THE PRESENCE OF VARIOUS CYTOTOXINS**

<table>
<thead>
<tr>
<th>t</th>
<th>0</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>10</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-7} M Vincristine Sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>120±1</td>
<td>115±2</td>
<td>94±1*</td>
<td>101±3*</td>
<td>108±2*</td>
<td>115±3</td>
<td>102±2*</td>
<td>111±1</td>
<td>93±3*</td>
</tr>
<tr>
<td>24</td>
<td>138±1</td>
<td>123±1*</td>
<td>119±2*</td>
<td>123±2*</td>
<td>134±4*</td>
<td>145±2</td>
<td>136±2</td>
<td>133±2</td>
<td>132±2</td>
</tr>
<tr>
<td>48</td>
<td>198±2</td>
<td>189±2</td>
<td>194±3</td>
<td>188±3</td>
<td>209±4*</td>
<td>218±1*</td>
<td>212±2*</td>
<td>213±2*</td>
<td>226±2*</td>
</tr>
<tr>
<td>10^{-5} M Vincristine Sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>106±2</td>
<td>98±2</td>
<td>97±1</td>
<td>89±1*</td>
<td>95±4*</td>
<td>100±1</td>
<td>100±4</td>
<td>93±3*</td>
<td>91±1*</td>
</tr>
<tr>
<td>24</td>
<td>125±2</td>
<td>124±1</td>
<td>131±2</td>
<td>120±3</td>
<td>125±6</td>
<td>135±2</td>
<td>135±3</td>
<td>127±2</td>
<td>123±1</td>
</tr>
<tr>
<td>48</td>
<td>256±2</td>
<td>247±3</td>
<td>248±3</td>
<td>244±2</td>
<td>254±6</td>
<td>262±3</td>
<td>262±2</td>
<td>254±4</td>
<td>250±1</td>
</tr>
<tr>
<td>10^{-7} M Colchicine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>113±1</td>
<td>100±3*</td>
<td>108±1</td>
<td>104±2*</td>
<td>94±2*</td>
<td>117±1</td>
<td>117±1</td>
<td>110±1</td>
<td>97±2*</td>
</tr>
<tr>
<td>24</td>
<td>160±3</td>
<td>151±2</td>
<td>145±2*</td>
<td>149±2*</td>
<td>138±2*</td>
<td>164±2</td>
<td>173±2*</td>
<td>175±2*</td>
<td>147±3*</td>
</tr>
<tr>
<td>48</td>
<td>350±7</td>
<td>351±3</td>
<td>335±6</td>
<td>339±3</td>
<td>329±3</td>
<td>369±2</td>
<td>399±7*</td>
<td>399±6*</td>
<td>358±6</td>
</tr>
<tr>
<td>10^{-5} M Colchicine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>82±2</td>
<td>67±1*</td>
<td>66±2*</td>
<td>65±3*</td>
<td>61±1*</td>
<td>63±2*</td>
<td>64±1*</td>
<td>59±2*</td>
<td>62±1*</td>
</tr>
<tr>
<td>24</td>
<td>130±2</td>
<td>128±2</td>
<td>122±3</td>
<td>115±5*</td>
<td>117±1*</td>
<td>132±3</td>
<td>131±3</td>
<td>121±1</td>
<td>124±2</td>
</tr>
<tr>
<td>48</td>
<td>221±5</td>
<td>217±4</td>
<td>215±1</td>
<td>193±10* 205±3</td>
<td>236±6</td>
<td>229±3</td>
<td>211±4</td>
<td>231±2</td>
<td></td>
</tr>
<tr>
<td>10^{-7} M Cytochalasin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>74±1</td>
<td>69±3</td>
<td>65±2*</td>
<td>72±2</td>
<td>67±2</td>
<td>57±2</td>
<td>80±1</td>
<td>73±2</td>
<td>74±1</td>
</tr>
<tr>
<td>24</td>
<td>121±2</td>
<td>114±1</td>
<td>121±2</td>
<td>120±1</td>
<td>121±4</td>
<td>125±6</td>
<td>126±3</td>
<td>127±1</td>
<td>135±3*</td>
</tr>
<tr>
<td>48</td>
<td>314±4</td>
<td>292±2</td>
<td>296±4</td>
<td>294±4</td>
<td>277±6*</td>
<td>305±12</td>
<td>286±4*</td>
<td>273±4*</td>
<td>268±2*</td>
</tr>
<tr>
<td>10^{-5} M Cytochalasin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>91±1</td>
<td>93±1</td>
<td>70±1*</td>
<td>69±1*</td>
<td>74±2*</td>
<td>75±2*</td>
<td>80±2*</td>
<td>76±2*</td>
<td>68±2*</td>
</tr>
<tr>
<td>24</td>
<td>222±1</td>
<td>206±2*</td>
<td>179±4*</td>
<td>180±3*</td>
<td>201±4*</td>
<td>225±2</td>
<td>211±3</td>
<td>212±4</td>
<td>215±4</td>
</tr>
<tr>
<td>48</td>
<td>386±4</td>
<td>391±8</td>
<td>326±9*</td>
<td>337±2*</td>
<td>381±4</td>
<td>412±5*</td>
<td>412±4*</td>
<td>398±5</td>
<td>406±2</td>
</tr>
<tr>
<td>10^{-7} M A23187</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>70±2</td>
<td>73±1</td>
<td>67±4</td>
<td>67±2</td>
<td>55±2*</td>
<td>62±1</td>
<td>74±2</td>
<td>76±2</td>
<td>57±2*</td>
</tr>
<tr>
<td>24</td>
<td>142±3</td>
<td>146±2</td>
<td>141±6</td>
<td>129±2</td>
<td>126±4*</td>
<td>142±2</td>
<td>148±2</td>
<td>153±2</td>
<td>123±5*</td>
</tr>
<tr>
<td>48</td>
<td>247±4</td>
<td>234±2</td>
<td>226±4*</td>
<td>220±3*</td>
<td>228±2*</td>
<td>247±4</td>
<td>256±3</td>
<td>267±2</td>
<td>232±7</td>
</tr>
</tbody>
</table>
**TABLE 4.1 — Continued.**

<table>
<thead>
<tr>
<th>t</th>
<th>0</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>10</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^{-6} M A23187</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>115.±5</td>
<td>104.±3</td>
<td>107.±3</td>
<td>112.±3</td>
<td>87.±1*</td>
<td>102.±2</td>
<td>114.±4</td>
<td>112.±4</td>
<td>91.±1*</td>
</tr>
<tr>
<td>24</td>
<td>183.±4</td>
<td>168.±3*</td>
<td>185.±2</td>
<td>189.±2</td>
<td>143.±2*</td>
<td>180.±2</td>
<td>207.±2*</td>
<td>207.±5*</td>
<td>178.±3</td>
</tr>
<tr>
<td>48</td>
<td>357.±9</td>
<td>329.±5*</td>
<td>358.±8</td>
<td>387.±2*</td>
<td>282.±6*</td>
<td>343.±7</td>
<td>391.±6*</td>
<td>372±2</td>
<td>292.±6*</td>
</tr>
</tbody>
</table>

* Significantly different vs. control using a 1-way ANOVA with a Tukey HSD follow-up where p ≤ .05.

Values equal lactate dehydrogenase activity x 10^4 as determined by spectrophotometric kinetic analysis (mean±SEM; n=5).
TABLE 4.2

EFFECT OF VARIOUS CYTOTOXINS ON % CHANGE IN LDH ACTIVITY VS. CONTROL IN THE ABSENCE OF ORG2766

<table>
<thead>
<tr>
<th>t</th>
<th>dose (M)</th>
<th>Vincristine</th>
<th>Colchicine</th>
<th>Cytochalasin D</th>
<th>A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>$10^{-7}$</td>
<td>32%*</td>
<td>8%*</td>
<td>0%</td>
<td>24%*</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>39%*</td>
<td>48%*</td>
<td>40%*</td>
<td>39%*</td>
</tr>
<tr>
<td>24</td>
<td>$10^{-7}$</td>
<td>30%*</td>
<td>14%*</td>
<td>-10%</td>
<td>18%*</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>30%*</td>
<td>23%*</td>
<td>50%*</td>
<td>41%*</td>
</tr>
<tr>
<td>48</td>
<td>$10^{-7}$</td>
<td>2%</td>
<td>17%*</td>
<td>23%*</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>10%*</td>
<td>6%</td>
<td>33%*</td>
<td>34%*</td>
</tr>
</tbody>
</table>

* Significantly different from control using a 1-way ANOVA with a Tukey HSD follow-up where $p \leq .05$.

Values equal per cent change in LDH activity vs. control in the absence of Org2766.
**TABLE 4.3**

EFFECT OF ORG2766 VS. CONTROL ON % CHANGE IN LDH ACTIVITY

<table>
<thead>
<tr>
<th>Org 2766 [-log M]</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>10</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4.2±2</td>
<td>-5.8±3</td>
<td>-12.3±3*</td>
<td>-18.0±4*</td>
<td>-12.3±3*</td>
<td>-12.3±2*</td>
<td>-11.8±2</td>
<td>-18.0±2*</td>
</tr>
<tr>
<td>24</td>
<td>3.5±1</td>
<td>-11.8±2*</td>
<td>-10.4±2*</td>
<td>-14.8±2*</td>
<td>-15.7±3*</td>
<td>-15.1±1*</td>
<td>-4.0±2</td>
<td>-7.0±2</td>
</tr>
<tr>
<td>48</td>
<td>-2.8±2</td>
<td>-14.0±1*</td>
<td>-15.5±1*</td>
<td>-11.3±2*</td>
<td>8.8±3*</td>
<td>-9.8±1*</td>
<td>-9.7±2*</td>
<td>-11.2±2*</td>
</tr>
</tbody>
</table>

* Significantly different from control using a 1-way ANOVA with a Tukey HSD follow-up where p < .05.

Values equal per cent change in LDH activity±SEM vs. control.
Fig. 5.1. Per cent change in $^{35}$S-Met incorporation vs. Control in the presence of graded doses of Org2766. Each culture (n=5) was pulsed with $^{35}$S-Met and the indicated dose of Org2766 following 24 hrs of pre-treatment with Org2766. *Significantly different vs. Control using a 1-way ANOVA with a Duncan follow-up where $p \leq .05$. 
### TABLE 5.1

**EFFECTS OF ORG 2766 ON DE NOVO PROTEIN SYNTHESIS**

<table>
<thead>
<tr>
<th>Org 2766 [-log M]</th>
<th>cpm/µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.4±1.9</td>
</tr>
<tr>
<td>13</td>
<td>51.5±2.0</td>
</tr>
<tr>
<td>12</td>
<td>56.2±3.0</td>
</tr>
<tr>
<td>11</td>
<td>60.5±4.6*</td>
</tr>
<tr>
<td>10</td>
<td>63.3±1.2*</td>
</tr>
<tr>
<td>9</td>
<td>61.8±5.3*</td>
</tr>
<tr>
<td>8</td>
<td>83.7±4.5*</td>
</tr>
<tr>
<td>7</td>
<td>80.7±5.0*</td>
</tr>
<tr>
<td>6</td>
<td>75.2±5.6*</td>
</tr>
</tbody>
</table>

* Significantly different versus Control using a 1-way ANOVA and Duncan follow-up (p < .05; n=5). Values equal mean±SEM.
Fig. 6.1. Average % change in area of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media. Each group has 10 cells with 9 measurements per cell. There are no significant group differences using a Mann-Whitney U-test where $p \leq .05$. 

- Control
- 10 nM Org2766

Time Post- Org2766

Mean % Change Area

8 h  24 h  48 h
Fig. 6.2. Average % change in perimeter of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media. Each group has 10 cells with 9 measurements per cell. There are no significant group differences using a Mann-Whitney U-test where $p \leq .05$. 
Fig. 6.3. Average % change in form factor of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media. Each group has 10 cells with 9 measurements per cell. There are no significant group differences using a Mann-Whitney U-test where $p \leq .05$. 
Fig. 6.4. Average % change in the fractal dimension ($D_f$) of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media. Each group has 10 cells with 9 measurements per cell. There are no significant group differences using a Mann-Whitney U-test where $p \leq .05$. 
Fig. 6.5. Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hrs after addition of Org2766 or control media. Each group has 10 cells with 9 measurements per cell. The algorithm for computing dS/dt is described in Methods. There are no significant group differences using a Mann-Whitney U-test where p ≤ .05.
Fig. 6.6. Average % change in area of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out. Each group has five cells with nine measurements per cell. *Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where p ≤ .05.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
Fig. 6.7. Average % change in perimeter of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out. Each group has five cells with nine measurements per cell. *Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where p ≤ .05.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
Fig. 6.8. Average % change in form factor of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out. Each group has five cells with nine measurements per cell. *Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where $p \leq .05$.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
Fig. 6.9. Average % change in the fractal dimension (D_f) of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out. Each group has five cells with nine measurements per cell. *Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where $p \leq .05$.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
Fig. 6.10. Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out (n=5). The algorithm for computing dS/dt is described in Methods. *Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where p ≤ .05.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
Fig. 6.11. Net % change in area of cultured Neuro-2a-C8 cells 8, 24 and 48 hours after colchicine wash-out. There are no significant group differences.
Fig. 6.12. Average rate of change in shape of cultured Neuro-2a-C8 cells per 5 min period 8, 24 and 48 hours after colchicine wash-out (n=5). The control group received no colchicine in this case.

P1: 24 h period prior to addition of colchicine.
P2: 12 h colchicine treatment period.
P3: 48 h period following colchicine wash-out.
TABLE 6.1

AVERAGE % CHANGE IN SIZE AND SHAPE PARAMETERS
AND MEAN dS/dt

<table>
<thead>
<tr>
<th>Group</th>
<th>t</th>
<th>Area</th>
<th>Perimeter</th>
<th>Form Factor</th>
<th>Dr</th>
<th>Mean dS/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Org2766</td>
<td>8</td>
<td>4.2±0.5</td>
<td>7.4±0.7</td>
<td>14.0±1.7</td>
<td>3.3±0.3</td>
<td>545±6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.8±0.3</td>
<td>7.5±0.9</td>
<td>13.0±1.6</td>
<td>2.8±0.3</td>
<td>529±8</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.4±0.5</td>
<td>7.1±0.5</td>
<td>15.3±1.3</td>
<td>2.9±0.3</td>
<td>524±7</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>4.0±0.4</td>
<td>7.6±1.2</td>
<td>13.3±2.0</td>
<td>3.9±0.5</td>
<td>538±9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.3±0.3</td>
<td>8.5±1.0</td>
<td>15.7±1.8</td>
<td>3.7±0.4</td>
<td>539±7</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.0±0.5</td>
<td>7.1±0.6</td>
<td>13.2±1.2</td>
<td>3.5±0.3</td>
<td>530±8</td>
</tr>
</tbody>
</table>

There are no significant between group differences for any parameters at 8, 24 or 48 hrs.
<table>
<thead>
<tr>
<th>Group</th>
<th>t</th>
<th>Area</th>
<th>Perimeter</th>
<th>Form Factor</th>
<th>Df</th>
<th>Mean dS/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>5.8±1.0</td>
<td>8.3±1.2</td>
<td>17.1±3.9</td>
<td>3.9±0.1</td>
<td>555±7*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.1±1.4</td>
<td>8.6±0.5</td>
<td>15.9±0.7</td>
<td>2.7±0.3</td>
<td>508±9*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>6.0±0.6</td>
<td>7.9±1.8</td>
<td>14.6±4.7</td>
<td>3.2±0.3</td>
<td>543±9*</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>6.0±0.6</td>
<td>10.3±1.4</td>
<td>18.9±1.6</td>
<td>4.5±0.7</td>
<td>567±8*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.8±1.1</td>
<td>7.4±1.9</td>
<td>17.1±4.6</td>
<td>3.1±0.6</td>
<td>515±17*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.8±0.4</td>
<td>6.1±0.7</td>
<td>12.9±1.3</td>
<td>2.9±0.3</td>
<td>546±9*</td>
</tr>
<tr>
<td>C</td>
<td>8</td>
<td>4.7±0.7</td>
<td>7.2±1.1</td>
<td>15.2±2.3</td>
<td>3.4±0.4</td>
<td>544±9*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.8±0.5</td>
<td>7.0±0.6</td>
<td>12.3±1.4</td>
<td>3.8±0.3</td>
<td>533±5*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.6±0.7</td>
<td>6.3±0.8</td>
<td>12.5±1.7</td>
<td>4.3±1.1</td>
<td>542±4*</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>5.3±1.3</td>
<td>8.0±1.8</td>
<td>15.7±4.0</td>
<td>4.1±0.4</td>
<td>535±10*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.8±1.0</td>
<td>7.2±1.2</td>
<td>15.6±2.0</td>
<td>2.7±0.4</td>
<td>528±8*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.3±0.9</td>
<td>8.3±1.4</td>
<td>16.1±2.7</td>
<td>3.0±0.5</td>
<td>518±14*</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>5.0±0.6</td>
<td>8.2±2.1</td>
<td>18.9±4.5</td>
<td>3.4±0.7</td>
<td>534±7*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.6±1.0</td>
<td>8.6±1.5</td>
<td>15.6±2.4</td>
<td>3.2±0.3</td>
<td>519±20*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.4±0.6</td>
<td>8.0±1.9</td>
<td>16.7±3.9</td>
<td>2.8±0.4</td>
<td>544±12*</td>
</tr>
<tr>
<td>F</td>
<td>8</td>
<td>5.0±0.5</td>
<td>9.9±1.9</td>
<td>22.0±5.0</td>
<td>4.0±0.9</td>
<td>540±8*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.2±0.4</td>
<td>7.9±0.7</td>
<td>16.8±1.6</td>
<td>3.8±0.5</td>
<td>546±9*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>5.1±0.8</td>
<td>6.9±1.0</td>
<td>16.4±2.2</td>
<td>3.1±0.4</td>
<td>550±3*</td>
</tr>
<tr>
<td>G</td>
<td>8</td>
<td>4.3±0.9</td>
<td>8.7±1.0</td>
<td>17.7±1.6</td>
<td>4.1±0.5</td>
<td>552±6*</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.4±0.7</td>
<td>4.6±0.5</td>
<td>9.8±1.3</td>
<td>2.6±0.5</td>
<td>520±14*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.9±1.0</td>
<td>7.5±1.2</td>
<td>15.9±2.2</td>
<td>3.2±0.3</td>
<td>526±19*</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>3.4±0.5</td>
<td>8.1±1.7</td>
<td>14.7±2.6</td>
<td>2.9±0.7</td>
<td>483±31</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.7±0.6</td>
<td>7.4±1.4</td>
<td>13.2±2.3</td>
<td>3.6±0.5</td>
<td>454±25</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.9±0.5</td>
<td>6.8±0.7</td>
<td>13.3±2.1</td>
<td>3.1±0.5</td>
<td>488±8</td>
</tr>
</tbody>
</table>

* Significantly different from control using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc where p ≤ .05.
APPENDICES
Fig. A.1. Fourier magnitudes (M) of the first 300 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. A.2. Fourier magnitudes (M) of the first 300 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. A.3. Fourier magnitudes (M) of the first 300 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. A.4. Fourier magnitudes (M) of the first 50 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. A.5. Fourier magnitudes (M) of the first 50 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. A.6. Fourier magnitudes (M) of the first 50 harmonics of the superimposed contour. These contours are actual data from Group A 48 hrs after colchicine washout. Temporal spacing between contours equals 5 min.
Fig. B. Reduction in per cent error (ε) with the calculation of increasing number of harmonics. See Chapter III for a discussion of ε and the corresponding algorithm.
Fig. C1. Change in cell shape as a function of time. Time progresses from left to right and down in 5 min intervals. This Group A cell was recorded 48 hrs after colchicine washout. $dS/dt$ is equal to 541, where the group mean equals 543. Note shape change of one particular process indicated by arrow.
Fig. C2. Change in cell shape as a function of time. Time progresses from left to right and down in 5 min intervals. This control cell was recorded 48 hrs after colchicine washout. $dS/dt$ is equal to 482, where the group mean equals 488. Note shape change of one particular process indicated by arrow.
Fig. C4. Change in cell shape as a function of time. Time progresses from left to right and down in 5 min intervals. This control cell was recorded 8 hrs after colchicine washout. See text in Chapter VI for discussion.
Fig. C5. Change in cell shape as a function of time. Time progresses from left to right and down in 5 min intervals. This Group E cell was recorded 8 hrs after colchicine washout. See text in Chapter VI for discussion.
Fig. C6. A single time-lapse image of each of the five cells of Group AP3.
Fig. C7. A single time-lapse image of each of the five cells of control Group at P3.
Fig. C8. A single time-lapse image of each of the five cells of control Group at P1.
Fig. C9. A single time-lapse image of each of the five cells of control Group at P2.
Fig. C10. A single time-lapse image of each of the five cells of Group AP1.
Fig. C11. A single time-lapse image of each of the five cells of Group AP2.
Fig. C12. A single time-lapse image of each of the five cells of Group GP2.
<table>
<thead>
<tr>
<th>Harmonic Range</th>
<th>$A^a$</th>
<th>$B^b$</th>
<th>$C^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 → 10</td>
<td>6.0</td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td>11 → 20</td>
<td>5.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>21 → 30</td>
<td>5.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>31 → 40</td>
<td>4.5</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>41 → 50</td>
<td>4.0</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>51 → 60</td>
<td>3.9</td>
<td>1.0</td>
<td>3.6</td>
</tr>
<tr>
<td>61 → 70</td>
<td>3.8</td>
<td>1.0</td>
<td>3.7</td>
</tr>
<tr>
<td>71 → 80</td>
<td>3.7</td>
<td>1.0</td>
<td>3.8</td>
</tr>
<tr>
<td>81 → 90</td>
<td>3.6</td>
<td>1.0</td>
<td>3.9</td>
</tr>
<tr>
<td>91 → 100</td>
<td>3.5</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>101 → 150</td>
<td>3.0</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>151 → 200</td>
<td>2.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>201 → 250</td>
<td>1.5</td>
<td>1.0</td>
<td>5.5</td>
</tr>
<tr>
<td>251 → 300</td>
<td>1.25</td>
<td>1.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

$^a$ These are the same weights used in the computation of $dS/dt$ in Chapter VI.

$^b$ All values are equally weighted.

$^c$ Weights are reversed so that the higher harmonics contribute to a greater extent vs. $A$ in the determination of $dS/dt$. 
### TABLE C2

**dS/dt AS A FUNCTION OF MODIFICATIONS IN WEIGHTING SCHEME**

<table>
<thead>
<tr>
<th>Weighting scheme</th>
<th>dS/dt</th>
<th>%↑ vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Cell AP3</td>
<td>541</td>
<td>12.2%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>482</td>
<td>-</td>
</tr>
<tr>
<td><strong>B</strong> Cell AP3</td>
<td>155</td>
<td>7.6%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>144</td>
<td>-</td>
</tr>
<tr>
<td><strong>C</strong> Cell AP3</td>
<td>581</td>
<td>3.0%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>564</td>
<td>-</td>
</tr>
</tbody>
</table>

* dS/dt values in one experimental and one control cell as a function of change in the weighting scheme.
## TABLE C3

dS/dt AS A FUNCTION OF MODIFICATIONS IN WEIGHTING SCHEME

<table>
<thead>
<tr>
<th>Weighting scheme</th>
<th>dS/dt</th>
<th>% ↑ vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Group AP3</td>
<td>543±9*</td>
<td>11.3%</td>
</tr>
<tr>
<td>Control</td>
<td>488±7</td>
<td></td>
</tr>
<tr>
<td>B Group AP3</td>
<td>155±3</td>
<td>9.9%</td>
</tr>
<tr>
<td>Control</td>
<td>141±4</td>
<td></td>
</tr>
<tr>
<td>C Group AP3</td>
<td>581±12</td>
<td>6.8%</td>
</tr>
<tr>
<td>Control</td>
<td>544±25</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different vs. control using a Mann-Whitney Rank Sum test (*p < .05*). Values equal mean±SEM.
<table>
<thead>
<tr>
<th>Group</th>
<th>Period</th>
<th>Weighting scheme</th>
<th>dS/dt</th>
<th>% ↑ vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>P1</td>
<td>A</td>
<td>555±7*</td>
<td>14.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>581±9</td>
<td>24.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>587±9</td>
<td>24.6%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>508±9*</td>
<td>11.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>143±3</td>
<td>10.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>534±15</td>
<td>11.0%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>543±9*</td>
<td>11.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>155±3</td>
<td>9.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>581±12</td>
<td>6.8%</td>
</tr>
<tr>
<td>B</td>
<td>P1</td>
<td>A</td>
<td>567±8*</td>
<td>17.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>158±2</td>
<td>20.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>578±9</td>
<td>22.7%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>515±17*</td>
<td>13.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>147±5</td>
<td>13.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>549±19</td>
<td>14.1%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>546±9*</td>
<td>11.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>155±2</td>
<td>9.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>580±5</td>
<td>6.6%</td>
</tr>
<tr>
<td>C</td>
<td>P1</td>
<td>A</td>
<td>543±9*</td>
<td>12.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>154±3</td>
<td>17.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>575±14</td>
<td>22.1%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>533±5*</td>
<td>17.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>151±2</td>
<td>17.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>561±11</td>
<td>16.8%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>542±4*</td>
<td>11.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>153±2</td>
<td>8.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>571±8</td>
<td>5.0%</td>
</tr>
<tr>
<td>Group</td>
<td>Period</td>
<td>Weighting scheme</td>
<td>$dS/dt$</td>
<td>% ↑ vs. Control</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>-----------------</td>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td>D</td>
<td>P1</td>
<td>A</td>
<td>535±10*</td>
<td>10.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>150±3</td>
<td>14.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>554±18</td>
<td>17.6%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>528±8*</td>
<td>16.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>150±2</td>
<td>16.2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>561±8</td>
<td>16.6%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>518±14*</td>
<td>6.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>147±3</td>
<td>4.2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>552±9</td>
<td>1.5%</td>
</tr>
<tr>
<td>E</td>
<td>P1</td>
<td>A</td>
<td>534±7*</td>
<td>10.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>150±2</td>
<td>14.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>560±4</td>
<td>18.9%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>519±20*</td>
<td>14.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>147±5</td>
<td>13.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>545±21</td>
<td>13.3%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>544±12*</td>
<td>11.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>156±3</td>
<td>10.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>590±16</td>
<td>8.5%</td>
</tr>
<tr>
<td>F</td>
<td>P1</td>
<td>A</td>
<td>540±8*</td>
<td>11.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>153±2</td>
<td>16.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>567±8</td>
<td>20.4%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>546±9*</td>
<td>20.2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>154±3</td>
<td>19.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>574±11</td>
<td>19.3%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>550±3*</td>
<td>12.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>155±1</td>
<td>9.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>578±8</td>
<td>6.2%</td>
</tr>
</tbody>
</table>
### TABLE C4 — Continued.

<table>
<thead>
<tr>
<th>Group</th>
<th>Period</th>
<th>Weighting scheme</th>
<th>dS/dt</th>
<th>% ↑ vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>P1</td>
<td>A</td>
<td>553±6*</td>
<td>14.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>157±3</td>
<td>19.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>587±19</td>
<td>24.6%</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>520±14*</td>
<td>14.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>152±4</td>
<td>17.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>582±15</td>
<td>21.0%</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>526±19*</td>
<td>7.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>152±5</td>
<td>7.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>577±18</td>
<td>6.0%</td>
</tr>
<tr>
<td>Control</td>
<td>P1</td>
<td>A</td>
<td>483±31</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>131±11</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>471±45</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>A</td>
<td>454±25</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>129±7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>481±31</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>A</td>
<td>488±7</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>141±4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>544±25</td>
<td>--</td>
</tr>
</tbody>
</table>

* Significantly different vs. Control group (received no Org2766 at any time) using a Kruskal-Wallis 1-way ANOVA on ranks with a Student-Newman-Keuls post hoc test where p ≤ .05.
<table>
<thead>
<tr>
<th>Weighting scheme</th>
<th>dS/dt</th>
<th>%↑ vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell EP1</td>
<td>563</td>
<td>5.6%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>533</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell EP1</td>
<td>162</td>
<td>7.3%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>151</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell EP1</td>
<td>618</td>
<td>10.0%</td>
</tr>
<tr>
<td>Control Cell</td>
<td>562</td>
<td>-</td>
</tr>
</tbody>
</table>

* dS/dt values in one experimental and one control cell as a function of change in the weighting scheme.
**TABLE C6**

EFFECTS OF WEIGHT MODIFICATIONS

Net % Change vs. Control When Switching from Scheme A to C

<table>
<thead>
<tr>
<th>Group</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1 (^a)</td>
<td>9.7</td>
</tr>
<tr>
<td>BP1</td>
<td>5.3</td>
</tr>
<tr>
<td>CP1</td>
<td>9.7</td>
</tr>
<tr>
<td>DP1</td>
<td>6.8</td>
</tr>
<tr>
<td>EP1</td>
<td>8.3</td>
</tr>
<tr>
<td>FP1</td>
<td>8.6</td>
</tr>
<tr>
<td>GP1</td>
<td>10.1</td>
</tr>
<tr>
<td>mean</td>
<td>8.4</td>
</tr>
<tr>
<td>AP2</td>
<td>-0.9</td>
</tr>
<tr>
<td>BP2</td>
<td>0.7</td>
</tr>
<tr>
<td>CP2</td>
<td>-0.8</td>
</tr>
<tr>
<td>DP2</td>
<td>0.3</td>
</tr>
<tr>
<td>EP2</td>
<td>-1.0</td>
</tr>
<tr>
<td>FP2</td>
<td>-0.9</td>
</tr>
<tr>
<td>GP2</td>
<td>6.5</td>
</tr>
<tr>
<td>mean</td>
<td>0.6</td>
</tr>
<tr>
<td>AP3</td>
<td>-4.5</td>
</tr>
<tr>
<td>BP3</td>
<td>-5.3</td>
</tr>
<tr>
<td>CP3</td>
<td>-6.1</td>
</tr>
<tr>
<td>DP3</td>
<td>-4.6</td>
</tr>
<tr>
<td>EP3</td>
<td>-3.0</td>
</tr>
<tr>
<td>FP3</td>
<td>-6.5</td>
</tr>
<tr>
<td>GP3</td>
<td>-1.8</td>
</tr>
<tr>
<td>mean</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

\(^a\) These values were obtained by calculating the difference between the % \(\uparrow\) in \(dS/dt\) vs. Control values using scheme C vs. scheme A listed in Table C4.
### TABLE C7

**EFFECTS OF WEIGHT MODIFICATIONS**

<table>
<thead>
<tr>
<th>Group</th>
<th>% Change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Net % Change&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>5.8</td>
<td>1.2</td>
</tr>
<tr>
<td>AP2</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>AP3</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>BP1</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td>BP2</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>BP3</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>CP1</td>
<td>5.9</td>
<td>-0.5</td>
</tr>
<tr>
<td>CP2</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>CP3</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>DP1</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>DP2</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>DP3</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>EP1</td>
<td>4.9</td>
<td>3.6</td>
</tr>
<tr>
<td>EP2</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>EP3</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>FP1</td>
<td>5.0</td>
<td>0.1</td>
</tr>
<tr>
<td>FP2</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>FP3</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>GP1</td>
<td>6.1</td>
<td>3.6</td>
</tr>
<tr>
<td>GP2</td>
<td>11.9</td>
<td>mean 2.2</td>
</tr>
<tr>
<td>GP3</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Control P1</td>
<td>-2.5</td>
<td>14.0</td>
</tr>
<tr>
<td>P2</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>11.5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> These values were obtained by calculating the within groups % increase in dS/dt when switching from weighting scheme A to C.

<sup>b</sup> These values were obtained by subtracting the within groups % increase in dS/dt when switching from weighting scheme A to C (seen in middle column) at P1 from P3.
Legend Figures D1 & D2

A: Original 8-bit images.

B: Image in A following high-pass filter.

C: Tracing of image in B yielding a 1-bit image.

D: The (x,y) co-ordinate points of the contour of the image in C, obtained from the output of the boundary tracking routine, were stored in an ASCII file for analysis by the various morphometric routines. The reconstruction of the image from these co-ordinate points is shown in D. The reflection of the image is due to a difference in the designation of point (0,0) between the original image and the boundary-tracked image. Note that all analyses are reflection invariant. The flattening of the reconstructed image at this stage is due to the correction for the non-square pixels in the light sensing chip of the video camera. The slight rounding of the smallest detail is a function of the reconstructed image being printed out from a Postscript format vs. the pixel for pixel print format of the top three images.
Fig. D1. See legend.
Fig. D2. See legend.
REFERENCES


VITA

Ralph was born to James E. and Martha D. Murry on the fifth of February, 1951, in Ottawa, Illinois. After attending Catholic primary and secondary school in Ottawa, Ralph served in the armed forces and worked in various aspects of the building trades. Ralph began full-time attendance at Northern Illinois University in 1984 and graduated with a Bachelor of Arts summa cum laude in 1986. Following eighteen months in the graduate program in physiological psychology at NIU Ralph transferred to Loyola University of Chicago's Graduate Program in Neuroscience in 1988. Ralph received a Loyola University Dissertation Fellowship and completed his dissertation work in the laboratories of Drs. Jerry A. McLane and Robert D. Wurster.
APPRAVAL SHEET

The dissertation manuscript submitted by Ralph F. Murry has been read and approved by the following committee:

Jerry A. McLane, Ph.D., Director
Associate Professor, Biochemistry
Loyola University of Chicago

Samuel P. Bowen, Ph.D.
Professor, Theoretical Physics
Argonne National Laboratory

Robert D. Wurster, Ph.D.
Professor, Physiology
Loyola University of Chicago

Mary Druse Manteuffel, Ph.D.
Professor, Biochemistry
Loyola University of Chicago

Talat Khan, Ph.D.
Principal Investigator, RR&D
Hines Veterans Hospital

The final copies have been examined by the director of the dissertation committee and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

11/2/93
Date

Signature