MORPHOLOGICAL CHANGES IN CHICK EMBRYO NEURAL TISSUE ASSOCIATED WITH HYDROCORTISONE USE DURING PRENATAL DEVELOPMENT

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Abstract

Glucocorticoids known to be such powerful agents that cell growth, differentiation and cell death are influenced in the brain of mammals throughout life. Despite this, relatively little toxicological information regarding prenatal exposure is available. The aim of this study was to determine the effect of prenatal hydrocortisone exposure on cell viability and cell morphology in chick embryonic neurons. Four different histological staining techniques namely, Hematoxylin and Eosin (H&E), Cresyl Fast Violet, Silver impregnation and a combination of Gold Chloride and Toluidine Blue were used to evaluate chick embryo neural tissue exposed to 0.137μM or 0.685μM hydrocortisone on day 3.75 (Carnegie stage 16) and day 5.5 (Carnegie stage 18) of development. Histological processing was optimized and neural tissue evaluated for any changes in neuron morphology and cell number. Specific ultrastructural changes to membraneous structures were evaluated by transmission electron microscopy (TEM). Fixation procedures that resulted in little to no disruption of these structures were optimized and used in studies evaluating the effect of hydrocortisone on neuron morphology. Primary chick embryonic neuronal cultures were prepared and increasing concentrations of hydrocortisone (26.3nM, 0.16μM, 0.63μM, 3.8μM, and 22.8μM) added. Fluorescence microscopy was applied to the *in vitro* hydrocortisone exposed primary neuronal

cultures. A combination of fluorescein diacetate (FDA) and propidium iodide (PI) was used to evaluate the effect of hydrocortisone on cell viability, whereas dichlorodihydrofluorescein diacetate (DCH_2FDA) was used to visualize reactive oxygen species (ROS) generation in neurons.

Histological evaluation of the neural tissue of chick embryos exposed to 0.137μM and 0.685μM hydrocortisone showed reduced neuron density and morphological changes associated with cell death. Glutaraldehyde with added magnesium chloride ($MgCl_2$) as stabilizing chemical and potassium permangenate were two fixatives that caused minimal disruption to neural tissue. These two fixating methods were applied to control neural tissue as well as tissues exposed to 0.137μM and 0.685μM hydrocortisone. When evaluated by TEM, the control tissue appeared to be intact with no displacement. Exposure of neurons to 0.137μM hydrocortisone appeared to have severe effects on the morphology of the mitochondria, endoplasmic reticulum (ER), nuclear and plasma membranes. More extensive damage was noted with 0.685μM hydrocortisone, leaving almost no cellular structure. Both concentrations of hydrocortisone indicated cell death associated with apoptosis and necrosis. *In vitro* studies using primary cultures of chick neurons indicated that hydrocortisone is non-toxic at low concentrations (26.3nM – 3.8μM) with the percentage viability ranging between 73% and 88%. A more toxic effect was seen at high concentrations (22.8μM). Cell death at the higher concentrations (22.8μM and 3.8μM) of hydrocortisone occurred due to ROS generation, as indicated by DCH_2FDA fluorescence

In conclusion, hydrocortisone indicated neurotoxicity at high concentrations of exposure. Although cell death could be detected, the exact mechanism (apoptosis or necrosis) still needs to be investigated. Since the developing brain is so susceptible to chemical insults care should be taken when administering this drug to pregnant mothers or young children.

Declaration

I, Eureka Smit hereby declare that this research dissertation is my own work and has not been presented for any degree of another University;

Signed:.....

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List of Abbreviations, Symbols and Chemical Formulae

%	Percentage
°C	Degrees centigrade
α	Alpha
β	Beta
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
µg/ml	Microgram per millilitre
$AgNO_3$	Silver nitrate
AO	Acridine orange
Apaf-1	Apoptosis protease activating factor-1
$AuCl_2$	Gold chloride
ATP	Adenosine triphosphate
Bax	Pro-apoptotic protein from the Bcl-family
Bcl-2	Anti-apoptotic protein from the Bcl-family
Bcl-X_L	Anti-apoptotic protein from the Bcl-family
Bid	Pro-apoptotic protein from the Bcl-family
BSA	Bovine serum albumin
C1	Carbon atom number one of ring A in the cortisol central skeleton
C2	Carbon atom number two of ring A in the cortisol central skeleton
C11	Carbon atom number eleven of ring B in the cortisol central skeleton

C16	Carbon atom number sixteen of ring B in in the cortisol central skeleton
$CaCl_2$	Calcium chloride
CAM	Chorioallantoic membrane
CH_3COONa	Sodium acetate
cm^2	Centimetres squared
CNS	Central nervous system
Co	Company
CO_2	Carbon dioxide
$Cu(NO_3)_2$	Copper (II) nitrate
dATP	2'-deoxyadenosine 5'-triphosphate
ddH_20	Double distilled water
DCF	Diclorofluorescein
DCH_2FDA	2'7'-diclorodihydrofluorescein diacetate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
Dr	Doctor
EDTA	Ethylene diamine tetra acetic acid ($C_{10}H_{16}N_2O_8$)
e.g	For example
ER	Endoplasmic reticulum
Etc.	Etcetera (and so forth)
et al.	et alibi (and others)
Fas	Receptor from the TNF family
FasL	Fas Ligand

FCS	Foetal Calf Serum
FDA	Fluorescein diacetate
g	gram
g/L	grams per litre
HBSS	Hanks Buffered Salt Solution
H&E	Hematoxylin and Eosin
$HgCl_2$	Mercuric chloride
H_2O	Water
H_2O_2	Hydrogen peroxide
i.e.	That is
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KMnO4	Potassium permangenate
L	Litre
Ltd.	Limited
M	Molar
mdm-2	Cellular proto-oncogene
mdr1a	Transcriptional P-glycoprotein
mg	milligrams
$MgCl_2$	Magnesium chloride
mg/ml	milligrams per millilitres
ml	millilitre
mM	millimolar
mm^3	cubic millimeter
MPT	Mitochondria permeability transition pore
mRNA	Messenger ribonucleic acid

NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
$NaH_2PO_4 \cdot 2H_2O$	Sodium phosphate dihydrate
Na_2HPO_4	Disodium Hydrogen Phosphate
No.	Number
O_2	Oxygen
OsO_4	Osmium tetroxide
p53	Tumor suppressor gene
pH	Logarithmic scale for the measurement of the acidity or alkalinity of an aqueous solution
PI	Propidium iodide
Post-	After
Pre-	Before
Pty.	Proprietary
Reg.	Registration
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SA	South Africa
TEM	Transmission electron microscopy
TNF	Tumor nectosis factor
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UK	United Kingdom
USA	United States of America
UTHSCSA	University of Texas Health Science Center at San Antonio
x	times

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Chapter 1 : Introduction

Glucocorticoids have long been known to influence cell proliferation and cell fate (Crochemore *et al*., 2002). Despite this, many of the actions of glucocorticoids are still unexplained (Smith *et al*., 1991). Since the observation by Hench in 1949, of a dramatic response to cortisone in patients with rheumatoid arthritis, adrenal steroids and synthetic glucocorticoids have become widely used in medicine (Clark *et al*., 1992). However, soon after Dr Hench and Kendall received the Nobel prize for Medicine for cortisone as treatment, reports of unfavourable side effects from pharmacological doses of glucocorticoids administered for extended periods of time were reported (Rubin *et al*., 1997).

Glucocorticoids can cause certain cellular responses by direct interaction with cell membranes, or more commonly, complex with specific receptors to modulate gene expression and protein synthesis (Smith *et al*., 1991). When administered in high pharmacological doses, steroids generally produce severe side effects that are extension of their pharmacological actions. No route or preparation is free from these diverse side effects. Because of the severity of these side effects, a number of factors must be considered when prolonged use of steroids is contemplated. These factors include the age of the individual, since side effects such as hypertension occur in older individuals, and the possibility of administering the lowest possible dose without compromising the desired therapeutic effect. Relationships of dosage, duration and host response should therefore be carefully considered. Since glucocorticoids mostly do not cure any disease, but are predominantly used for symptomatic relief, the effect of a possible glucocorticoid induced Cushing's syndrome should also be taken into consideration when administering these steroids (Rubin, 1982) (Clark *et al*., 1992). However, when used at the recommended dosages glucocorticoids are predictably effective, safe, well tolerated and have few systemic effects (Abisheganaden *et al*., 2000).

Glucocorticoids are increasingly being recognized as key players in the development and maintenance of brain structures (Crochemore *et al*., 2002). Exposure of a fetus to levels of cortisol that are inappropriately high for the current stage of fetal development

has been proposed to be a major link between adverse intrauterine conditions and altered development of fetal tissues and organs (Antonow-Schlorke *et al*., 2003). This medication however, is still prescribed to pregnant women suffering from diseases such as rheumatoid arthritis and asthma or women who are at risk for preterm delivery. Furthermore children, at critical stages of neurodevelopent, are also frequently administered glucocorticoids for various diseases.

The British National Formulary chapter determined that during the year 2000, 5.3 million prescriptions of hydrocortisone were filled, and that number showed an increase of 1.7% from the previous year (http://www.doh.gov.uk/public/stats1.htm). Also in the UK, 98% of obstetric units are prescribing repeated courses of glucocorticoids when the risk of preterm delivery is judged to persist. In South Africa it was found that overall glucocorticoid unit production every three months range between 654 460 units and 1253 569 units from 2001 to 2005. While for hydrocortisone alone, the highest production was noted during October to December of 2004 with 550 560 units (Personal communication with company, Pharmacia).This trend is disturbing, since there is no evidence to support an advantage of multiple over single courses, whereas an increasing body of evidence suggests that fetal exposure to glucocorticoids may have long-term adverse consequences for childhood and adult development (Smith *et al*., 2000). Therefore it would appear as if physicians administer this medication with ease and little thought is given to the consequences.

From here the question arise as to how cognitive development will be affected in babies exposed to prenatal and postnatal glucocorticoids. To better understand the effects, neuron development was investigated at cellular level by evaluating the effect of hydrocortisone on neuron morphology. The chick embryo *in ovo* model was utilized as this very closely reflects the intrauterine environment of a human developing fetus (Butler and Juurlink, 1987).

The mechanisms of action of glucocorticoids and the previously investigated effects of glucocorticoids on the developing nervous system were reviewed in Chapter 2. The effect of hydrocortisone on neuron morphology was investigated in Chapter 3 by using histology that enables the general visualization of neurons. Four different staining methods namely Hematoxylin and Eosin, Cresyl Fast Violet, Silver Impregnation and a combination of Gold Chloride and Toluidine Blue were used. The histological processing

of chick embryo heads were optimized and the optimal procedures applied to further experimental samples. Samples were evaluated for any changes in cell number and membrane morphology after hydrocortisone exposure. Due to limited magnification of light microscopy, transmission electron microscopy (TEM) was used in chapter 4 to evaluate the effect of hydrocortisone on the ultrastructure of the plasma membrane, nuclear membrane and organelles such as the mitochondria and endoplasmic reticulum (ER). Optimal fixation procedures for TEM was determined in order to better visualize the membranous structures. Fixatives that caused little to no disruptions to the membranes were used in further studies. Since a decrease in cell number could not be completely confirmed by light microscopy, fluorescence microscopy was applied to determine neuron cell viability in chapter 5. The fluorescent dyes fluorescein diacetate (FDA) and propidium iodide (PI) were used to determine the concentrations of hydrocortisone that has an effect on cell viability. FDA, measuring esterase activity of the cell indicated viable cells and PI that can only cross a membrane that has lost its integrity, were used in combination. The fluorescent probe dichlorodihydrofluorescein diacetate (DCH_2FDA) was also used to determine whether cell death occurred due to reactive oxygen species (ROS) generation in the neurons. DCH_2FDA only shows fluorescence in the presence of the ROS, hydrogen peroxide (H_2O_2) a reported trigger for cell death. All results were taken into consideration and a hypothesis with regard to mechanism of cell death and consequences of prenatal glucocorticoid exposure were discussed in Chapter 6.

The scientist finds his reward in the joy of comprehension, and not in the possibilities of application to which any discovery may lead. (Albert Einstein, (1932) Epilogue to Planck: "Where is Science Going?" p211)

The overall aim of this study was to understand the effects of hydrocortisone on prenatal neural development and to highlight the importance of developing alternative therapeutic strategies.

Chapter 2 : Literature Review

2.1 INTRODUCTION

Cortisol is a steroid hormone that is produced by the cortex of the adrenal gland and has potent anti-inflammatory action (Alberts *et al*.,1998). Technology allowed the development of hydrocortisone, a synthetic hormone that also exhibits the anti-inflammatory properties of cortisol (Guyton, 1987) (Brocklebank *et al*., 2001). Conditions that are treated with hydrocortisone can be divided into three groups namely (i) auto-immune such as rheumatoid arthritis (Cole and Schumacher, 2005); (ii) allergy such as asthma and eczema (Macdessi *et al*., 2003) (Goustas *et al.*, 2003); and (iii) inflammatory conditions such as sport injuries (Read and Motto, 1992).

The long term neurological effects of hydrocortisone and the effect on brain development in fetuses are not well known (Flagel *et al*., 2002) and yet this medication is used by children and pregnant women.

Damage to or cell loss in the brain during early development may cause severe problems later in life. Although hydrocortisone is a very useful drug, it should be investigated for the teratogenic effects it may cause to the developing nervous system. This would lead to the development of safer alternative therapeutic strategies for pregnant women and developing children.

2.2 GLUCOCORTICOIDS

The adrenal cortex produces a variety of hormones that are classified according to their origin and chemical structure. These hormones are collectively known as corticoids (coticosteroids). The adrenal cortex contains a large amount of cholesterol which is utilized in the synthesis of the corticoids. Functionally, the corticoids are classified in three categories: the glucocorticoids, mineralcorticoids and the sex hormones (e.g. estrogen). The glucocorticoids are synthesized in the zona fasciculata, the intermediate layer, of the adrenal cortex (Meyer *et al*., 1996). Adrenal corticoids are not stored, but are synthesized as needed (Clark *et al*., 1992).

2.2.1 The discovery of cortisone and the development of glucocorticoids as a therapeutic agent

During the 1930's six hormones from the adrenal cortex were isolated and identified by Dr E.C. Kendall of the Mayo Clinic in the United States. At the same institution Dr P.H. Hench studied the possible effects of adrenal hormones on rheumatoid arthritis. However, it was not until 1948 when Merck, Sharp and Dohme synthesized a sufficient quantity of cortisone that Dr Hench could demonstrate, during clinical trails, the potent anti-inflammatory activities that cortisone possess. One year after the first publications on the efficacy of cortisone in the treatment of rheumatoid arthritis, Dr Kendall and Hench were awarded the Nobel Prize for medicine. Consequently cortisone, as well as other glucocorticoids were developed and used as therapeutic agents for a variety of diseases (Rubin *et al*., 1997).

2.2.2 Biosynthesis of glucocorticoids and synthetic corticoids

The various steroids are structurally similar, all being synthesized from the same chemical precursor, cholesterol. In the zona fasiculata and zona reticularis, cholesterol is changed to pregnolone, which is metabolized to progesterone then to desoxyhydrocortisone and finally to cortisol (cortisone) (Clark *et al*., 1992). Cortisol (cortisone) consists of a central skeleton of three interconnected rings, each of six carbon atoms and another ring, consisting of five carbon atoms (Figure 2.1) (Jasani, 1979).

Cortisol (cortisone) is the most prominent glucocorticoid found in the human. It is a colorless, crystalline steroid with a molecular weight of 362.5, a melting point of 217-220°C and is slightly soluble in water (Meyers *et al*., 1970). At supraphysiological (pharmacological) dosages, the glucocorticoids are anti-inflammatory, anti-allergic and anti-rheumatic. Cortisone is biologically inactive and is converted in the liver to hydrocortisone by changing the keto-group on C11 to a hydroxyl group (Sommers, 2000). Modification of the structure of hydrocortisone resulted in derivatives, which are highly effective treatment agents (Abisheganaden *et al*., 2000). Although natural corticoids can be obtained from animal adrenals, synthetic analogues are usually synthesized from cholic acid (obtained from cattle) or steroid sapogenins found in plants of *Lilaceae* and *Dioscoreaceae* families (Meyers *et al*., 1970).

Biosynthesis of the steroid hormone involves alteration of the side group of cholesterol with the aid of specific enzymes present in cells (Jasani, 1979). Addition of double bonds, methylation or hydroxylation to the basic structure of hydrocortisone yields synthetic analogues of cortisol that will increase anti-inflammatory potency (Rubin, 1982). The ultimate aim in altering the steroid molecule, is to decrease sodium-retaining activity and to increase anti-inflammatory glucocorticoid activity (Swingle *et al*., 1982). The addition of a double bond at the C1,C2 position of hydrocortisone yields prednisone. Hydroxylation or methylation at the C16 position, give rise to bethamethasone or dexamethasone (Rubin, 1982) (Sommers, 2000).

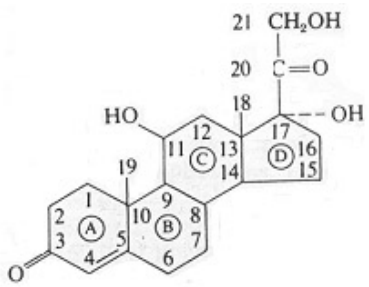


Figure 2.1 Basic corticosteroid nucleus (Rubin, 1997)

After the early era of synthetic glucocorticoids, new formulations and routes of delivery were developed as strategies for achieving a high therapeutic index in the treatment of dermatologic, respiratory and articular inflammatory conditions (Miller, 2000). Glucocorticoids are available in a wide range of preparations and can be administered parenterally, topically or by inhalation (Rubin, 1982).

2.2.3 Metabolism of glucocorticoids

Glucocorticoids are well absorbed by the gastro-intestinal tract (McPhillips, 1982). During passage through the liver, glucocorticoids are removed from the circulation. Hepatic metabolism involves the reduction and conjugation of glucocorticoids to form water-soluble compounds, which can be excreted into the urine. An alpha$_2$ globulin, called transcortin, synthesized by the liver binds approximately 95% of the circulating glucocorticoids under normal circumstances. The remaining 5% is the metabolically

active fraction, and any excess glucocorticoids may be loosely bound to albumin (Meyers *et al*., 1970).

During physiological conditions, 25-30mg glucocorticoids, specifically hydrocortisone and cortisol, are produced by the adrenal cortex and secreted daily. At pharmacological levels, depending on the severity of the disease, and whether acute or chronic, glucocorticoid administration can range from 5mg per day to 300mg per day (Pachorek, 2002). The synthetic analogues are approximately 75% bound to transcortin and it is probably only the free unbound form that is biologically active (Sommers, 2000). If the patient does not suffer from adrenal hormone insufficiency, the administration of these glucocorticoids will result in daily glucocorticoid concentrations much higher than that in normal individuals (Miller, 2000).

The half-life of glucocorticoids in the circulation is normally about 90 - 110 minutes, and may be increased when large amounts (pharmacological levels) are present (Meyers *et al*., 1970). Hepatic degradation of glucocorticoids reduces plasma concentrations fairly rapidly, after 8 hours only 25% of the peak value remains, and the active drug disappears in about 12 hours (Clark *et al*., 1992). Although the glucocorticoids disappear out of the blood, the metabolic changes which they initiate at tissue level persist for hours or maybe even days. Therefore, their biological half-life is much longer than their plasma half-life (Sommers, 2000).

Glucocorticoids can be divided into three groups according to the half-life of each compound. Hydrocortisone is considered a short-acting steroid, prednisone an intermediate-acting steroid and bethamethasone or dexamethasone long-acting steroids. Although the half-life of these compounds differ, the potency of these glucocorticoids remains equal when dose and rate of administration are taken into account (Rubin, 1997).

2.2.4 Mechanism of action of glucocorticoids

Glucocorticoids do not cure any disease, they are predominantly used for their anti-inflammatory properties and provide only symptomatic relief (Clark *et al*., 1992). Because of their anti-inflammatory activity, glucocorticoids have been used in a variety of diseased states, including: respiratory diseases, rheumatic and collagen diseases,

skin diseases, oncotherapy, organ transplantation, shock and liver disease (Sommers, 2000) (Meyers *et al*., 1970). Glucocorticoids can cause certain cellular responses by direct interaction with cell membranes, or more commonly, they complex with specific receptors to modulate gene expression and protein synthesis (Smith *et al*., 1991).

Glucocortiocid receptors are found in most cell types (Sommers *et al*., 2003) (Smith *et al*., 1991). Because glucocorticoids are steroid hormones, they are hydrophobic and able to pass freely through the lipid bilayer of the cell membrane. Once inside the cell, the glucocorticoid interacts with a specific cytoplasmic receptor. A conformational change activates the receptor protein and the receptor-steroid complex is translocated via nuclear pores into the nucleus. In the nucleus, the activated receptor-steroid complex binds to the regulatory sequence on the target gene of the DNA. Transcription is initiated and complementary messenger ribonucleic acid (mRNA) produced. Translation of the mRNA results in the production of various proteins or enzymes. It is important to note that not all genes which are modulated by glucocorticoids are up-regulated (Alberts *et al*., 1998) (Smith *et al*., 1991) (Abisheganaden *et al*., 2000).

Glucocorticoids promote the synthesis of lipocortin. Lipocortin is a protein that inhibits phopholipase A_2, the rate limiting enzyme in the production of eicosanoids. Inhibition of phospholipase A_2 via lipocortin prevents the release of arachidonic acid and the synthesis of eicosanoids such as the prostaglandins and leukotrienes (Smith *et al*., 1991) (Clark *et al*., 1992) (Swingle *et al*., 1982) (Abisheganaden *et al*., 2000). Prostaglandins and leukotrienes are both compounds derived from arachidonic acid that induce inflammation by various mechanisms, including vasodilation, increased vascular permeability and chemo-attraction of neutrophils (Campbell, 1999). Glucocortiocids also inhibit the induction of cyclo-oxygenase by cytokines and thereby block cytokine enhancement of prostaglandin synthesis (Clark *et al*., 1992).

Most physiological effects of glucocorticoids can be detected within 30 minutes (Jasani,1979). However, according to the half-life of the product and the action of the glucocorticoid following nuclear binding, glucocorticoids sometimes do not lead to immediate symptom relief, as the synthesis of certain proteins e.g. lipocortin take at least 6 hours before a significant effect can be seen (Sommers *et al*., 2003).

2.2.5 The effects of glucocorticoids

Great controversy regarding the effects of glucocorticoids on the body exists. The side effects of glucocorticoids range from minor to severe and life threatening. The biological consequences vary both qualitatively and quantitatively depending on the route, frequency and type of steroid employed (Torphy *et al*., 1997)(Jasani, 1979). When administered in pharmacological doses for prolonged periods, steroids generally produce toxic effects that are extensions of their pharmacological actions (Rubin, 1982) (Rubin, 1997). Toxic side effects are also more prevalent with systemic administration and careful consideration should be employed when prolonged use of steroids is contemplated (Park, 1986).

Because of their chemical similarity to cortisol, the anti-inflammatory steroids exert important metabolic and endocrine effects in addition to the anti-inflammatory action. Like cortisol, their metabolic effects tend to be wide-spread, affecting many organs and tissues that possess the specific cytoplasmic receptor proteins. However, the net effect that glucocorticoids exert varies considerably depending on the type of tissue (Jasani, 1979) (Meyers *et al*., 1970). Long term exposure of glucocorticoids has been shown to affect almost all tissue and therefore an effect will be seen in basically every physiological system; for a detailed list of the system it affects as wells as the effects itself, as presented in Table 2.1.

Table 2.1 Effects of prolonged glucocorticoid therapy (compiled from various sources)

SYSTEM / AREA	EFFECT
IMMUNE	Glucocorticoids cause a heightened susceptibility to serious bacterial, viral and fungal infections. Poor wound healing with decreased scar tissue formation may also be seen (Rubin, 1997).
SKIN	Excessive thinning of the skin due to atrophy induced by glucocorticoids (Miller, 2000).
CARDIO-VASCULAR	Increased artherosclerotic risk, especially in elder individuals (Miller, 2000). Glucocorticoids induce hypertension specifically in people with underlying cardiovascular disease (Rubin, 1982). Capillary fragility has also been seen in long-term glucocorticoid exposure (Smith *et al*., 1991).
MUSCULAR	Various myopathies have been observed with prolonged glucocorticoid exposure (Miller, 2000). Glucocorticoids may also exert an effect at neuromuscular junctions, although the exact mechanism remains unclear (Rubin, 1997).
OPTHALMIC	Glucocorticoids may induce cataract formation and glaucomas (Rubin, 1997).
REPRODUCTIVE	Hypogonadism has been observed in individuals exposed to glucoccorticoids for a long period of time (Miller, 2000).
GASTRO-INTESTINAL AND METABOLISM	Glucocorticoids may lead to the formation of peptic ulcers, or the reactivation of previously healed ulcers. Glucocorticoids, due to their ability to induce tissue atrophy, can therefore enhance ulcerogenic potential. Glucocorticoids have been shown to stimulate gluconeogenesis (Rubin, 1997). Obesity, dyslipidemia and glucose intolerance with hyperglycemic action (diabetic symptoms) occur in about one forth to one third of the patients who receive steroid therapy for extended periods of time (Miller, 2000).
FLUIDS AND ELECTROLYTES	Administration of glucocorticoids increases sodium retention and potassium excretion and if prolonged, can cause hypokalemic alkalosis (Clark *et al*., 1992).
SKELETAL AND GROWTH	Increased renal calcium loss, together with inhibition of renal reabsorption and gastro-intestinal absorption of calcium ions are seen with prolonged glucocorticoid use (Clark *et al*., 1992). These reduced levels in calcium may have severe effects on osteoblasts (Rubin, 1997). Osteoporosis is a well known effect of long-term glucocorticoid use, and in children growth retardation due to cessation of long-bone growth occur (Silva *et al*., 1997) (Abisheganaden, 2000).
NEUROLOGICAL	Treated individuals may experience euphoria and other behavioral abnormalities (Clark *et al*., 1992). Psychological abnormalities occur in 4% to 36% of patients (Sommers, 2000). This varies from anxiety and agitation to manic depressive psychosis or schizophrenia. Insomnia may be a forerunner of severe psychotic reactions. The hippocampus is the principle neural target for glucocorticoids (Rubin, 1997). It contains high concentrations of glucocorticoid receptors and has a marked sensitivity to these hormones. In the developing individual, glucocorticoids prevent the increase in forebrain DNA and depresses DNA synthesis in virtually all cell types (Jasani, 1979).

As shown in Table 2.1, the effects of glucocorticoids may influence the growth and development of an individual as so little is known about the effects that glucocorticoids may have on the development of a fetus, most researchers advise that care should be taken during pregnancy as glucocorticoids exhibit teratogenic properties (Torphy *et al*., 1997). Physicians usually monitor neonates born to women taking long-term corticosteroids for adrenal insufficiency, physical and psychological development (Taddio, 2002). Experimental work has repeatedly shown that glucocorticoids (specifically hydrocortisone) administered to pregnant animals cause a high risk of cleft palates in the offspring (Peterka *et al*., 1983). To understand the teratological effects of glucocorticoids, the development of the nervous system will be discussed.

2.3 THE DEVELOPING NERVOUS SYSTEM

During embryonic morphogenesis, a collection of individual neurons turns into a functioning network with unique characteristics (Ronen *et al*., 1998). This functioning network will undergo various alterations and modifications to finally form a functional brain. However, during the whole gestational age (the age of an embryo counting from the time of fertilization) (http://www.thefreedictionary.com), the developing brain has a vulnerability that is easily influenced by factors such as hypoxia and chemical stimulation resulting in specific neurodevelopmental consequences (McQuillen *et al*., 2005). The different stages of development will now be discussed.

2.3.1 Development from primitive cell layers to the neural tube

At the third week of gestation three germ layers are established in the embryo. These three layers are made up of different types of tissue, namely the ectoderm, mesoderm and endoderm. The nervous system and the skin (with accessory structures i.e. hair, nails, etc.) develop from the ectoderm layer (Bergman *et al*., 1999). During this time, the ectoderm thickens and forms a slipper shaped plate, called the neural plate. A crease or fold in the neural plate appears and deepens to become known as the neural groove (Scheibel, 1999). The lateral edges of the neural plate soon become elevated to form the neural folds. As these neural folds become more elevated, they approach each other and fuse in the midline to form the first rudiment of the entire nervous system – the

neural tube (Gray, 1918). The coordinated movements of the cells during this time of development are a result of neurulation genes that assist in the shaping and bending of the neural plate and closure of the neural groove (Colas *et al*., 2001). The fusion of the neural tube begins in the cephalic regions and proceeds in cephalic and caudal directions. However, at the cranial and caudal ends of the embryo, fusion is delayed leaving residual openings called neuropores. These neuropores temporarily form open connections between the lumen of the neural tube and the amniotic cavity. Closure of the cranial neuropore occurs at approximately the 25th day near the rostral region of the neural tube. The caudal neuropore closes almost 2 days after cranial neuropore closure (O'Rahilly *et al*., 1994).

As the rostral neuropore closes, the thickened neurofolds fuse to form three dilations, known as the primary brain vesicles, (Table 2.2) known as the prosencephalon or forebrain, the mesencephalon or midbrain and the rhombencephalon or hindbrain. When the embryo reaches the fifth week of development, the prosencephalon consists of two parts, the telencephalon – which will later develop into the cerebral hemispheres and the diencephalon, later becoming the thalami. At the same time, the rhombencephalon can be divided into the metencephalon, later known as the pons and cerebellum, and the meylencephalon, which later forms the medulla oblongata. The midbrain and cerebral aqueduct in the adult human brain will develop from the brain vesicle known as the mesencephalon (Sadler, 2000) (Gray, 1918).

Table 2.2 Primary brain vesicles in the chick embryo and their derivatives (Sadler, 2000)

Brain	**Primary Vesicles**	**Secondary Vesicles**	**Derivatives**
Forebrain	Prosencephalon	Telencephalon	Cerebral Hemispheres
		Diencephalon	Thalami
Midbrain	Mesencephalon	Mesencephalon	Midbrain – Cerebral aqueduct
Hindbrain	Rhombencephalon	Metencephalon	Pons
			Cerebellum
		Myelencephalon	Medulla Oblongata

2.3.2 General histogenesis

The entire wall of the recently closed neural tube which consists of neuroepithelial cells, is formed by a thick pseudostratified epithelium. These neuroepithelial cells are the progenitors of all neurons and glial cells (Aaku-Saraste *et al*., 1997).

Once the neural tube closes, neuroepithelial cells begin to give rise to a cell type characterized by a large round nucleus – the primitive nerve cells, also called the neuroblasts. At this stage of development, neuroepithelial cells are very susceptible to respond to nonspecific mechanico-chemical stimulation that may cause abnormalities during further neurulation (Savel'ev, 1993). The dividing neuroepithelial cells found next to the lumen of the neural tube are surrounded by neuroblasts that create a mantle layer, just within the external limiting membrane (Figure 2.2). Neuroepithelial cells are constantly dividing, giving rise to neuroblasts. However, once neuroblasts form, they lose the ability to divide (Sadler, 2000).

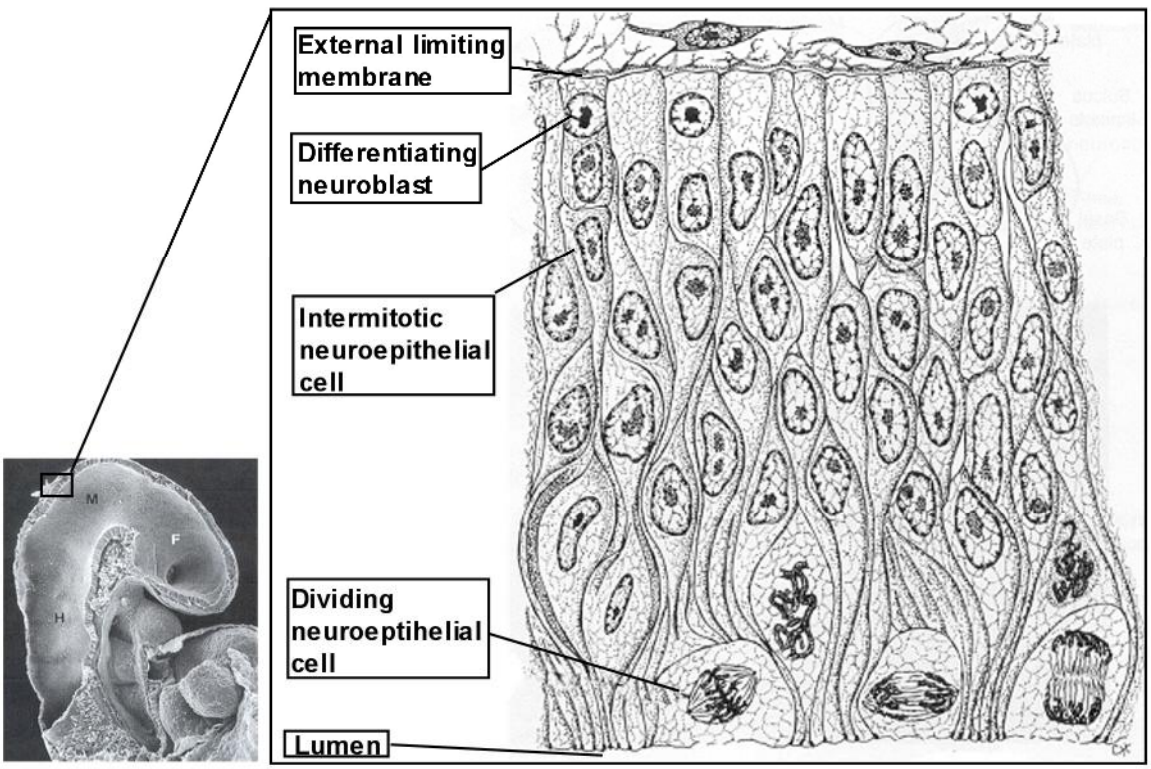


Figure 2.2 Section of the neural tube illustrating the location of neuroepithelial cells and

neuroblasts. In the scanning microcraph the three brain vesicles hindbrain (H), midbrain (M) and forebrain (F) can be seen (Sadler, 2000).

Neuroblasts initially show a central process, called a transient dendrite that extends to the lumen. With time, as the neuroblasts migrate from the lumen to the mantle layer, this transient dendrite disappears and gives rise to neuroblasts with a rounded and apolar appearance (Bergman *et al*., 1999). This rounded apolar appearance of the neuroblast are only temporary, since further differentiation give rise to two cytoplasmic processes on opposite sides of the cell body. Neuroblasts with these two processes are known as bipolar neuroblasts. Each process develops individually to either form a primitive axon or primitive dendrites. At this stage the bipolar neuroblast has transformed into a multipolar neuroblast. Further development of multipolar neuroblasts results in adult neurons (Sadler, 2000).

Primitive supporting cells, the gliablasts, are also formed by neuroepithelial cells; this happens after production of the neuroblasts ceases. Like the neuroblasts, gliablasts migrate to the mantle and marginal layers. Once there, they differentiate to protoplasmic astrocytes, fibrillary astrocytes and oligodendroglial cells. When neuroepithelial cells cease to produce neuroblasts and gliablasts, they differentiate into ependymal cells (Sadler, 2000).

2.3.3 Histological differentiation of the brain

Due to divisions, the walls of the neural tube are progressively thickened and also result in the enlargement of the brain vesicles at the anterior end of the tube. Preceding neural tube closure, the plasma membrane of neuroepithelial cells shows a progressive downregulation in polarity, indicating the onset of neurogenesis (Aaku-Saraste *et al*., 1997). The neuroepithelial cells at the lumen, or ventricular border, multiply and give rise to neuroblasts that migrate to the external border of the thickening neural tube vesicle or telencephalon, which will later develop into the cortex of the brain (Scheibel, 1999).

The cerebral cortex of the adult is made up of six cell layers. Each layer has its own distinct pattern of organization and connections (Burkitt *et al*., 1993). The migrating neuroblasts form these layers in an inside-out development. The cells initially move to form the deepest or sixth layer. Each successive migration ascends farther to form the more superficial layer. Of the primitive supporting cells, the radial glial guide cells

develops that assist neuroblasts in migrating through the layers already laid down. It is believed that anomalies in these radial glial guide cells may result in developmental abnormalities, such as epilepsy and dyslexia (Scheibel, 1999).

Decoy neural cells locate themselves at the margin between the cortical or grey matter and the white matter, as well as at the outermost edge of the neural tube wall, which will later develop into the most superficial layer of the cortex. Temporary fiber connections or holding patterns from structures below the cerebral cortex, specifically the thalamus, begin to grow into the primitive cortex. Synaptic connections are established between these temporary fibers and the decoy neural cells. As the neuroblasts migration nears an end and all the layers are successfully accomplished the decoy cells disappear and permanent synaptic connections with the neuroblasts, later to be neurons, are created (Scheibel, 1999). Although connectivity at this stage is rather limited, it has been shown that ionic channels are present and that primitive neuronal cells display excitability characteristics (Hendriks *et al*. 1997)(Morest *et al*. 1999).

Since the developing brain is so vulnerable to endogenous and exogenous stimulation, either physical or chemical, and because these factors may influence neurodevelopment (Barbazanges *et al*., 1996), a drug such as hydrocortisone should be investigated for the effect that it may exert on the developing nervous system. Because it is known that glucocorticoids are powerful agents that influence cell growth and differentiation (Cortez *et al*., 2003), and because these steroids are frequently used by the mother during the prenatal period, the mechanisms, influences and characteristics it exerts on the developing nervous system needs to be investigated.

2.4 GLUCOCORTICOIDS AND THE DEVELOPING NERVOUS SYSTEM

Recent advances in neurobiology have heightened interest in the effects of glucocorticoids on the brain and behavior (Starkman *et al*., 2001). In fact, glucocorticoids are such powerful agents that parameters such as cell growth, differentiation and cell death are influenced throughout life in the mammalian brain (McCarthy *et al*., 2002). Despite the serious influences that glucocorticoids have on the developing nervous system, little is known about the long term effects of glucocorticoids in the pre-and postnatal period in humans (Edwards *et al*., 2001).

2.4.1 Prenatal exposure to glucocorticoids and the postnatal effects

Prenatal exposure to elevated levels of glucocorticoids (whether endogenous or exogenous) may cause severe complications later in life (Edwards *et al*., 2001). A few studies have been carried out to evaluate the effect that endogenous glucocorticoids may have on fetal development. It is well known that severe depression is associated with elevated glucocorticoid (specifically cortisol) levels (Belanoff *et al*., 2001). These studies found that when pregnant individuals undergo prolonged stress, an increase in glucocorticoid levels can be detected in mother and fetus (Buitelaar *et al*., 2003).

In some instances, due to diseased states in the mother (asthma sufferer) or fetus (risk of preterm birth) systemic glucocorticoids are administered to the pregnant mother. These exogenous glucocorticoids readily cross the placenta to influence the developing fetus (Seckl, 2004). It was found that glucocorticoids administered during the third trimester of pregnancy had little to no effect on children, aged 1-12 years, when evaluated by standardized tests (Veszelovszky *et al*., 1981) (MacArthur *et al*., 1981,1982) (Collaborative Group on Antenatal Steroid Therapy) (NIH Consensus Conference). Children between 1-7 years that were exposed to synthetic glucocorticoids through the entire gestational age were evaluated with no formal tests (simply by reports from their mothers and teachers) and no lasting effects were reported (Tincini *et al*., 1992) (Wagoner, 1993). Troutman *et al*., (1995) however, found during a pilot study with standardized tests, that children whose mothers were treated with glucocorticoids throughout pregnancy were shy, emotional, and less sociable and have a greater trend for avoidance. Gaillard *et al*., (2001) found that neonates who received antenatal steroid treatment after preterm birth had a 15% chance of neurodevelopmental disability. From these studies it can be seen that very few results regarding the psychological outcome of glucocortiocids on the developing nervous system correlate. And since neurogenesis occur in certain areas of the brain well into adult life, and appears to be modulated by glucocorticoids, the effects that glucocorticoids may have psychologically, morphologically and physiologically, should be carefully considered.

Glucocorticoid administration during pregnancy reduced offspring birth weight and low-birth-weight babies have higher plasma cortisol levels throughout adult life. These adults, who have been exposed to glucocorticoids prenatally then also exhibit behavior

reminiscent of anxiety (Seckl, 2004). Steroid hormones contribute to shaping behavioral function including attention, perception, memory and emotional processing during early development and act as risk factors for psychopathology (Goodyer *et al*., 2001) (Erickson *et al*, 2003). It is therefore obvious that elevated glucocorticoids may have downstream effects on the brain and play a facilitatory role in the development of adaptive effects. In fact, glucocorticoids can exert maladaptive rather than adaptive effects thereby causing the exacerbation of certain psychological conditions, including depressive mood states (Wolkowitz *et al*., 1999) (Korte, 2001).

Changes in cellular structure due to glucocorticoid exposure usually develop over the course of several days, whereas effects on energy metabolism or signal transduction may become apparent within hours (de Kloet *et al*., 1998). High levels of glucocorticoids during early development result in morphological, physiological and behavioral modifications later in life (Edwards *et al*., 2001). In a study by Flagel *et al*., (2002) a 30% reduction could be seen in cerebral tissue volume in neonates treated with glucocorticoids. Flagel *et al*., (2002) evaluated the effect of dexamethasone on neurodevelopment in newborn rat pups between day 3 and day 6 after birth. This treatment regimen was used to mimic a prolonged 42-day treatment commonly used in intensive care settings for premature infants to lessen chronic lung disease. The treatment group received 0.5mg/kg dexamethasone on postnatal day 3, followed by 0.25mg/kg dexamethasone on postnatal day 4, with 0.125mg/kg on postnatal day 5 and 0.05 mg/kg on postnatal day 6. Brain weights and measurements were obtained during necropsy. Elevated glucocorticoids affect brain function through two general mechanisms: interaction through the genome and interaction with cell membranes (Wolkowitz *et al*., 1999).

Neurotoxic changes include alterations of neuronal differentiation, growth, migration and neurotransmitter turnover with possible underlying mechanisms being energy deprivation, exitotoxicity (ion channel overreactivity contributing to neurodegeneration) and disturbed calcium homeostasis (Edwards *et al*., 2001) (Cortez *et al*., 2003). Glucocorticoids mainly induce premature development of certain structures and although this might be an attempt to protect the nervous system, such drastic changes may cause irreversible damage. Examples of premature development include: (i) initiation of adrenergic differentiation in the embryo by stimulation of catecholamine producing cells

from the premigratory crest cells, (ii) premature induction of secretion of the enzyme glutamine synthetase and (iii) induction of premature Growth Hormone cells to become mature and respond to Growth Hormone Releasing Hormone (Smith *et al*., 1984) (Moscona, 1975) (Inoue *et al*., 2002). These neurotoxic processes may accentuate morphological changes to such an extent that cell death may occur in certain vulnerable neurons, resulting in brain damage (Wolkowitz *et al*., 1999) (Goodyer *et al*., 2001) (Simon *et al*., 1998).

In the developing central nervous system, glucocorticoids have been shown to have detrimental effects on neurons and glial cells. Researchers found that glucocorticoids retard differentiation and maturation of pyramidal neurons, oligodendrocytes and astrocytes (Huang *et al*., 2001). A great deal of research has been done on the short-term consequences of glucocorticoid administration, and it has been found that atrophy in these neurons is reversible when the hormone is removed. However, after long term exposure it would appear that the cells begin to die (Lambroso *et al*., 1998).

2.4.2 The hippocampus and glucocorticoid receptors

Glucocorticoid actions in the brain are mediated by glucocorticoid receptors and mineralocorticoid receptors. Glucocorticoid receptors occur throughout the brain but are most abundant in the hippocampal, hypothalamic and pituitary area (Belyi, 1980). The hippocampus contains non-selective mineralocorticoid receptors that bind cortisone with an affinity, approximately 10-fold higher than colocalized glucocorticoid receptors (Kawata *et al*., 2002). The glucocorticoid receptors themselves also show differential affinity in pyramidal neurons of the hippocampus (Joels *et al*., 1991). Thus, in the hippocampus, glucocorticoids can activate both glucocorticoid and mineralocorticoid receptors. This progressive activation of mineralocorticoid receptors and additional activation of glucocorticoid receptors can cause profound changes in neuronal integrity, neuronal function and changes in neuroendocrine regulation (de Kloet *et al*., 1998).

Access of natural and synthetic glucocorticoids to brain steroid receptors is controlled by three factors. The first is the bioavailability of glucocorticoids due to corticosteroid binding globulin. Dexamethasone does not bind corticosteroid binding globulin and is available in high amounts. The second determinant is the enzyme 11β-hydroxysteroid dehydrogenase, which plays a role in the modulation of access of high amount of

cortisone. A third determinant of availability, which particularly pertains to synthetic corticosteroid, is the mdr1a P-glycoprotein. Animals with a disruption of the mdr1a gene show increased accumulation of glucocorticoids in the brain (de Kloet *et al*., 1998).

The cellular effects of steroids will have consequences for the functional processes involving the hippocampus. Both excitatory and inhibitory information are affected when corticosteroid levels are altered. When glucocorticoid receptors become occupied, additionally to the mineralcorticoid receptors, excitatory transmission (and thus the hippocampal output) is reduced. At very high glucocorticoid levels, inhibitory networks are impaired (de Kloet *et al*., 1998). Glucocorticoid elevation synergizes with stress-induced activation of serotonergic, dopaminergic, and noradrenergic neurons in the brain stem and thus increases the sensitivity of limbic-forebrain areas (Laaris *et al*., 1995) (de Kloet *et al*., 1998). In fact, elevated glucocorticoids may even cause changes in the serotonin receptor (Lopez et al., 1997). All these morphological and physiological changes in the neurons, culminates in cognitive impairment, such as memory loss seen in elder individuals (Starkman *et al*., 2001) (Lambroso *et al*., 1998) (Newcomer *et al*., 1999).

In a study using liver cells, it was found that glucocorticoids increase the activity of membrane proteins (Rosseau *et al*., 1980). Some researchers also illustrated that possible membrane glucocorticoid receptors exist in amphibian neurons (Moore, 2002). It is therefore a possibility that glucocorticoids may have severe effects on the membrane structure of neurons in other experimental animals and even humans.

Ample evidence shows that chronic elevation of glucocorticoid levels lead to neurodegeneration or suppressed neurogenesis in the hippocampus (Lambroso *et al*., 1998). The degree of hippocampal atrophy, can be correlated to the degree of elevated glucocorticoid levels (Lupien *et al*., 1998). Hortnagl *et al*., (1993) found that the same amount of damage can be seen in elevated glucocorticoid levels, independent of the time exposure. Thus, any increase in glucocorticoids may result in severe damage, disregarding the period of exposure. Elevated glucocorticoid levels will not only affect hippocampal cell responses but also compromise the viability of the cells, particularly when the exposure persists and neuroendocrine dysfunction are being disrupted (de Kloet *et al*., 1998) (McEwen, 1998).

2.4.3 The effect of glucocorticoids on neuronal calcium

Calcium plays an important role in the homeostatis of neuronal growth (McCarthy *et al*., 2002). Neuronal calcium stores associated with specialized intracellular organelles, such as the ER and mitochondria, dynamically participate in generation of cytoplasmic calcium signals which accompany neuronal activity (Verkhratsky *et al*., 1998). Any dysfunction in the movement from outside to inside the cell or between organelles inside the cell may have fundamentally negative effects and the disturbance may even lead to apoptosis and/or necrosis (Pretorius *et al*., 2005).

As already mentioned, long term glucocorticoid exposure causes loss of hippocampal pyramidal neurons, as well as reduced level of excitability in a calcium-mediated mechanism (McEwen, 1998). When the glucocorticoid receptors are activated to a large extend (high doses of glucocorticoids), intracellular calcium levels were found to be increased (de Kloet *et al*., 1998). The differential activation of particular signal transduction pathways by highly localized changes in calcium may determine whether a cell survives or dies (McCarthy *et al*., 2002).

Chronic over-induction of certain enzymes due to elevated glucocorticoid levels would affect many ATP-demanding processes, such as the maintenance of membrane potentials and transport across the membrane. In fact, one of the most prominent features seen with elevations in glucocorticoid levels, is the long-term enhancement of calcium influx into neurons. Potassium and sodium fluxes were on the other hand little affected by increased steroid receptor occupation (de Kloet *et al*., 1998). Other researchers found that potassium channels are inhibited, due to interaction between the ion channel and the glucocorticoid, thereby leading to neuromodulatory effects (Zaki *et al*., 2002). These alterations in ionic flow, may account for the changes in excitability and possible membrane damage seen in tissue exposed to glucocorticoids.

2.5 CELL DEATH MECHANISMS DUE TO GLUCOCORTICOID EXPOSURE

Although it has been established that exposure to hydrocortisone may cause cell death of neural tissue, the exact mechanism of death remain unclear. Lee *et al*. (2002), found a persistent loss of hippocampal volume during major depression. Hippocampal atrophy

due to depression typically involves significant hyper-secretion of glucocorticoids, and these glucocorticoids may play a role in neuron cell death. Other factors that could also have a possible role include: intracellular calcium movement across organelle membranes, oxygen radical generation, neuronal defenses against insults and energy availability (ATP) (Lee *et al*., 2002). Most of these factors play a crucial role in, and can be associated with, apoptosis. However, death by necrosis, or a combination of the two death mechanisms aponecrosis, should be considered. Various studies have indicated that glucocorticoids may be responsible for cell death in different types of tissue via apoptosis (Motyka *et al*., 1995) (Balakumaran *et al*., 1996) (Hoetzenecker *et al*., 2004). Other researchers simply stated that cells exposed to glucocorticoids suffer severe damage and die (Vijayan *et al*., 1987) (Lambroso *et al*., 1998) (Goodyer *et al*., 2001) (Erickson *et al*., 2003) (Cortez *et al*., 2003).

2.5.1 Apoptosis and glucocorticoids

Apoptosis, also known as programmed cell death, is involved in the morphogenesis of embryonic tissues as well as in homeostasis of adult tissues. Apoptosis can be regarded as an injury-limiting mode of cell disposal since the formation of apoptotic bodies avoids inflammation (Huppertz *et al*., 1999). During the process of apoptotic body formation, several structural changes in the cells occur. In summary, the cell shrinks and becomes denser, the chromatin becomes pyknotic (margination of chromatin), the nucleus can also undergo lysis (karyorhexis) and the cell emits processes that often contain nuclear material – the budding phenomenon, resulting in apoptotic bodies (Majno *et al*., 1995). In contrast to previous descriptions, it is now postulated that the mitochondria may undergo swelling and changes to the ER may occur (Jaeschke *et al*., 2003). Physiologically, the cell undergoes a certain cascade of events (Huppertz *et al*., 1999) (Jaeschke *et al*., 2003). Induction of this cascade can be the result of certain death activator proteins binding to receptor sites. Increased calcium ion concentrations, as well as reactive oxygen species (ROS) may be responsible for initiating the apoptotic cascade (Pretorius *et al*., 2005).

A schematic representation of how glucocorticoids activate apoptosis is shown in Diagram 2.1. Glucocorticoids such as hydrocortisone can activate the intrinsic and extrinsic pathway of apoptosis (Konopleva *et al*., 1999) (Pretorius *et al*., 2005). Glucocorticoids can directly activate the p53 tumor suppressor gene by phosphorylation.

Phosphorylation of the gene causes it to become stable and active (Schafer, 1998). The oncogene mdm-2 prevents phosphorylation of p53 by binding to the N-terminal domain on the protein resulting in the degredation of p53. However, the moment that p53 becomes phosphorylated, it will inhibit mdm-2 in an auto-regulatory loop (Burns *et al*., 1999). Glucorticoids may induce reactive oxygen species (ROS), and thereby also causing p53 to become phosphorylated (Lowe *et al*., 2000). The active and stable phosphorylated p53 leads to an increased transcription of Bax, a pro-apoptotic protein from the Bcl-family. Other members of the Bcl-family affected by activated p53 are Bcl-2 and Bcl-X_L. Bcl-2 and Bcl-X_L are anti-apoptotic proteins localized in the outer mitochondrial membrane. When Bcl-2 and Bcl-X_L become activated they inhibit the mitochondria from releasing cytochrome *c*. However, activated p53 prevents the anti-apoptotic effects of Bcl-2 and Bcl-X_L by inhibiting their activity (Burns *et al*., 1999) (Konopleva *et al*., 1999).

Glucocorticoids can also follow the Fas extrinsic apoptotic pathway (Woodle *et al*., 1998) (Burns *et al*., 1999) (Konopleva *et al*., 1999). It has been indicated that some medications, including hydrocortisone, or cytotoxic drugs activate Fas (Konopleva *et al*., 1999). Fas is a receptor from the tumor necrosis factor (TNF) family (Woodle *et al*., 1998). Fas ligand (FasL) or cytotoxic drugs bind to Fas and activate Caspase 8. Stable and active p53 also cause a transcriptional increase in Fas levels, and ultimately also an increase in the activation of caspase-8 (Burns *et al*., 1999) (Konopleva *et al*., 1999). Caspase-8 in turn causes the activation of Bid, another pro-apoptotic Bcl-family member. Bid, like Bax is moved to the mitochondria where these pro-apoptotic factors induce the opening or formation of a mega-channel called the mitochondrial permeability transition pore (MPT). As soon as this megachannel is formed or opened, there is an influx of calcium ions. These calcium ions can be from the cytoplasm, or from a ruptured ER. ROS generated by glucocorticoids may cause severe damage to the ER resulting in the release of its contents into the cytosol, thereby increasing the level of calcium ions in the cytosol and ultimately also the mitochondria (Van Cruchten *et al*., 2002).

An influx of calcium ions into the mitochondria, will result in an increase in mitochondrial volume (Sukocheva *et al.*, 1997). This swelling of the mitochondria will put strain on the mitochondrial membranes, and eventually leading to the rupture of the outer membrane. Cytochrome *c*, which is located in the intermembrane space, will be released into the

cytosol. In the cytosol, cytochrome *c* together with apoptosis protease activating factor-1 (Apaf-1) forms a complex with dATP. This complex is responsible for the activation of pro-caspase 9. Pro-caspase 9 activates caspase 9, which in turn activates caspase 3 and the cell enters apoptosis (Van Cruchten *et al*., 2002) (Pretorius *et al*., 2005).

2.5.2 Necrosis and glucocorticoids

Although the apoptotic pathways seem clear, great controversy exists regarding necrosis. Most researchers are of the opinion that necrosis is not a form of cell death, but rather the end stage of any cell death process (Majno *et al*., 1995) (Van Cruchten *et al*., 2002). Necrosis is signaled by irreversible changes in the nucleus and cytoplasm. Therefore, just as apoptosis, a necrotic cell undergoes certain morphological changes. The formation of a mega-channel in the mitochondria (MPT) also occurs in necrosis and swollen mitochondria are present. Hydrocortisone has been shown to increase the mitochondrial calcium pool resulting in swollen mitochondria (Michea *et al*., 2002) (Van Cruchten *et al*., 2002). The swollen mitochondria lead to cellular swelling, a very typical feature of necrosis. This is usually accompanied by chromatin condensation and eventually lysis of the nuclei and the cell itself. Plasma membrane permeabilization causes release of all cellular enzymes and other contents, culminating in the destruction of the cell by necrosis (Jaeschke *et al*., 2003).

2.5.3 Aponecrosis and glucocorticoids

It would appear as if glucocorticoids, such as hydrocortisone can cause death in cells by both of these mechanisms. The more possible explanation, however, would be that not just one of these death mechanisms are involved, but both, simultaneously. For example, the MTP plays an important role in necrosis, as well as in apoptosis (Jaeschke *et al*., 2003). It has been stated that because apoptosis and necrosis share similar morphological and biochemical features, they are intertwined and more than one type of cell death is found in the same cell (Proskuryakov *et al*., 2003). The ability of a necrotic process to be converted to an apoptotic one and vice versa illustrates that apoptotic and necrotic cell death are not necessarily distinct and independent events. The sharing of these pathways is often described as aponecrosis or necrapoptosis. This concept offers an explanation as to why tissues sometimes exhibit features of apoptosis as well as necrosis (Jaeschke *et al*., 2003). Since so many researchers found that glucocorticoids

induced death by apoptosis or necrosis, it would be more appropriate to assume that death actually occurred by aponecrosis.

2.5.4 The Bcl-family

The induction of apoptosis by glucocorticoids has been proven in various cells. However, many researchers found quite the opposite – that glucocorticoids, specifically hydrocortisone protect the cell from entering apoptosis (Sasson *et al*., 2003)(Chang *et al*., 2004) (Chen *et al*., 2005). Some other research then showed that hydrocortisone had no significant effect on apoptosis (Thurner *et al*., 2001)(Kaplan *et al*., 2002). From here the question arises as to how and when hydrocortisone would activate cell death by apoptosis. Sasson *et al*., (2001) stated that the modulation of Bcl levels play an important role in mediating the glucocorticoid effect on cell survival. These authors reported that glucocorticoids can have opposite effects on Bcl levels in different kinds of cells. In other words, in some cells glucocorticoids may induce apoptosis whereas in other cells, it may inhibit apoptosis. Chang *et al*., (2004) also illustrated that glucocorticoids reduced surface Fas expression and could have negative effects on downstream apoptosis signaling pathways, namely the Bcl family member Bid.

It would seem that the Bcl family members are the central control stage of the apoptotic machinery (Konopleva *et al*., 1999). As mentioned previously, the Bcl family consist of pro-apoptotic factors, such as Bax and Bid, and apoptotic inhibitors Bcl-2 and Bcl-X_L. The ratio of the Bax and Bid transcripts to the Bcl-2 and Bcl-X_L transcripts will determine whether cytochrome *c* is released or not, thereby determining whether the cell will enter apoptosis (Konopleva *et al*., 1999) (Van Cruchten *et al*., 2002).

In the developing nervous system, researchers also found that glucocorticoids play an important role in regulating the Bcl family (Vekrellis *et al*., 1997) (Almeida *et al*., 2000). During development, the sensitivity of neurons to signals that induce apoptosis may be regulated by modulating levels of Bax (Vekrellis *et al*., 1997). Throughout embryonic life, Bcl-X_L can be found in the brain and appears to be located exclusively in the neurons (Merry *et al*., 1997).

The proteins encoded by the Bcl-family are associated with mitochondrial or nuclear membranes, distribution of intracellular ions, mitochondria permeability transition

phenomena and free radical generation (Almeida *et al*., 2000). It could therefore be argued that in the developing nervous system, glucocorticoids may regulate cell death, possibly aponecrosis, by the ratio between pro- and anti- apoptotic molecules.

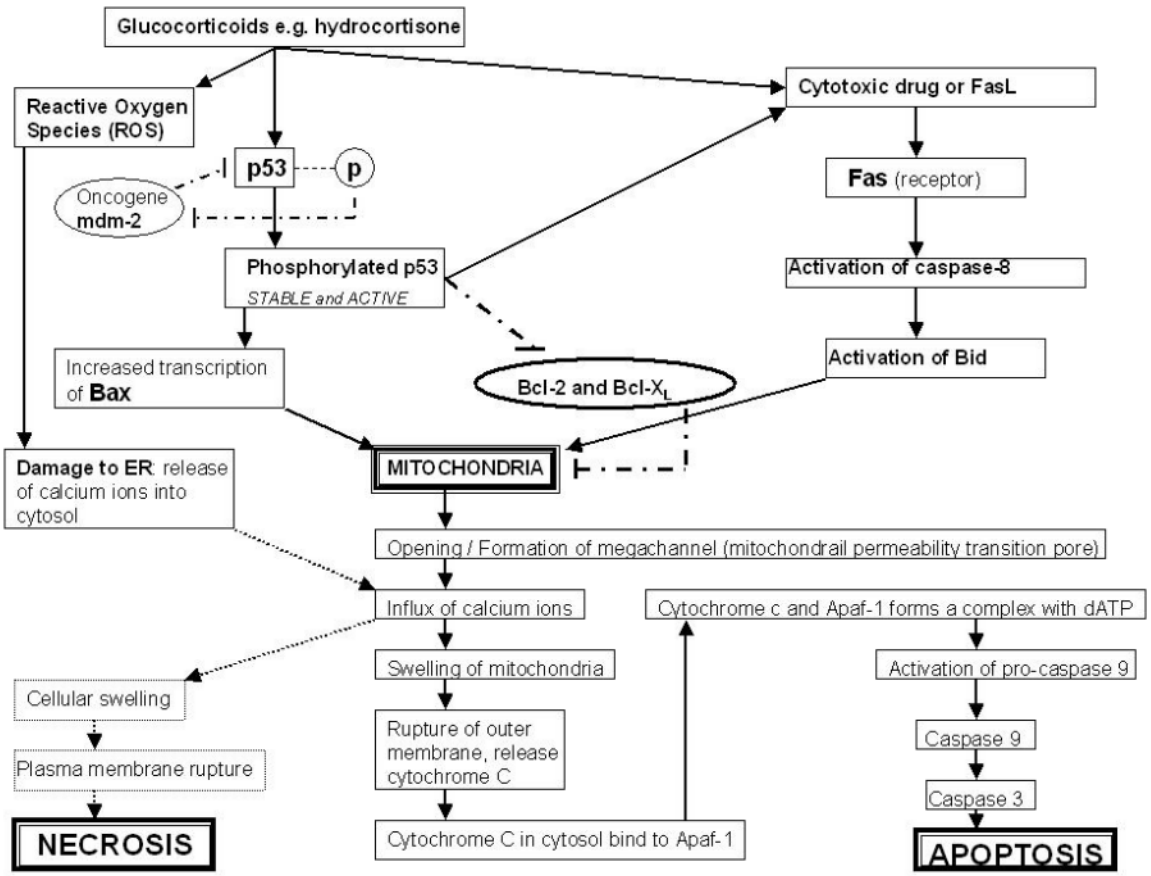


Diagram 2.1 Different pathways by which glucorticoids can induce apoptosis or necrosis. (compiled from various literature sources used)

2.6 HYDROCORTISONE AND FUTURE IMPLICATIONS OF GLUCOCORTICOIDS

Hydrocortisone is the steroid of choice in emergency situations (McPhillips, 1982). Usually hydrocortisone sodium succinate is utilized as it has the most rapid onset – peak plasma levels are reached within 1 hour and persist for up to 6 hours (Smith *et al*., 1991). Hydrocortisone sodium succinate is a water-soluble ester that is usually administered intramuscularly but may be given intravenously (Rubin, 1982).

Hydrocortisone is also the most frequently used glucocorticoid for diseases such as asthma and rheumatoid arthritis (Swingle 1982) (Abisheganaden *et al*, 2000). In children it is recommended to keep the dose of hydrocortisone below 500 μg a day to reduce any risk in growth, physical and psychological retardation (Smith *et al*., 1991).

When all the effects are taken into consideration, it would be advantageous to develop analogues of glucocorticoids that minimize the systemic effects and has no teratological effects (McPhillips, 1982). Development of novel therapeutic agents with the same anti-inflammatory, anti-allergic and anti-rheumatic potential should also be considered as alternative therapeutic strategies.

2.7 SUMMARY

The effects of glucocorticoids may influence the growth and development of an individual. In individuals who received steroid treatment after preterm birth neurodevelopmental disability could be observed. Steroid hormones contribute to shaping behavioral function including attention, perception, memory and emotional processing during early development and act as risk factors for psychopathology. It is therefore obvious that elevated glucocorticoids may have downstream effects on the brain and play a facilitatory role in the development of adaptive effects. Both excitatory and inhibitory information are affected when corticosteroid levels are altered. When administered in pharmacological doses for prolonged periods, steroids generally produce toxic effects that are extensions of their pharmacological actions. It was also found that after long term exposure cells began to die.

Glucocorticoids can activate cell death either via apoptosis or necrosis. Glucocorticoids have been shown to directly activate the p53 tumor suppressor gene by phosphorylation causing it to become stable and active. Active p53 leads to an increased transcription of Bax which activates the rest of the apoptotic cascade. Hydrocortisone or cytotoxic drugs may activate Fas, which activates Caspase 8 and eventually Bid, also resulting in apoptosis. ROS generation due to glucocorticoids could also activate apoptosis, either by stabilizing p53 or by causing damage to the ER. A damaged ER may cause severe disruptions to the calcium pool and cellular swelling with death by necrosis may follow. All these events appears to accumulate in the formation of the MPT which results in

death of the cell. MTP plays an important role in necrosis, as well as in apoptosis. Since so many researchers found that glucocorticoids induced death by apoptosis or necrosis, it would be more appropriate to assume that death actually occurred by aponecrosis.

Because of all the above mentioned implications of hydrocortisone, the aim of this study was to determine the effect of prenatal hydrocortisone exposure on cell viability and cell morphology in chick embryonic neurons. The following research objectives were investigated.

2.8 RESEARCH OBJECTIVES

- To establish the stage of embryonic development, the fixation period, the pre-embedding processing and the section thickness optimal for light microscopy.

- To establish four different staining methods namely, Hematoxylin and Eosin (H&E), Cresyl Fast Violet, Silver impregnation and a combination of Gold Chloride and Toluidine Blue, and to use these methods to study the effect of hydrocortisone on neuronal tissue structure.

- To compare to the control the effects of 0.137μM and 685μM hydrocortisone on chick embryo neural tissue at Carnegie stage 20 (day 8) of development.

- To determine the best procedure for tissue collection and method for fixation to obtain optimal tissue integrity when evaluating chick embryo neural tissue by transmission electron microscopy (TEM).

- To apply the optimal fixation methods to the selected brain areas in the control sample and investigate neural tissue for any damage that might have occurred due to the processing.

- To evaluate samples exposed to hydrocortisone for any damage that may have occurred due to the action of the glucocorticoid.

- To establish primary cultures from chick embryonic neural tissue that will be suitable for fluorescence microscopy studies.

- To determine the effect of increasing concentrations of hydrocortisone (0, 26.3nM, 0.16μM, 0.63μM, 3.8μM, and 22.8μM) on cell viability by utilizing the FDA, PI double fluorescence staining method.

- Furthermore to use primary neuron cultures to determine the effect of hydrocortisone on ROS production and to determine the mechanism of cell death using the fluorescent probe DCH_2FDA

Chapter 3: Histological studies to determine the effect of hydrocortisone on brain development in the chick embryo model

3.1 INTRODUCTION

Synthetic glucocorticoids have been shown to exert neurotoxic effects on the human embryo *in utero* by crossing the placenta. High levels of maternal glucocorticoids also show marked long-term repercussions for the newborn individual well into adult life (Barbazanges *et al*., 1996). Preterm infants that were exposed to high doses of glucocorticoids prenatally showed stunted head growth with no gain in head circumference (Huysman *et al*., 2005). Smith *et al*., (2000) showed a glucocorticoid dose-dependent reduction in brain weight and volume.

Embryonic neurons are very sensitive and may undergo developmental changes due to a variety of stimuli (Hu *et al*., 1996). Although it is a well-known fact that glucocorticoids causes degeneration and depletion of neurons, it is difficult to isolate individual factors that contribute to neuronal damage (Yuen *et al*., 1981)(Schwab *et al*., 2001). Some researchers found that glucocorticoids caused a significant decrease in both cell number and length of cellular processes (Hu *et al*., 1996). Other researchers indicated that although no reduction in cell number could be observed, the relative size of the neurons decreased (Carlos *et al*., 1992). Glucocorticoids may also be responsible for reducing nerve cell connections and thereby also interrupt critical synaptic processes. Other insults such as lack of glucose uptake and oxygen due to reactive oxygen species (ROS) have also been noted (Seckl, 2005).

Even if glucocorticoids do not necessarily induce cell death, the extent of damage to the neurons would be worthy to investigate. Pathological examination of the nervous system by histology is an important component of neurotoxicology, however, the features of the brain together with problems experienced with histological procedures can make this assessment quite challenging (Jortner, 2005). Despite the challenges of histology, it is often used in research. Histology offers the investigation of microscopic anatomy or architecture of the tissue, as well as the relations of individual cells to each other. It is however, important to remember that certainly no one staining method allows the

visualization of all cells and individual components within a section. (Loots et al., 1993) In this study, histology with four different staining procedures was applied to evaluate all changes in cell morphology.

Therefore, the following research objectives were addressed in this chapter:

- To establish the stage of embryonic development, the fixation period, the pre-embedding processing and the section thickness optimal for light microscopy.

- To evaluate the effectiveness of the histological stains : Hematoxylin and Eosin (H&E), Cresyl Fast Violet, Silver impregnation and a combination of Gold Chloride and Toluidine Blue, on the morphology of neural tissue.

- To evaluate the effects of 0.137μM and 0.685μM hydrocortisone on chick embryo neural tissue at Carnegie stage 20 (day 8) of development.

3.2 MATERIALS

3.2.1 Chicken embryos

Fertilized Broiler hatching eggs were obtained from the National Chicks hatchery in Pretoria, (Astral Operations Limited Reg. No. 1947/027453/06 Lynnwood Ridge) South Africa.

3.2.2 Disposable items

The surgical blades were from MACMED, Johannesburg, South Africa.

3.2.3 Reagents

Ethanol, glacial acetic acid, hydrogen peroxide, oxalic acid, sodium thiosulphate, hydroquinone, copper (II) nitrate ($Cu(NO_3)_2$), gold chloride ($AuCl_2$), potassium aluminum sulphate, sodium iodide, chloral hydrate, Hematoxylin, Eosin, Toluidine blue and Albumin silver were all from Merck Johannesburg, South Africa. Cresyl fast violet and mercuric chloride ($HgCl_2$) were supplied by BDH Chemicals Ltd., Poole, England.

Formaldehyde, xylene and citric acid were obtained from Saarchem (Pty) Ltd., Muldersdrift, South Africa. Silver nitrate ($AgNO_3$) was supplied by Lennon Manufacturing, London, England. Tissue-Tek III paraffin wax was from Sakura, Zoeterwoude, Netherlands and Gurr's neutral mounting medium was from Gurr Ltd., London England. SOLU-CORTEF™ 100mg Act-O-Vial was obtained from Pharmacia, Midrand, South Africa.

The water in all studies was distilled and deionized (ddH_2O) with a Continental Water System.

3.3 METHODS

3.3.1 Chicken embryos

Eggs were incubated at 36ºC in a humidified Heraeus incubator with no additional CO_2 or O_2. Eggs were either placed into the incubator immediately (with rounded side to the top, where the chorioallantoic membrane (CAM) is situated) or were kept at 4ºC for no longer than 2 weeks before placing into the incubator. Eggs were turned longitudinally twice daily. Day of placement in incubator was taken as day 1, which corresponds to Carnegie stage 9. Table 3.1 illustrates a comparison between the Carnegie Stages of the human and chicken embryos.

Table 3.1: Comparison of Carnegie Stages between human and chicken embryo. (Butler and Juurlink, 1987)(Harkness et al., 1997)

	Stage	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Human	**Days**	20	22	24	28	30	33	36	40	42	44	48	52	54	55	58
Chicken	**Days**	1	1.5	2	2.25	2.5	3	3.25	3.75	4.75	5.5	6.25	7.25	7.75	8.5	10

3.3.2 The optimal stage of embryo development for histology

To determine the optimal stage of development for histology the eggs were removed from the incubator at days 7 (Carnegie stage 20), 8 (Carnegie stage 21), 9 (Carnegie stage 22) and 10 (Carnegie stage 23) of development. The eggshell was broken with a scalpel, the embryo removed by spoon spatula and decapitated.

3.3.3 The optimal fixation period of chick embryo heads for histological evaluation

Chick embryo heads were fixed in 4% formaldehyde fixative for a period of 1 day through to 14 days. The following parameters namely the size of the head and the hardness of the tissue, were evaluated for preparing sections and duration of fixation. The latter was evaluated following staining with H&E (for procedure see section 3.3.6.1) to determine at which period ultimate penetration of the tissue occurred.

3.3.4 Exposure of chick embryos to hydrocortisone

On day 3.75 (Carnegie stage 16) eggs were removed from the incubator and under aseptic conditions a small hole were made in the eggshell with a sterile pin. The eggs were inoculated through this hole with either 0.137μM or 0.685μM hydrocortisone in a volume of 1μl and 5μl (SOLU-CORTEF™ 100mg Act-O-Vial consist of a two compartment vial containing per 2ml when mixed, hydrocortisone sodium succinate equivalent to 100mg hydrocortisone, 0.9% benzyl alcohol and water for injection, final concentration 50mg/ml, 0.138M), before the hole was closed with melted candle wax. This procedure was repeated on day 5.5 (Carnegie stage 18). The embryos were terminated at a stage determined in Section 3.3.3. Eggs were removed from the incubator, the eggshell broken with a scalpel, the embryo removed and decapitated.

3.3.5 Pre-embedding processing of embryo tissue

Using the recommendation of Sheehan et al., (1980) various times and conditions were tested to optimize the procedure.

The tissue was dehydrated in 70% ethanol for 3-8 hours to initiate dehydration, it was then placed in 90% ethanol overnight, followed by emersion in absolute ethanol for 2-3 hours. The tissue was placed through another two changes of fresh absolute ethanol of 2-3 hours each before being submerged overnight in xylene for clearing. The tissue was placed in 30% paraffin wax and 70% xylene, followed by 70% paraffin wax and 30% xylene, then in a pure paraffin wax solution for 1 hour each before being embedded. Warm, melted paraffin wax was used to impregnate the tissue and was allowed to set by cooling at 4°C. The tissue was orientated to ensure that the specific characteristics which needed to be investigated were easily accessible during sectioning and that the sections provided the largest possible area for investigation.

3.3.6 Section Thickness

Tissue sections of 5μm, 7μm, 10μm and 12.5μm were prepared using a Reichert - Jung, Supercut 2050 microtome (Reichert - Jung, West Germany) and stained with H&E (for procedure see section 3.3.6.1) to the determine the correct tissue thickness required for evaluation of cellular morphology following exposure to hydrocortisone.

3.3.7 Histological staining procedures used to determine changes in the cellular morphology following exposure of chick embryos to hydrocortisone.

A variety of histological stains were evaluated to optimize the visualization of neural tissue in the chick embryo head in control embryos (not exposed to hydrocortisone) and embryo's exposed to 0.137μM or 0.685μM at day 3.75 and 5.5. These included: Hematoxylin and Eosin (H&E), Cresyl Fast Violet, Silver Impregnation and a combined Gold Chloride and Toluidine Blue staining method. All slides prepared from sections of the control, 0.137μM hydrocortisone exposure and 0.685μM hydrocortisone exposure were processed simultaneously.

3.3.7.1 Hematoxylin and Eosin (H&E)

Slides with sections of the chick embryo head were deparaffinized as follows. The slides were placed in two changes of xylene followed by two changes of 100% ethanol of 2 minutes each. Rehydration of the tissue was accomplished by washing the slides for 1 minute in 90% ethanol, followed by 70% ethanol and finally in ddH_2O. The tissue was stained by placing the slides in a hematoxylin solution for 5-10 minutes, prepared by dissolving 1g of hemotoxylin powder in 1L dH_2O before 50g potassium aluminum sulphate, 1g citric acid, 0.2g sodium iodate and 50g chloral hydrate was added. The Hematoxylin solution was ripened for 3 months at room temperature.

The slides were transferred from the hematoxylin solution to tap water for another 10 minutes, followed by a quick rinse in dH_2O. The tissue was then counterstained with 1% eosin for 3 minutes. An aqueous 1% eosin solution was prepared by dissolving 25g of eosin in 1250ml dH_2O containing 4ml glacial acetic acid. Finally, the slides were rinsed in dH_2O, dehydrated by increasing levels of ethanol, cleared in xylene and mounted in Gurr's neutral mounting medium. Photographs were taken by a digital camera attached

to the microscope (Nikon DXM 1200, Nikon Instech Co., Kanagawa, Japan) and captured by Nikon ACT-1 version 2 software for Nikon cameras.

3.3.7.2 Cresyl Fast Violet

Slides were deparrafinized and rehydrated as described above, rinsed with dH_2O and transferred to a 1% Cresyl Fast Violet solution for 30 minutes. The Cresyl Fast Violet solution was prepared by dissolving 1g of Cresyl Fast Violet powder in 100ml dH_2O containing 0.25ml glacial acetic acid. After staining, the slides were rinsed in dH_2O, dehydrated as described above, mounted and photographed.

3.3.7.3 Silver Impregnation

The slides were deparaffinized and hydrated before being placed in a protargol-peroxide solution for 3-4 days at 37°C in total darkness. The protargol-peroxide solution was prepared by dissolving 1g of albumin silver in 100ml dH_2O. A 2ml volume of a 1% $Cu(NO_3)_2$ solution and 2ml of a 1% $AgNO_3$ was added to the protargol solution followed by the addition of 3 drops of 30% H_2O_2 to complete the protargol-peroxide solution.

After 3-4 days in the protargol-peroxide solution, the slides were rinsed in dH_2O and transferred to a reducing bath for 10 minutes. The reducing bath constituted 0.5g hydroquinone dissolved in 100ml dH_2O containing 5ml of a 30% formaldehyde solution warmed to 60°C before use. The slides were moved from the reducing bath, rinsed rapidly in dH_2O before being toned in a 1% $AuCl_2$ solution for 10 minutes. The slides were rinsed again before being placed in another reducing solution for 10 minutes, prepared by dissolving 2g of oxalic acid in 100ml dH_2O containing 5ml of a 30% formaldehyde solution. The slides were rinsed with dH_2O and fixed for 2 minutes in a 5% sodium thiosulfate solution. Finally the slides were dehydrated, mounted and photographed.

3.3.7.4 Combination of gold chloride and Toluidine blue

The sections were deparaffinized and rehydrated as described in Section 3.3.6.1. The slides were then stained for 30 seconds using a 0.1% Toluidine blue solution prepared by dissolving 0.1g of Toluidine blue powder in 100ml dH_2O for 30 seconds. After staining

in Toluidine blue, the slides were quickly rinsed in dH_2O before being placed in the gold sublimate for 4 hours at room temperature in a dark place. The gold sublimate was prepared by adding together 5ml of a 1% $AuCl_2$ solution, 25ml of a 1% $HgCl_2$ solution and 5ml dH_2O to obtain a final volume of 30ml. The slides were then rinsed with dH_2O and transferred to a 5% sodium thiosulfate solution for 2 minutes. After staining the slides were again rinsed in H_2O, dehydrated, mounted and photographed.

3.4 RESULTS AND DISCUSSION

3.4.1 The stage of embryonic development, fixation period, pre-embedding procedure and section thickness

The first objectives of this chapter was to establish parameters such as the stage of embryonic development, the fixation period, the pre-embedding processing and the section thickness optimal for light microscopy of chick embryo neuronal tissue. These conditions together with several different staining methods were used to determine the effects of hydrocortisone on neuronal tissue in the developing embryo.

At Carnegie stage 20 (day 8 of development) of embryological development, the size of the head was easily manageable during fixation, processing, embedding and sectioning. Physically all external features were easily distinguished and it was possible to identify the different regions of the brain microscopically

All brain areas were identified with the assistance of 'The Atlas of Chick Development' (Bellairs and Osmond, 1998). Both the cranial and cephalic flexures are present at day 3. The general topography expected to be seen at day 4, when viewed coronally, include the secondary vesicles the myelencephalon, the metencephalon, the mesencephalon and the diencephalon. The optic cups and pharyngeal arches can also be identified. The structures posess the typical thin vesicle morphology and little detail can be distinguished. However, by day 7 the mesencepholon has grown so large that it projects over the metencephalon. The walls of the telencephalic vesicles have thickened to such a degree that the cerebral hemispheres can be distinguished. In a sagital section, the developing choroid plexus and pineal gland can be seen just posterior to the cerebral hemispheres. The thick floor and thin roof of the myelencephalon and metencephalon as

well as the mesencephalon can be distinguished. The isthmus, connecting the mesocoele and rhombocoele (forth ventricle) can also be seen. The eye chambers, nasal chonchae and oral cavity can easily be distinguished to orientate the embryo head. In a coronal section, both cerebral vesicles with the region between the two telencephalic vesicles are visible. The interorbital septum and larynx can also be identified at this stage of development.

The time needed for optimal fixation was determined to be 5 days, where all tissue was adequately fixed. Little was altered to the pre-embedding processing as described by Sheehan *et al.,* (1980). It was however found that a time period of 5 hours in 70% ethanol was sufficient to initiate dehydration and that 2 hours in each of the three changes of absolute ethanol were optimal.

Tissue sectioning may compromise the cellular membrane (Didenko *et al*., 2002). It is therefore recommended that slightly thicker section should be used, since thicker sections give a greater chance of whole cells being included in the plane of sectioning (Burkitt *et al*., 1993). However thick sections may decrease visualization, due to the obstruction of the light transmitted through the section during light microscopy. For this study the optimal tissue thickness was found to be 7μm and was used throughout this study.

3.4.2 The effect of hydrocortisone on chick embryo neural tissue

Once all parameters were optimized the effect of hydrocortisone on neuronal tissue was studied. Chick embryos were exposed to two dosages (0.137μM or 0.685μM) of hydrocortisone at day 3.75 and 5.5. At Carnegie stage 20 (day 8) the embryos were removed, the tissue was processed and histological sections were prepared. Microscopically all structures at this stage of development correlated to the 'The Atlas of Chick Development', with the only exeption being that the heads and brain structures of the embryos exposed to 0.137μM or 0.685μM hydrocortisone had a reduced size.

Slides were prepared and stained with H&E, a general stain that was used to visualize all cell types (Loots et al., 1993). Hematoxylin stains the nuclei blue to black while the counterstain, Eosin stains the cytoplasm pink (Sheehan *et al.,* 1980). In this study H&E staining was utilized to identify the different brain areas and to get an overall impression

of the neuronal morphology at Carnegie stage 20 of development. A very low magnification photograph of an H&E stained, coronal section of an 8-day-old chick embryo head is shown in Figure 3.1(a). The telencephalic vesicles, the interorbital septum and eye chambers could be identified. At a higher magnification, the region between the two telencephalic vesicles (Figure 3.1a label (A)) of control (Figure 3.1b and 3.1c) and experimental embryos exposed to 0.137μM hydrocortisone (Figure 3.1d and 3.1e), and 0.685μM hydrocortisone (Figure 3.1f and 3.1g) was evaluated.

In Figure 3.1(b) a high density of neuronal cells was observed. A higher magnification, Figure 3.1(c), revealed neurons with distinguishable plasma membranes and various synaptic processes. The neurons were densely packed together and most neuron cell bodies showed a typical round to oval shaped appearance.

Following exposure to 0.137μM hydrocortisone, low magnification of the intertelencephalic region revealed that the tissue was more fragile and tore easily during the preparation of sections when compared to the control samples. In fetal sheep exposed to glucocorticoids, it was found that glucocorticoid therapy lead to acute disturbances in the neuronal cytoskeleton (Schwab *et al*., 2001). Antonow-Schlorke *et al*., (2003) then also investigated the effect of glucocorticoid exposure in the fetal baboon brain and illustrated that neuronal cytoskeletal proteins are affected in such a way that acute loss of neuronal processes and neuronal damage could be observed. In an article that reviewed clinical trials of pharmacological doses of glucocorticoids in respiratory distress syndrome, Thompson (2003) found that glucocorticoids had severe effects on connective tissue and might even inhibit proliferation of fibroblasts and ground substance deposition.

In the control tissue, the neurons appeared larger compared to other cell types and were easily identified (Burkitt *et al*., 1993). At a higher magnification of the same area of tissue exposed to hydrocortisone (Figure 3.1 e), the neurons appeared to be smaller with fewer processes and had a more intensive staining than was observed for control sections. Banuelos *et al*., (2005) have found that this type of staining is associated with neuronal damage when brain damage due to crude alkaloid extracts in adult rat brain tissue was evaluated. Jortner (2005) has suggested that the ‘dark’ neuron staining is due to

inadequate fixation, however this would not be the case in this study as this parameter was carefully established as part of the first objective of this chapter.

At a low magnificationof neuronal tissue exposed to 0.685μM hydrocortisone (Figure 3.1 f), staining appeared to be darker compared to the control samples. At a higher magnification (Figure 3.1g) the neurons did not have clearly distinguishable plasma membranes, very few processes could be identified, most cells stained lighter and the intracellular space had a pinkish coloration possibly due to the loss of the cell membrane integrity and the leakage of cytoplasm and its contents into the intracellular space.

Figure 3.2 (a) to (m) shows sagital sections of a control, 8-day-old chick embryo head region. The region investigated, the floor of the myelencephalon is shown in Figure 3.2 (a) and the location of the eye chamber, isthmus, rhombocoele or fourth ventricle and mesocoele is shown. The neurons in the floor of the myelencephalon of the control sample appeared to have a relatively high density (Figure 3.2 b and c). Figure 3.2 (d) showed cells with typical neuronal morphology, oval to round shaped cell bodies with many neuronal connections. At a higher magnification (Figure 3.2 e) the neurons had intact cell membranes with clear nuclei and various synaptic processes.

In Figure 3.2 (f) to (I), the cell morphology of neuronal tissue exposed to 0.137μM hydrocortisone is presented. A decrease in cell number was observed when compared to the control tissue (Figure 3.1 f and g). Although several neuronal connections could be identified, the neurons had a darkly stained appearance. Turner *et al*., (2004) found that the degree of dark cell change and axonal swelling are histological indicators of apoptosis and are usually accompanied by and increase in caspase 3 expression. The darkly stained neurons observed in these photographs might therefore indicate neuronal stress. At a higher magnification these finding were confirmed. Exposure of neurons to 0.137μM hydrocortisone caused a decreased cell number and an increased neuronal staining while some other neurons stained lighter, with nuclei that appear to be fragmented. Murakami *et al*., (1997) found that an overall triangular appearance of the nucleus with small dark particles that appeared to be fragmented nuclei, was a morphological feature commonly associated with neuronal injury. After histological investigation (cresyl violet staining) of the hippocampal area in transgenic mice exposed

to ischemia these neurons were also evaluated by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL), confirming degraded DNA.
Exposure of chick embryos to a higher concentration of hydrocortisone namely 0.685μM, resulted in increased fragility of the tissue. In the myelencephalon region (Figure 3.2 j to m), darkly stained cells again gave the appearance of an increase in cell density, however the torn tissue and an increase in triangular shaped neurons were clear indicators that hydrocortisone at this concentration caused neuronal damage. (Thompson, 2003) (Schwab *et al*., 2001). At higher magnifications neurons were stained darkly, cell membranes were difficult to distinguish and some nuclei appeared fragmented indicating neuronal damage (Murakami *et al*., 1997).

Fix *et al*., (1996) used H&E staining for similar purposes by assessing morphological integrity of neurons in the central nervous system in neurotoxicity testing. H&E staining revealed necrotic nerve cell bodies after exposure of neurons to the neurotoxin dizocilpine maleate (MK-801). These necrotic neurons, however, required a high magnification for detection

The effects of hydrocortisone were further investigated using Cresyl Fast Violet staining. Cresyl Fast Violet is a specific neuron stain that stains the Nissl substance of the neurons dark blue to purple while the rest of the neuron including the nuclei stain pale purple to blue. In this study Cresyl Fast Violet was applied in the same manner as H&E, to evaluate neuronal morphology and density. Figure 3.3 shows samples stained with Cresyl Fast Violet. Figure 3.3 (a), taken at a low magnification, shows the region evaluated. The region that will later develop into the pineal gland (black square, labeled (A)) was evaluated at higher magnifications. A cerebral hemisphere, the floor of the mesencephalon, nasal chonchae, oral cavity, and eye chamber are indicated on the slide for orientation purposes. The control sample showed a high neuron density with numerous synaptic processes (Figure 3.3 b and c). At a higher magnification, intact cell membranes of the neurons could be seen with darkly stained nuclei. Overall, the neurons appeared to be morphologically well preserved with no membrane damage and typical normal neuronal morphology.

Following exposure to 0.137μM hydrocortisone a decrease in cell density (Figure 3.3 f to h) was observed. At a higher magnification (Figure 3.3 (i)), neuron density was

decreased when compared to the controls and disrupted cell membranes were observed. A decrease in cell density when stained with Cresyl Fast Violet is a clear indicator of cell loss. (Wall *et al*., 2000). Fragmented nuclei were also observed in some neurons, this feature together with the disrupted cell membranes indicated that hydrocortisone at this concentration was neurotoxic (Murakami *et al*., 1997) (Yuen *et al*., 1981).

The density of neurons exposed to 0.685μM hydrocortisone appeared to be only slightly less dense that the controls. However at higher magnifications, most neurons appeared to have fragmented nuclei, disrupted cell membranes and changes in the structure of synaptic processes could be observed (Figure 3.3 j to m).

To further investigate the effects of hydrocortisone on neuron morphology Silver Impregnation was utilized, as this method stains specific cell types with cell body neurofibrils appearing darkish red to black against a yellow to brown background while the nerve fibers in the grey and white matter appears red to black. (Loots *et al*., 1977). Whole neurons become selectively filled with silver, which precipitates within the matrix of the cytoplasm. As the precipitate accumulates, it forms a typical crystalline precipitate that appear dark in transmitted light. For some unknown reason, only a small portion of cells in a preparation are impregnated completely, so that the rest of the cells have a transparent golden appearance. (Peters *et al*., 1976). It is however a very difficult histological stain, since it is difficult to obtain consistent results. (Loots *et al*., 1977)

In this study, samples that were stained with Silver Impregnation, were evaluated based on the ‘dark’ neuron characteristic. Wall *et al*., (2000) also used this evaluation after staining with silver impregnation for the investigation of neuronal degeneration in rats exposed to a cholinergic agonist. Neuronal degeneration was visualized as darkly stained neurons together with cell loss as observed by Cresyl Violet staining. Figure 3.4 (a) was taken at a low magnification to illustrate the area evaluated, namely the floor of the myelencephalon (black square, labelled (A)). The isthmus, rhombocoele or fourth ventricle and eye chamber are indicated to orientate this sagital section of an 8-day-old chick embryo head.

Neurons from the control sample (Figure 3.4 b to e) are shown at four different magnifications. Both red to black and golden brown neurons could be distinguished. The lower magnification photographs illustrated a high density of neurons, with easily distinguishable and intact cell membranes (Figure 3.4 b to d). Most nuclei could be identified within the centre of the cell body and a number of synaptic processes could be seen (Figure 3.4 e).

Exposure of embryos to 0.137μM hydrocortisone resulted in neurons with a very darkly stained appearance, and reduced cell density (Figure 3.4 f to h). At a high magnification (Figure 3.4 i), intense staining of the neurons was observed indicating neuronal degeneration (Wall *et al*., 2000). In the neurons not stained as darkly, no nuclei could be distinguished. The synaptic processes also had a disrupted appearance. Tissue exposed to 0.685μM hydrocortisone did not appear to have suffered irreversible damage. Although an overall increase in dark cells could be seen when compared to the control sample the cells still appeared to possess a certain density (Figure 3.4 j to l). At a high magnification (Figure 3.4 m) the neurons clearly had fragmented nuclei while the intercellular space between cells appeared to be increased. The number of dark cells, however, were not as many as seen in the sample exposed to 0.137μM hydrocortisone.

Since Loots *et al*., (1993) stated that more that one staining method is necessary for proper evaluation in histology, a combination Gold Chloride and Toluidine Blue staining method was used to view both neurons and neuroglia. On its own, gold sublimate is a delicate procedure that requires extreme purity of reagents and materials. Toluidine Blue on the other hand is a general method used to evaluate neurons and glia. (Sheehan *et al*., 1980) By combining these two methods a midway was achieved that preserved the best of both methods. This method was applied in this study to confirm the results obtained by the other staining procedures.

Control samples all sectioned in exactly the same position were stained with gold sublimate (Figure 3.5 a), Toluidine blue (Figure 3.5 b) and a combination of the two methods (Figure 3.5 c). The morphology of the neurons was better visualized by using the combination method and the characteristics of each stain contributed to the overall improved visibility of the neurons.

A low magnification photograph (Figure 3.6 a) was again used to illustrate the area evaluated, namely the region between the two telencephalic vesicles. In the control samples the density of neurons in this region was high (Figure 3.6 b-d). The neurons, at a high magnification (Figure 3.6 e), showed no darkly stained areas and cell membranes could be easily distinguished. Numerous synaptic processes could also be identified. Following exposure to 0.137μM hydrocortisone (Figure 3.6 f-j), intensely dark stained neurons indicating neuronal damage were observed with disrupted synaptic processes. Breakages and torn areas, indicating tissue fragility, of the sample exposed to 0.685μM hydrocortisone were also seen (Figure 3.6 j to l).

The effects of hydrocortisone on chick embryo neuronal development as seen with H&E, Cresyl Fast Violet, Silver Impregnation and a combination of Gold Chloride and Toluidine Blue staining methods are summarized in Table 3.2 at the end of the chapter.

3.5 CONCLUSION

Optimal histological conditions for evaluation of chick embryo neural tissue were evaluated and it was found that the optimal stage of development was Carnegie stage 20 with 5 days fixation in formaldehyde. The optimal times and conditions for pre-embedding processing, using the recommendation of Sheehan *et al*., (1980) were found to be 5 hours in 70% ethanol to initiate dehydration and only 2 hours in each of the absolute ethanol solutions. These optimal conditions were applied for evaluation of the effect of hydrocortisone on neural morphology.

This study utilized histology to evaluate the effect of hydrocortisone on neuron morphology with four staining procedures namely a general staining method and specific staining methods for neurons. At a hydrocortisone concentration of 0.137μM, neuron density was reduced and changes in cell morphology, such as the numerous darkly stained neurons which is associated with cell death, was observed. Samples exposed to 0.685μM hydrocortisone mostly showed a reduced neuron density an indication of neuronal cell death.

Histology cannot, however, be the only procedure to determine cytotoxicity, and other procedures with better sensitivity must be applied to confirm the results. (Goldey *et al*., 1994) Other evaluation procedures were applied in the chapters to follow. Whether these neurons suffered cell death (apoptotic or necrotic) or simply damage that lead to cell cycle arrest will be discussed in chapter 6.

Table 3.2 Summary of results obtained by H&E, Cresyl Fast Violet, Silver Impregnation and a combination of Gold Chloride and Toluidine Blue staining methods in control samples, as well as samples exposed to 0.137μM and 0.685μM hydrocortisone

STAIN	GENERAL	DENSITY			CELL MEMBRANE AND SYNAPTIC PROCESSES			FRAGMENTATION OF NUCLEI		
		Control	0.137μM	0.685μM	Control	0.137μM	0.685μM	Control	0.137μM	0.685μM
H&E	Nuclei of all cells appear blue to black, cytoplasm pink	High	Decreased with increased 'dark' neurons	Slight decrease in density with increase in darkly stained cells	Distinguishable	Smaller synaptic processes, tissue very fragile	Increased fragility of tissue, difficulty in distinguishing membranes, possible loss of membrane integrity	None	Present	Present
Cresyl Fast Violet	Nissl substance appears dark blue, rest of neuron pale blue	High	Decrease	Slightly less dense than controls	Numerous synaptic processes visible, intact cell membranes	Disrupted cell membranes	Disrupted cell membranes, change in strucuture of synaptic processes	None	Present	Present
Silver Impregnation	Red to black neurons with yellow to brown background	High	Decreased with increased 'dark' neurons	Decrease	Easily distinguishable with intact cell membranes	Disrupted synaptic processes	Increase in intercellular space, disrupted membranes	None	None detected	Present
Combination of gold chloride and Toduidine blue	Neurons dark blue to black	High	Decreased with increased 'dark' neurons	Decrease	Easily distinguishable with intact cell membranes and various synaptic processes	Disrupted synaptic processes	Increased fragility of tissue, difficulty in distinguishing membranes	None	None detected	None detected

(a)

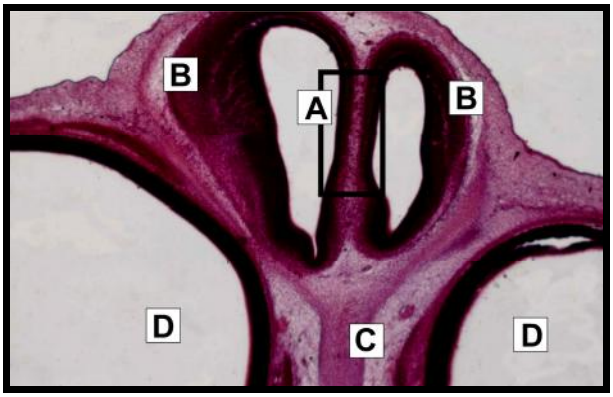
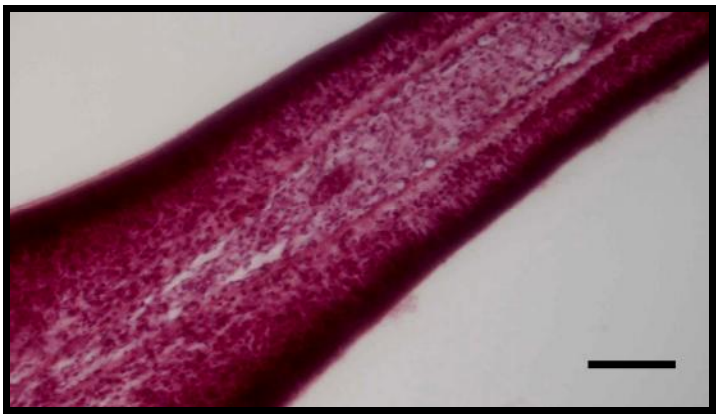


Figure 3.1 Small magnification photograph of a coronal section of an 8-day old chick embryo head to illustrate the area investigated. Area (A) – within black square, was enlarged to evaluate neuron morphology. (A) represents the region between the two telencephalic vesicles, labelled (B). The interorbital septum (C) and eye chambers (D) can also be identified. H&E staining used.

(b)



(c)

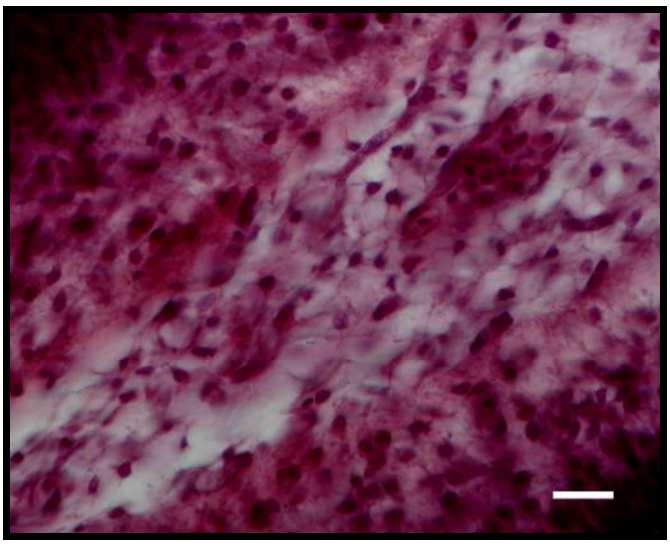
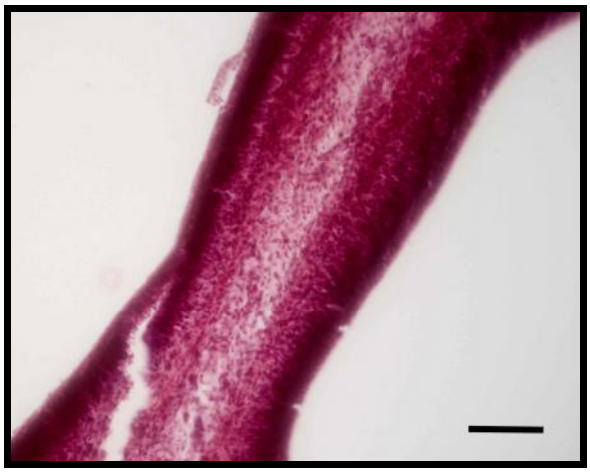
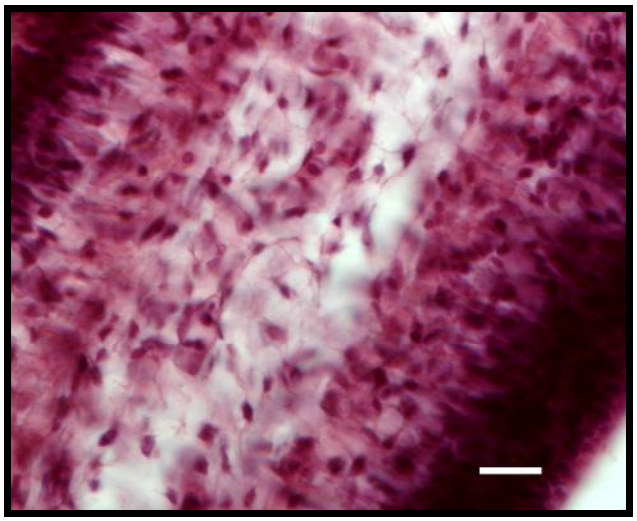


Figure 3.1 H&E stained tissue from the intertelencephalic region of chick embryos not exposed to hydrocortisone, shown at a low (a) and higher (b) magnification. (Black bar = 100μm) (White bar = 20μm)

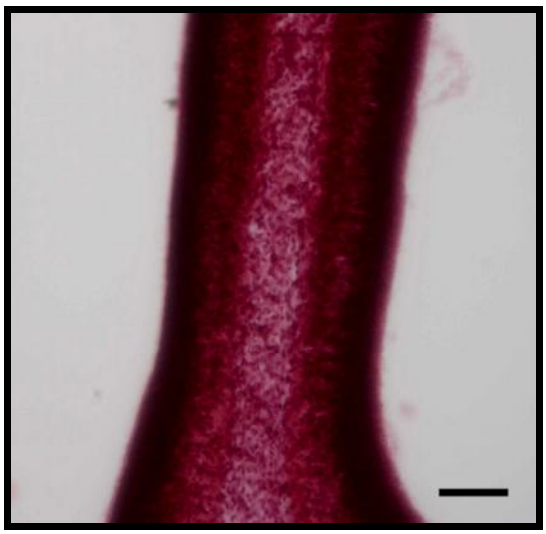
(d)



(e)



(f)



(g)

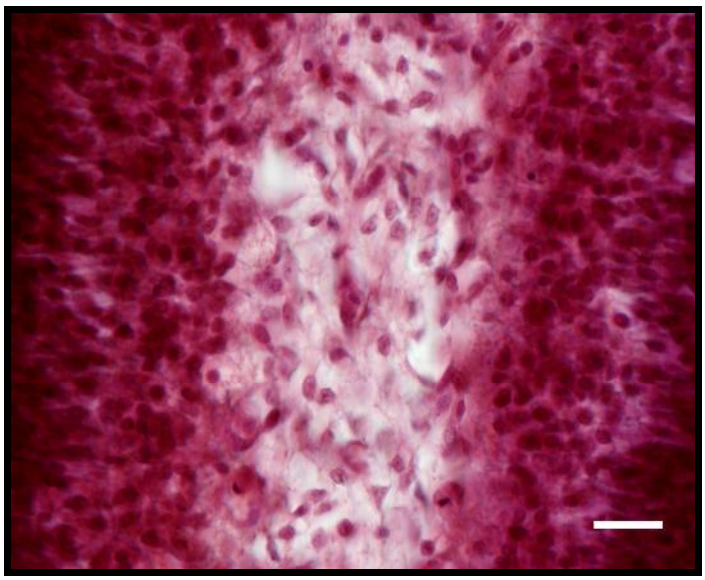


Figure 3.1 H&E staining of chick embryonic brain tissue from the intertelencephalic region exposed to 0.137μM hydrocortisone and lower (d) and higher (e) magnifications. The same area was investigated in neurons exposed to 0.685μM hydrocortisone, again at a lower (f) and higher (g) magnification. (Black bar = 100μm) (White bar = 20μm)

(a)

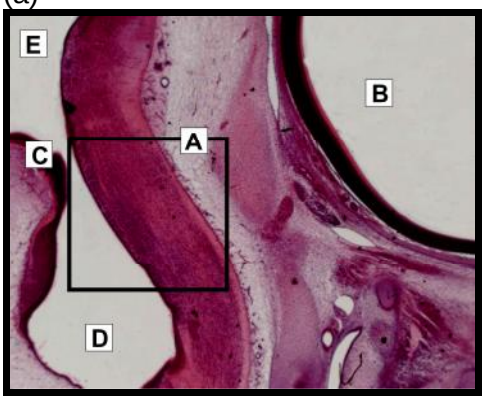
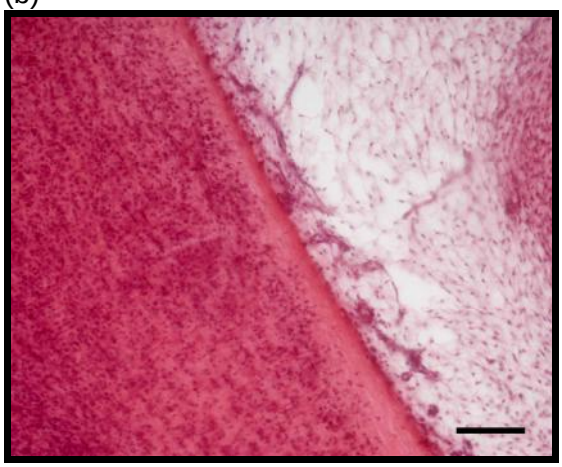
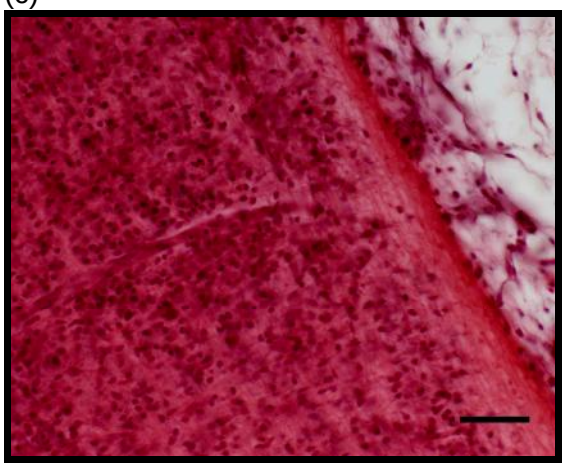


Figure 3.2 Small magnification photograph of a sagital section of the embryo head, to illustrate the area investigated. The floor of the myelencephalon (black square, labelled (A)) were enlarged to visualize neurons. The eye chamber (B), isthmus (C), rhombocoele or fourth ventricle (D) and mesocoele (E) are indicated for orientation. Staining done with H&E.

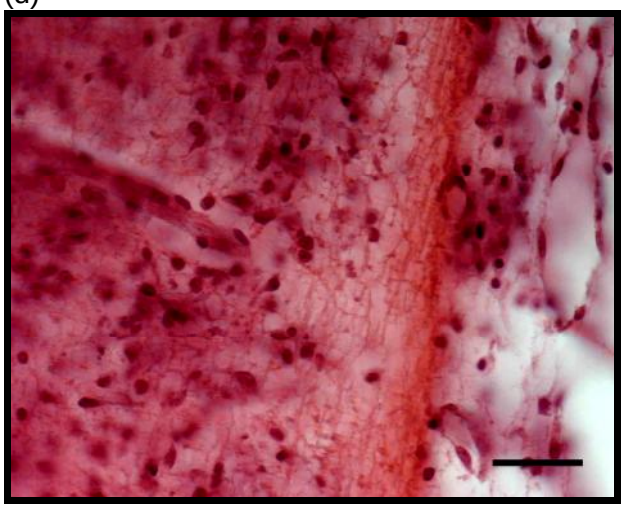
(b)



(c)



(d)



(e)

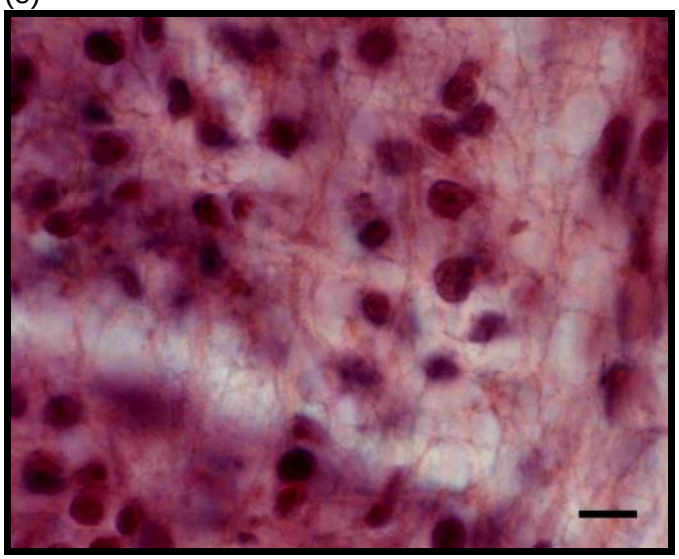
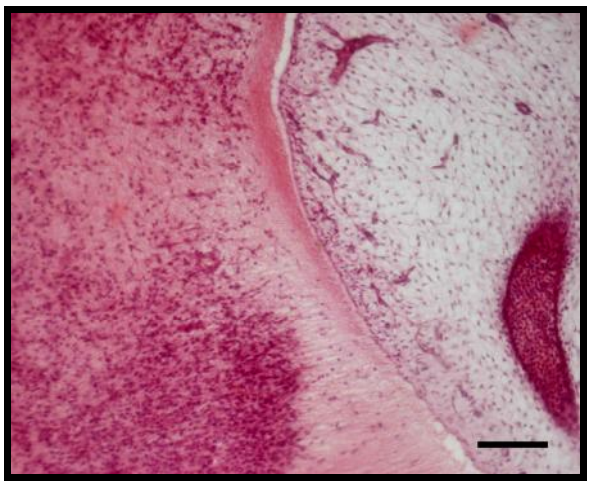
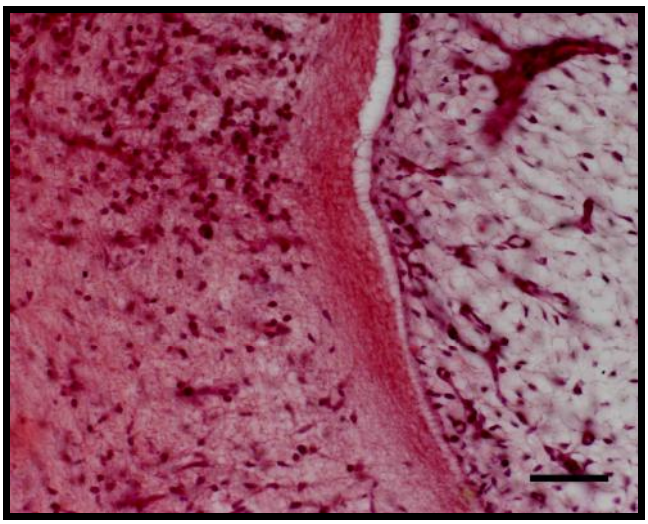


Figure 3.2 A series of magnifications of the floor of the myelencephalon of 8-day-old chick embryos not exposed to hydrocortisone and stained with H&E. Magnifications, Bar: (b) = 100μm, (c) = 50μm, (d) = 30μm and (e) = 10μm.

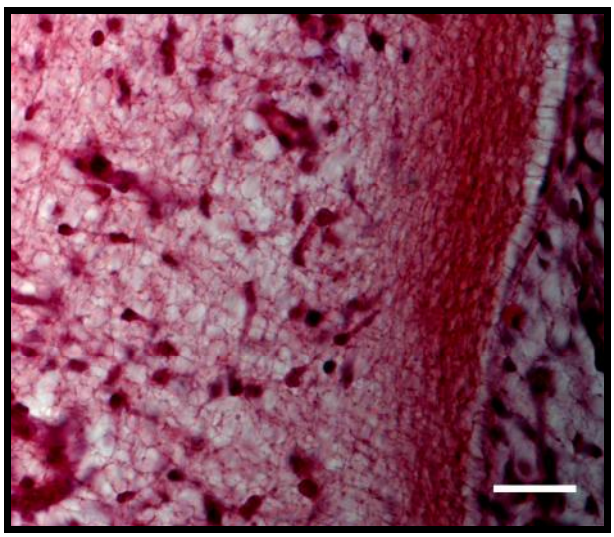
(f)



(g)



(h)



(i)

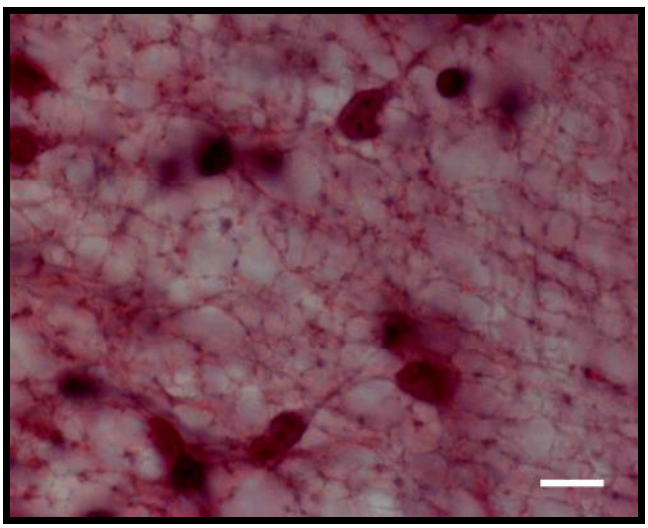
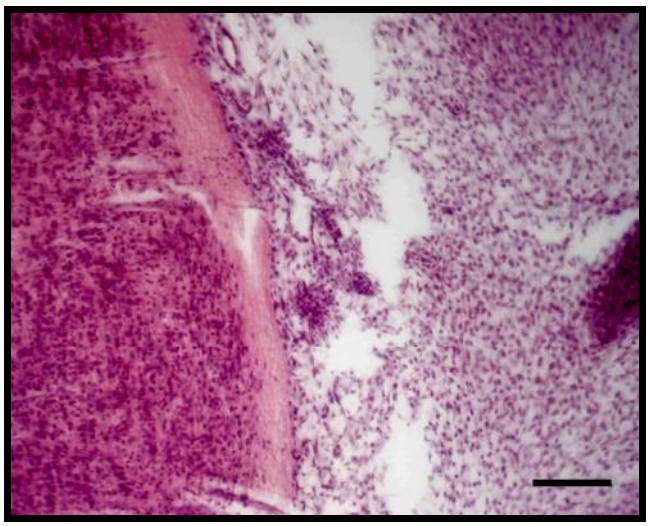
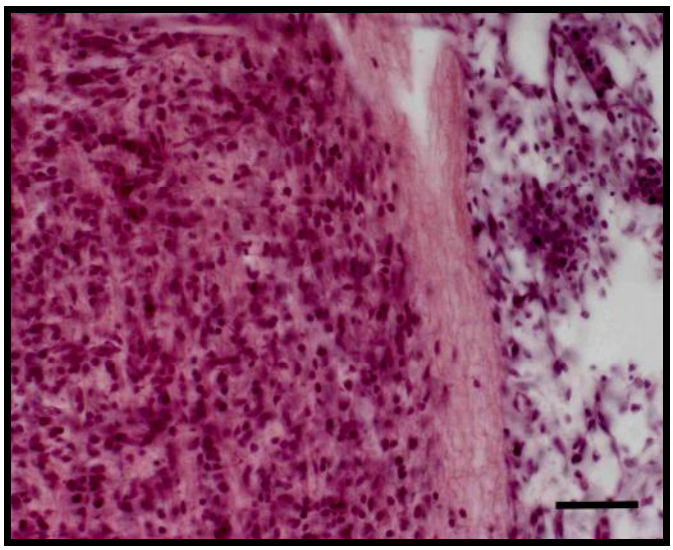


Figure 3.2 H&E staining of the myelencephalon of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (f) = 100μm, (g) = 50μm, (h) = 30μm and (i) = 10μm.

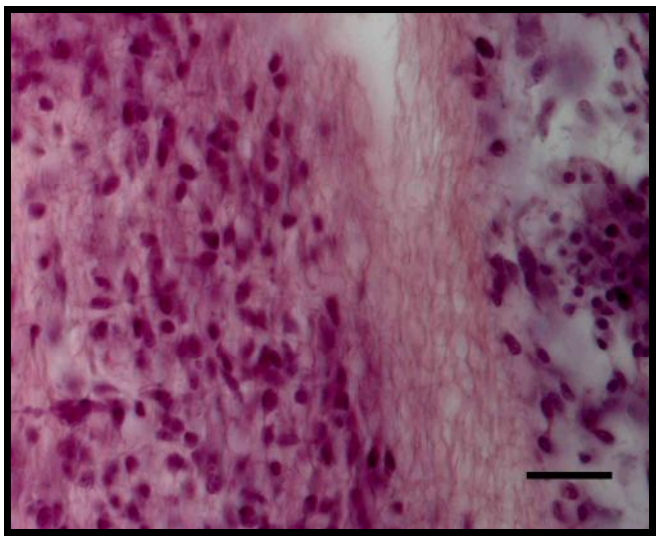
(j)



(k)



(l)



(m)

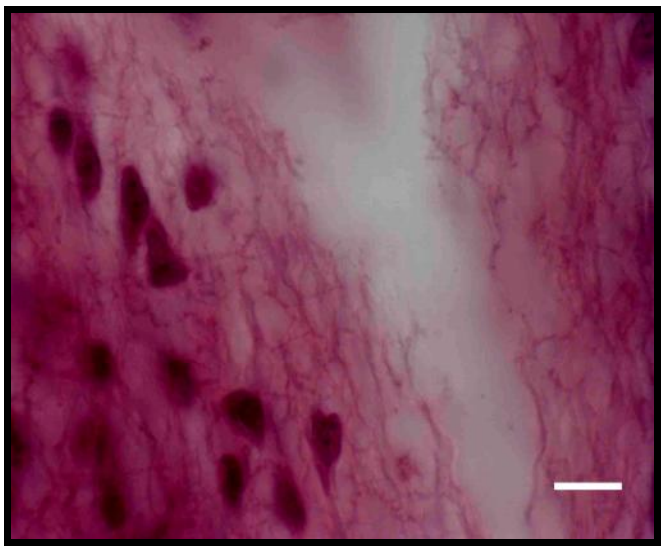


Figure 3.2 H&E staining of the myelencephalon of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (j) = 100μm, (k) = 50μm, (l) = 30μm and (m) = 10μm.

(a)

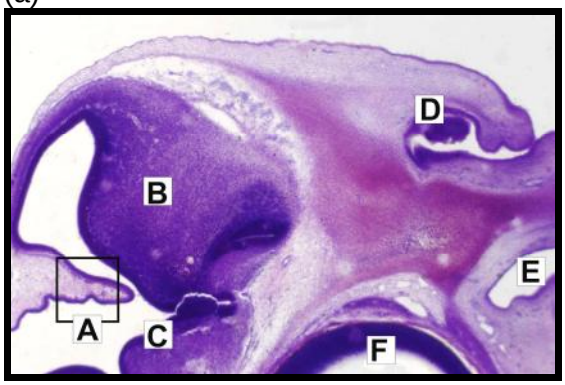
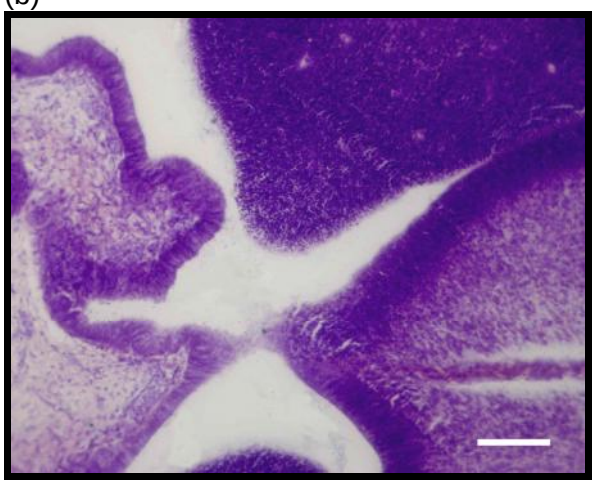
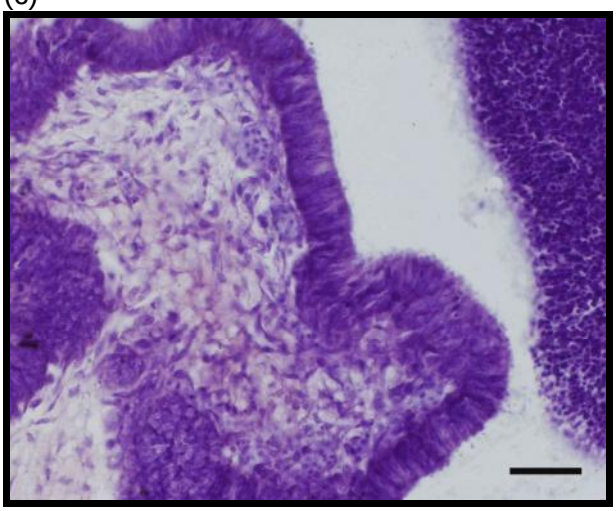


Figure 3.3 Small magnification photograph of 8-day-old chick embryo head in sagital section stained with Cresyl Fast Violet. Some areas are shown for orientation and to highlight area investigated. The area that will later develop into the pineal gland (black square, labelled (A)) were enlarged to visualize neurons. A cerebral hemisphere (B), floor of the mesencephalon (C), nasal chonchae (D), oral cavity (E), and eye chamber (F) are also indicated.

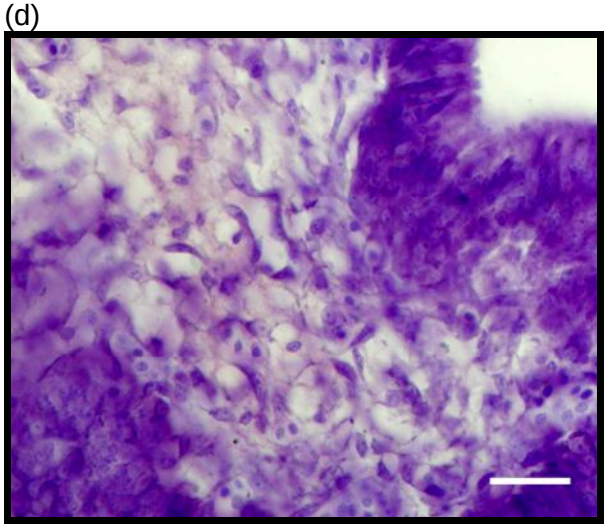
(b)



(c)



(d)



(e)

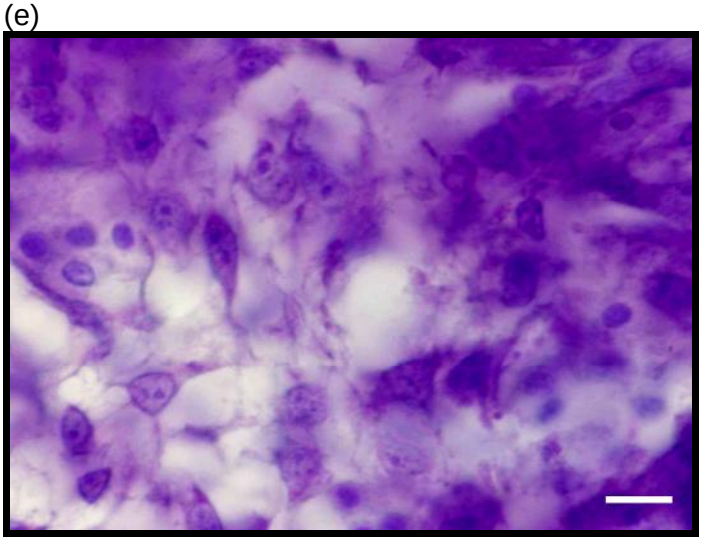
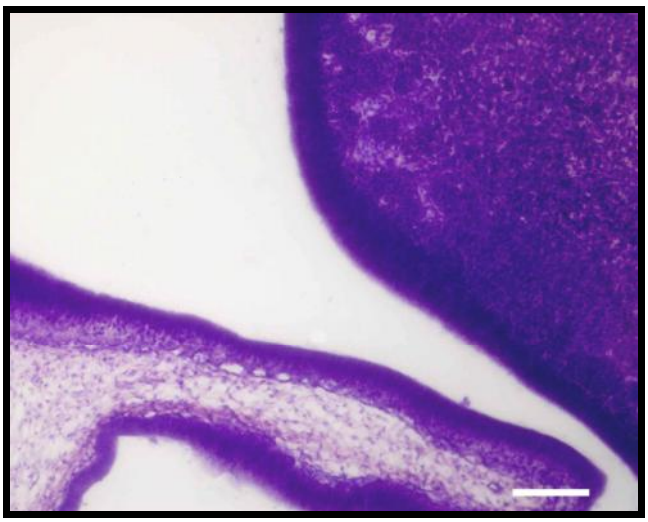
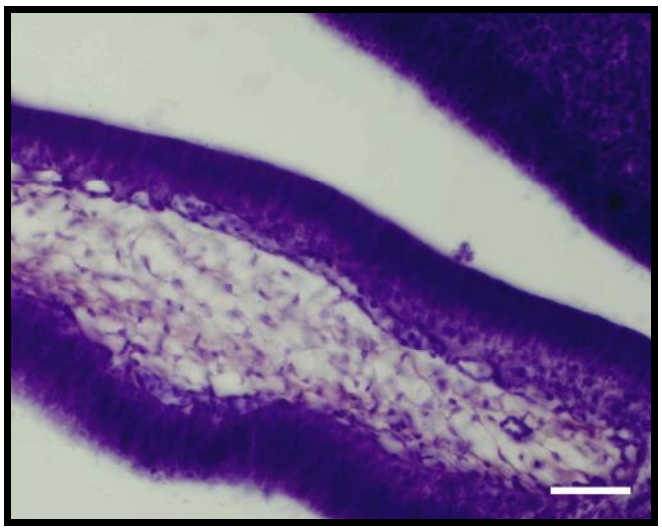


Figure 3.2 A series of magnifications of the area that will later develop into the pineal gland of an 8-day-old chick embryo not exposed to hydrocortisone and stained with Cresyl Fast Violet. Magnifications, Bar: (b) = 100μm, (c) = 50μm, (d) = 30μm and (e) = 10μm.

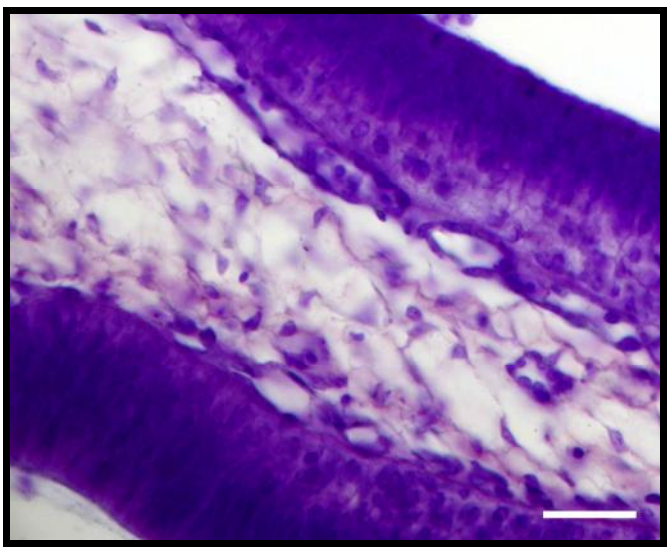
(f)



(g)



(h)



(i)

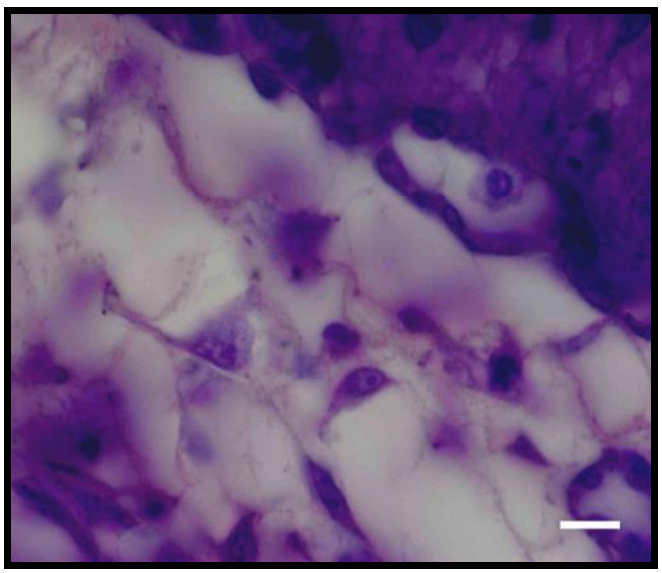
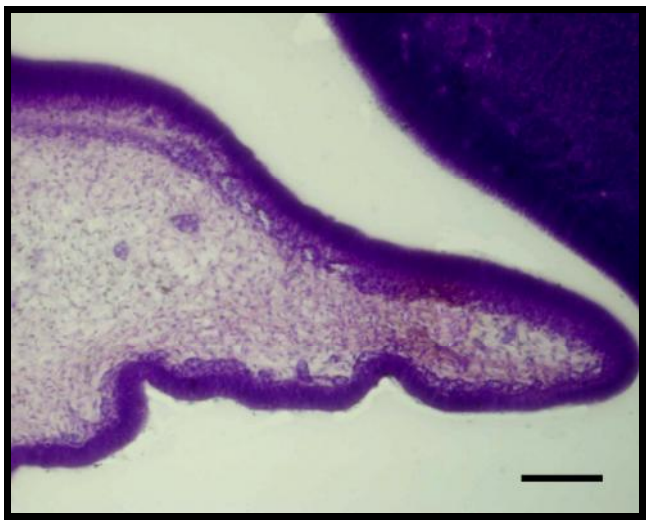
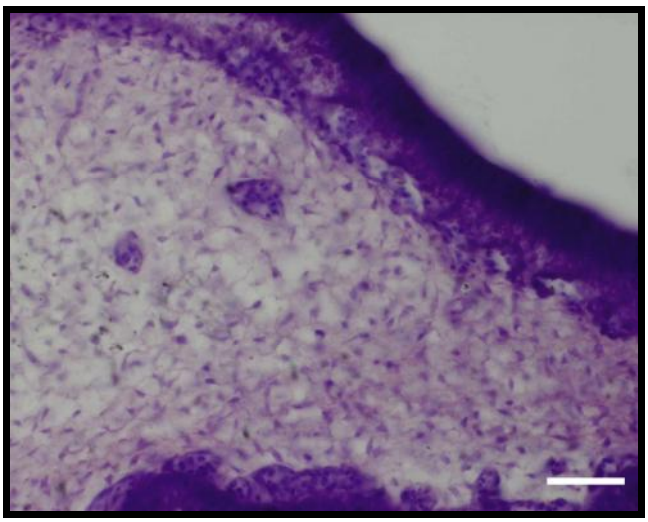


Figure 3.3 Cresyl Fast Violet staining of the primitive pineal gland of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (f) = 100μm, (g) = 50μm, (h) = 30μm and (i) = 10μm.

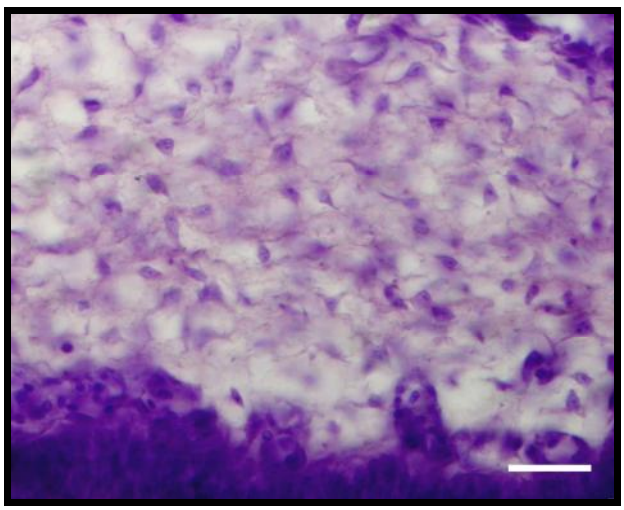
(j)



(k)



(l)



(m)

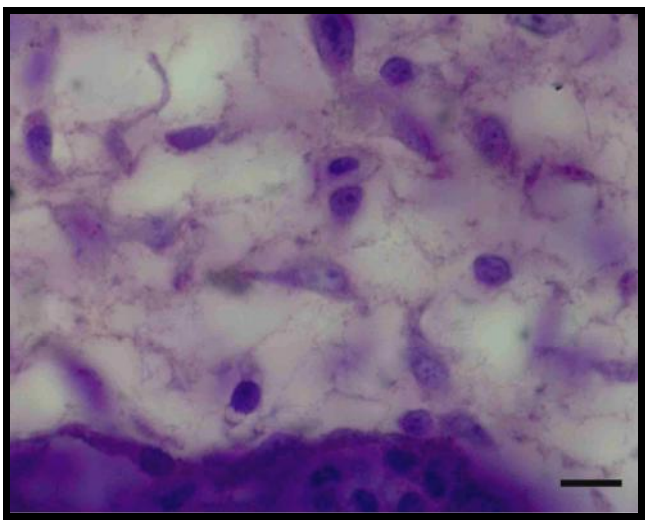


Figure 3.3 Cresyl Fast Violet staining of the area that will develop into the pineal gland of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (j) = 100μm, (k) = 50μm, (l) = 30μm and (m) = 10μm.

(a)

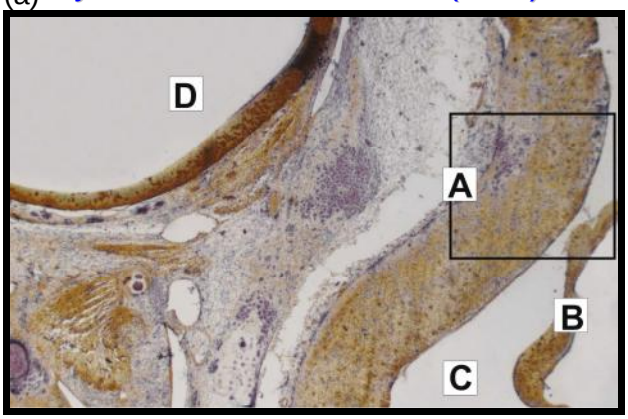
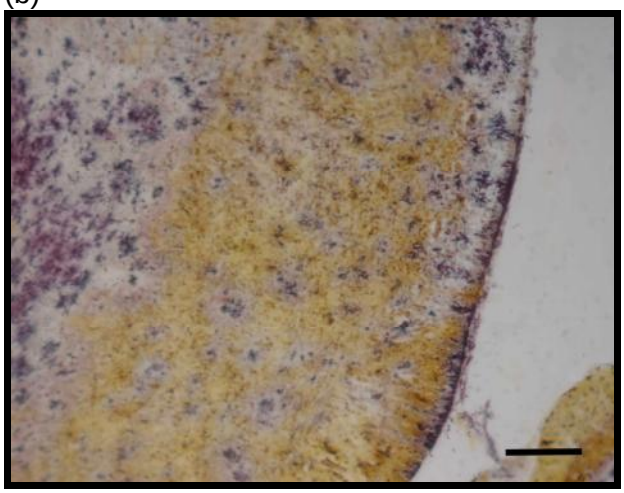
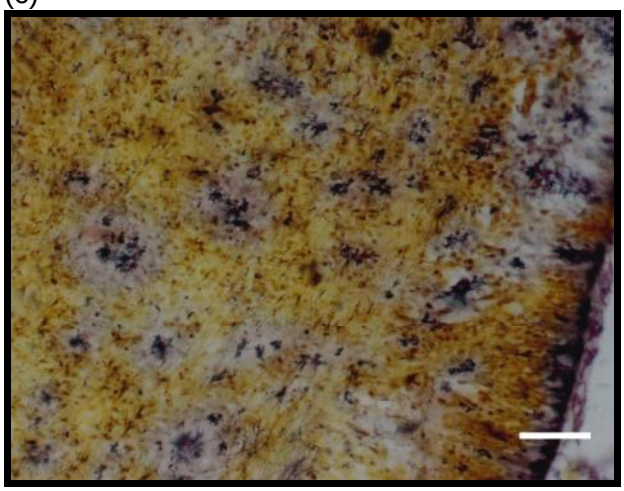


Figure 3.4 Small magnification photograph to illustrate area investigated. The floor of the myelencephalon (black square, labelled (A)) were enlarged to visualize neurons. The isthmus (B), rhombocoele or fourth ventricle (C) and eye chamber (D) are indicated for orientation. This 8-day-old chick embryo head was stained with Silver Impregnation.

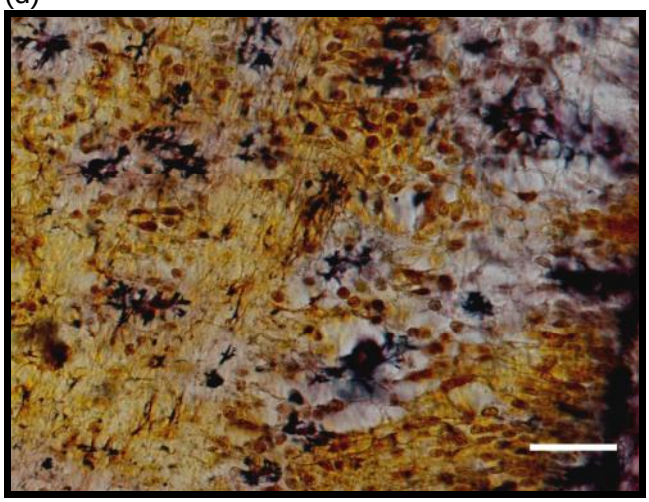
(b)



(c)



(d)



(e)

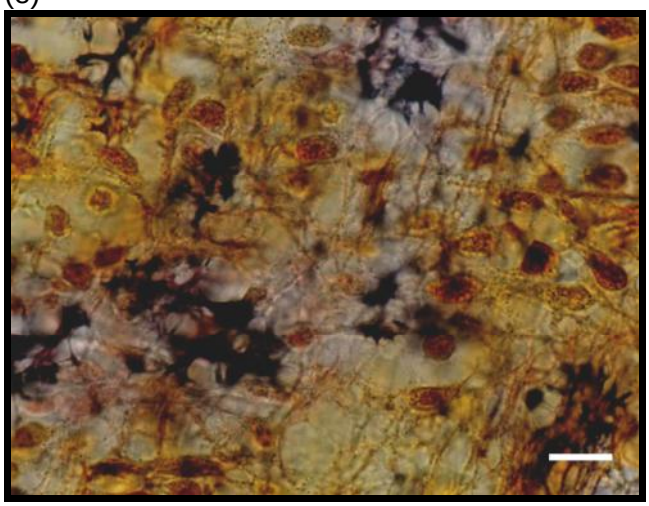
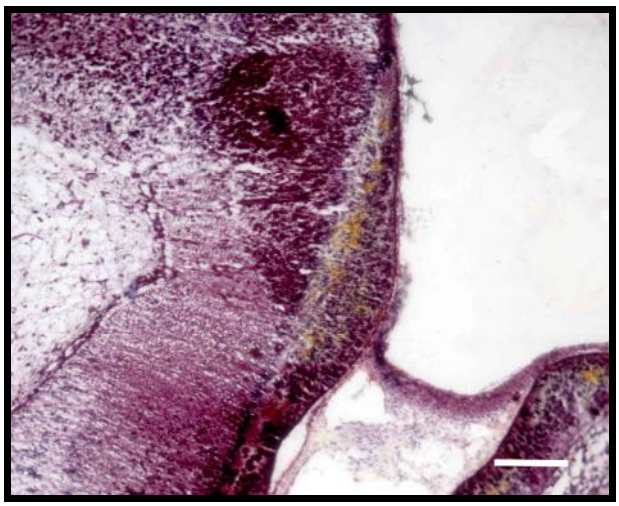
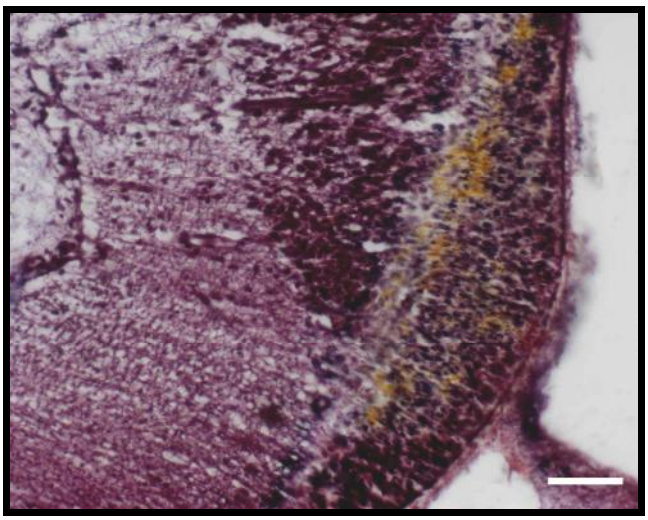


Figure 3.4 A series of magnifications of the floor of the myelencephalon of 8-day-old chick embryos not exposed to hydrocortisone and stained with Silver Impregnation. Magnifications, Bar: (b) = 100μm, (c) = 50μm, (d) = 30μm and (e) = 10μm.

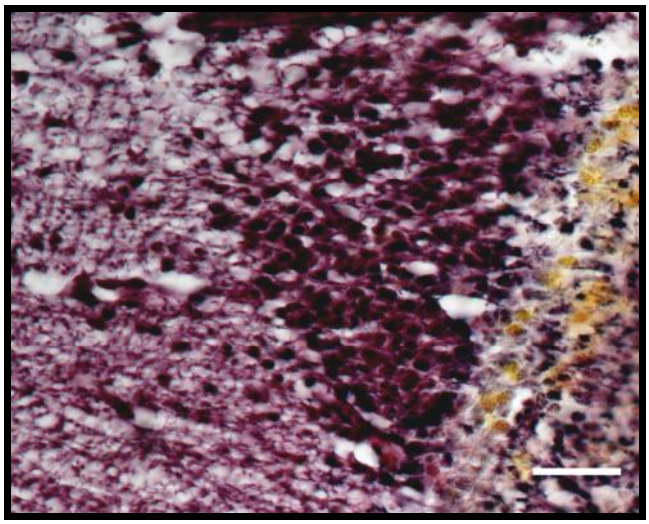
(f)



(g)



(h)



(i)

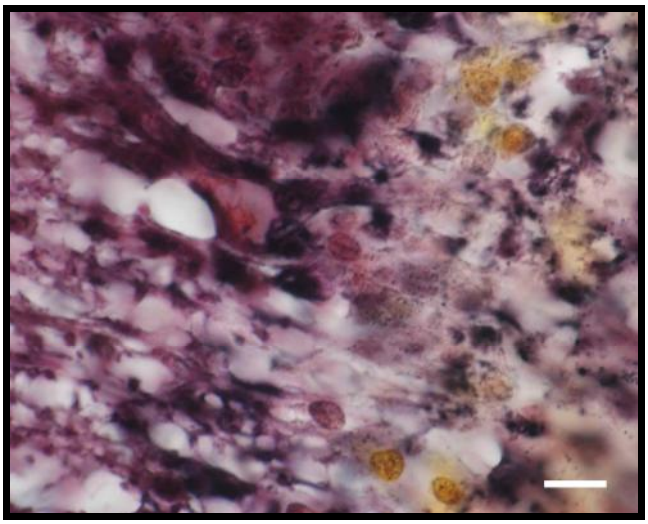
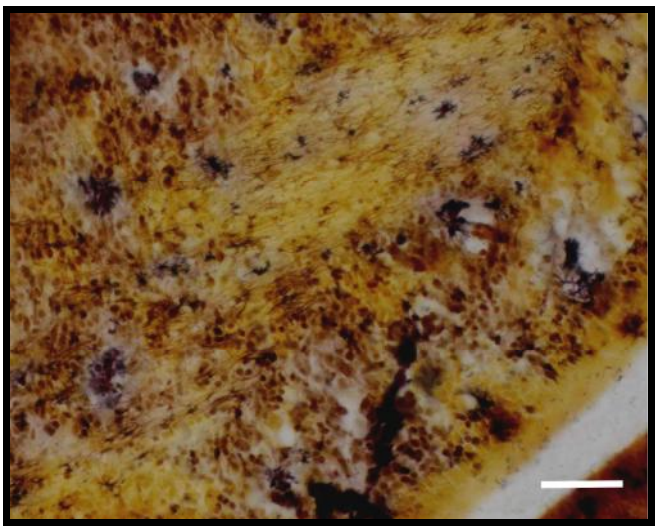


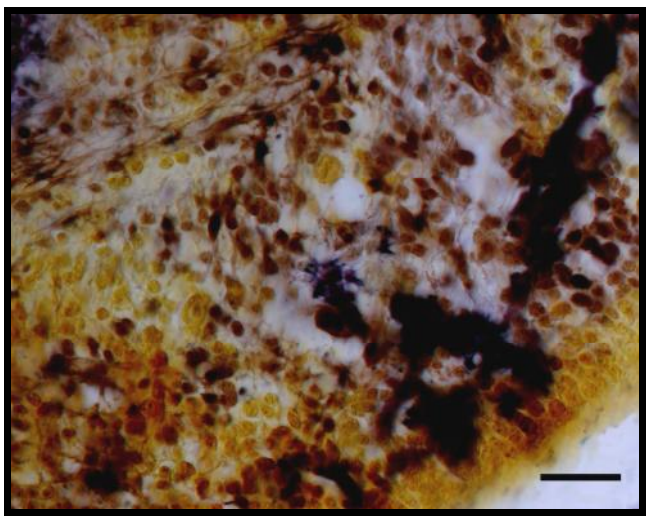
Figure 3.4 Silver Impregnation of the floor of the myelencephalon of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (f) = 100μm, (g) = 50μm, (h) = 30μm and (i) = 10μm.

(j)

(k)



(l)



(m)

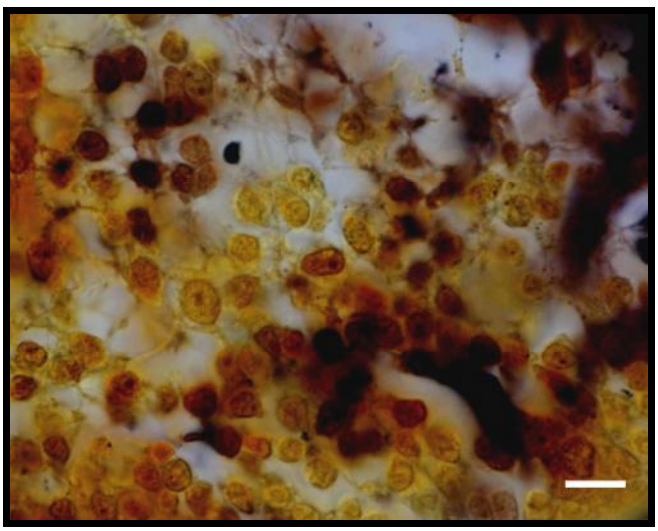
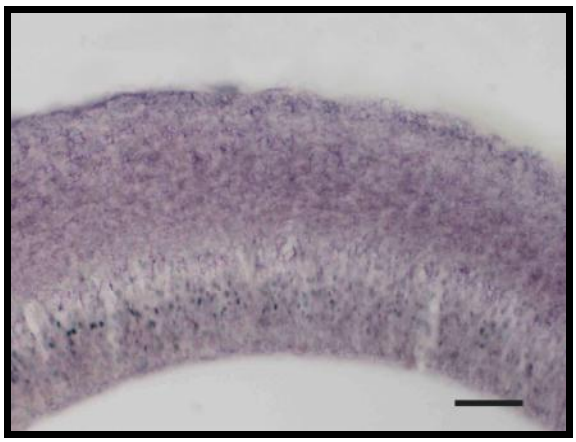
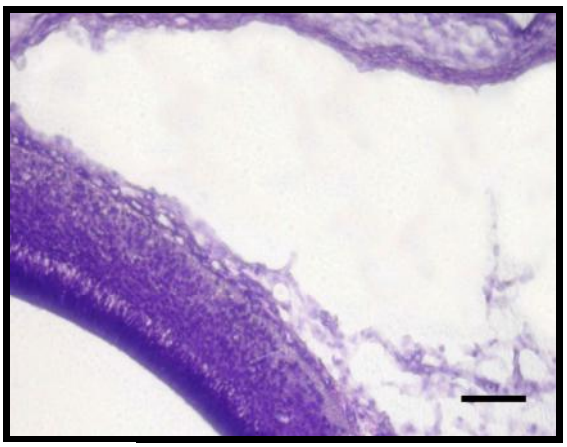


Figure 3.4 Silver Impregnation of the myelencephalon of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (j) = 100μm, (k) = 50μm, (l) = 30μm and (m) = 10μm.

(a)



(b)



(c)

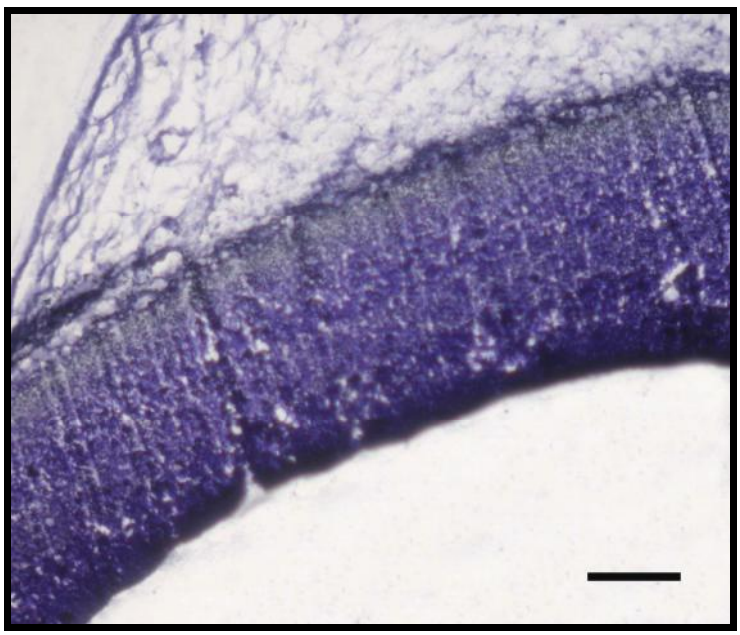


Figure 3.5 Different staining procedures to compare the combined Gold Chloride - Toluidine Blue stain. Control sample an 8-day-old chick embryo stained with only Gold Chloride (a), stained with only Toluidine Blue (b) and stained with a combination of the two; Gold Chloride - Toluidine Blue (c). Bar: (a),(b) and (c) = 100μm.

(a)

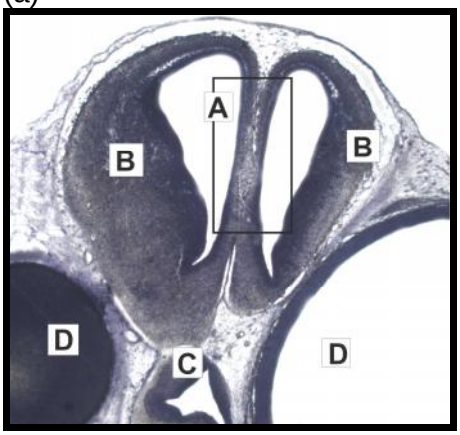
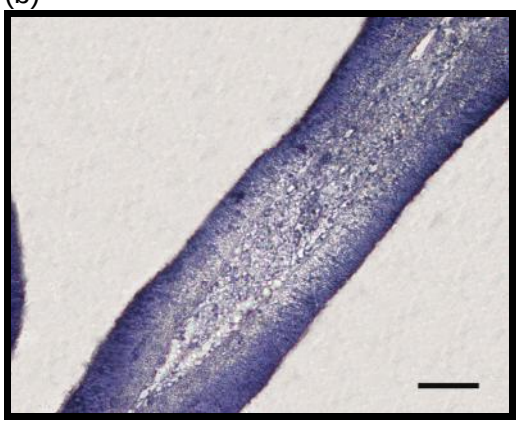
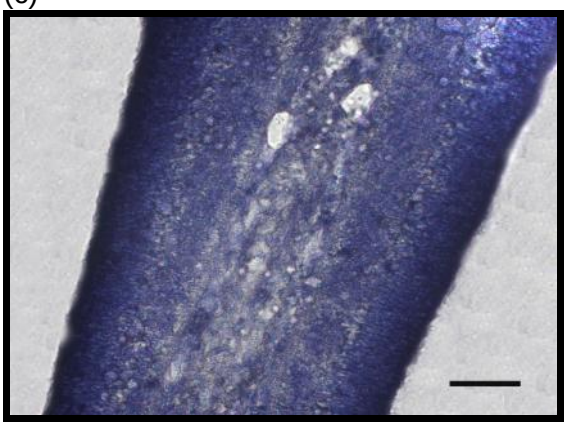


Figure 3.6 Small magnification photograph of and 8-day old chick embryo head, stained with Gold Chloride - Toluidine Blue, to illustrate the area investigated. Area (A) – within black square, was enlarged to evaluate neuron morphology. (A) represents the region between the two telencephalic vesicles. The two telencephalic vesicles are labelled (B). The diencephalon (C), chambers (D) and optic lobe (E) can also be identified.

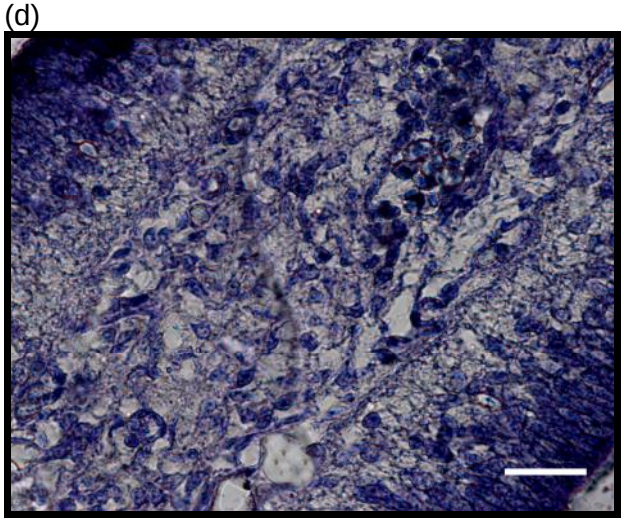
(b)



(c)



(d)



(e)

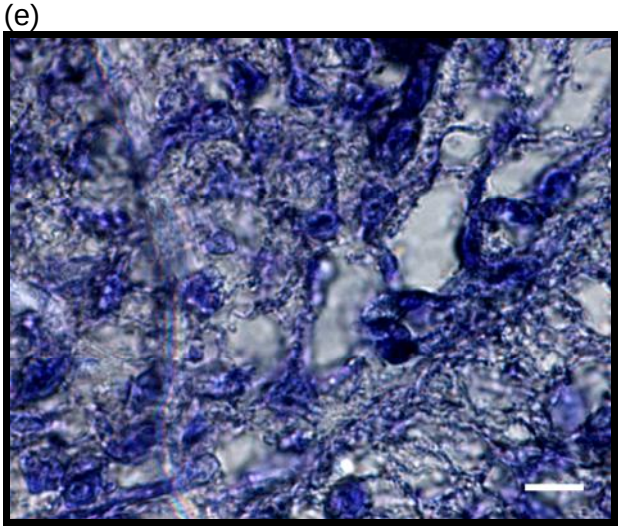
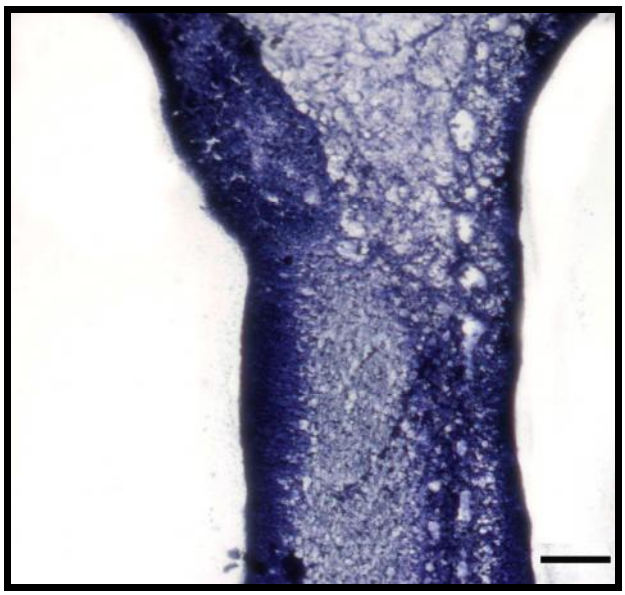
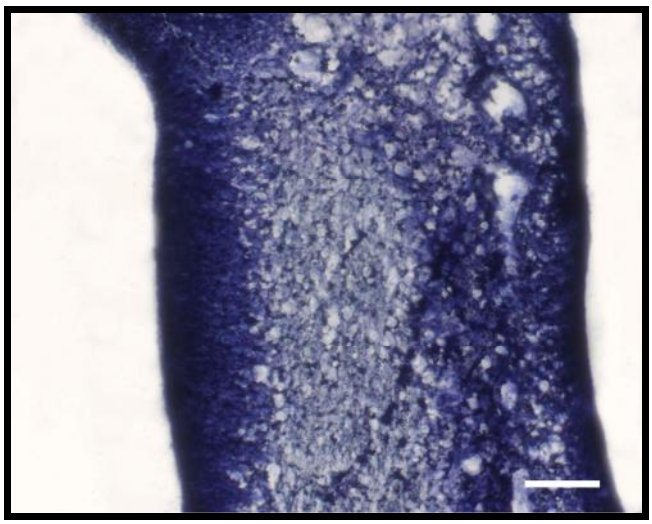


Figure 3.6. Gold Chloride - Toluidine Blue staining of chick embryonic brain tissue from the intertelencephalic region not exposed to hydrocortisone. A series of magnifications were taken to evaluate neuron morphology. Magnifications, Bar: (b) = 100μm, (c) = 50μm, (d) = 30μm and (e) = 10μm.

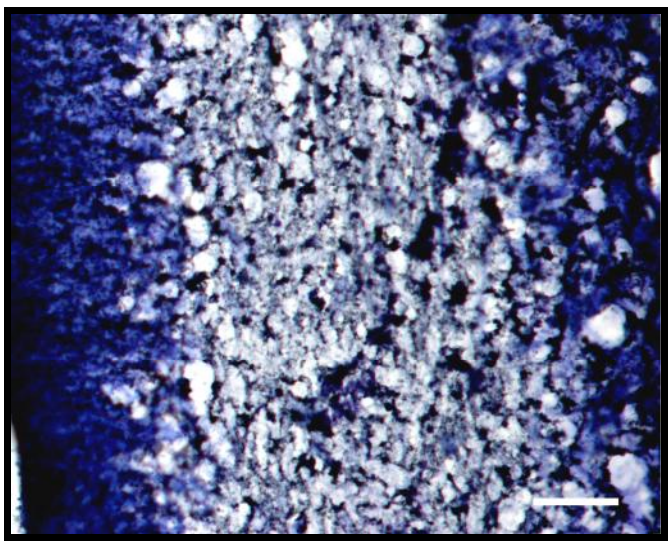
(f)



(g)



(h)



(i)

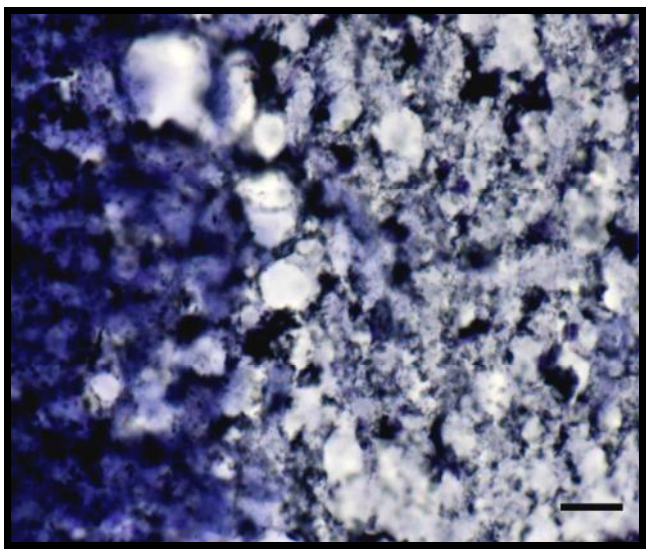
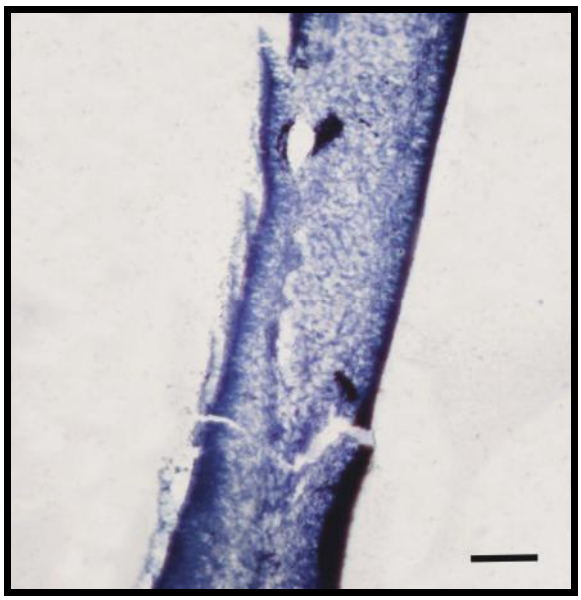
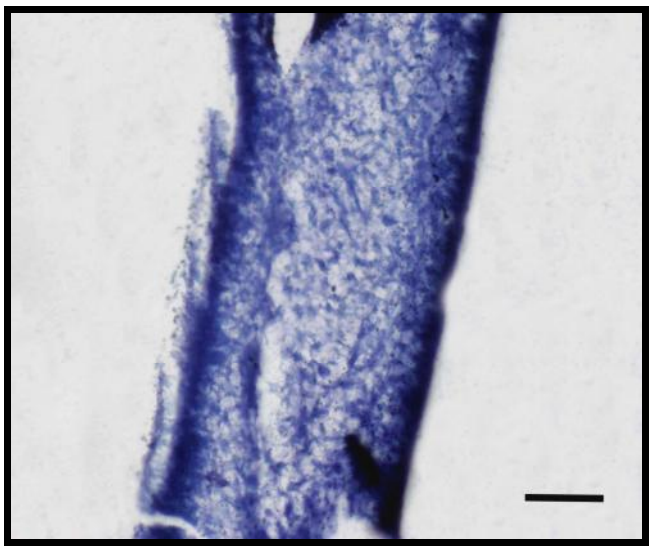


Figure 3.6 Gold Chloride - Tolduidine Blue of the region between the two telencephalic vesicles of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (f) = 100μm, (g) = 50μm, (h) = 30μm and (i) = 10μm.

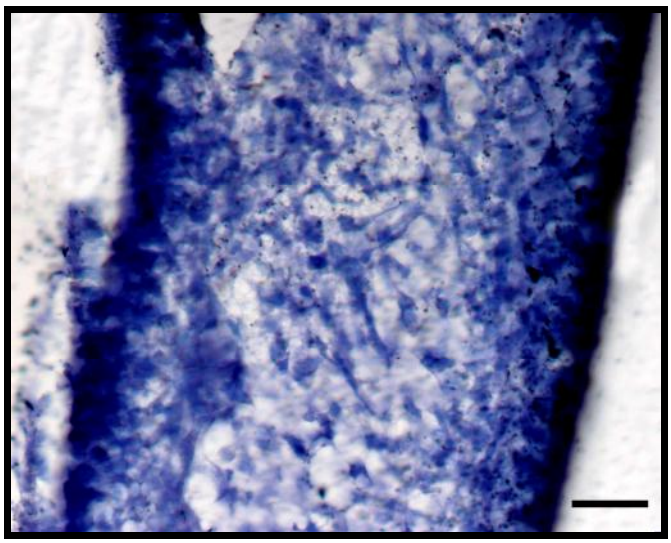
(j)



(k)



(l)



(m)

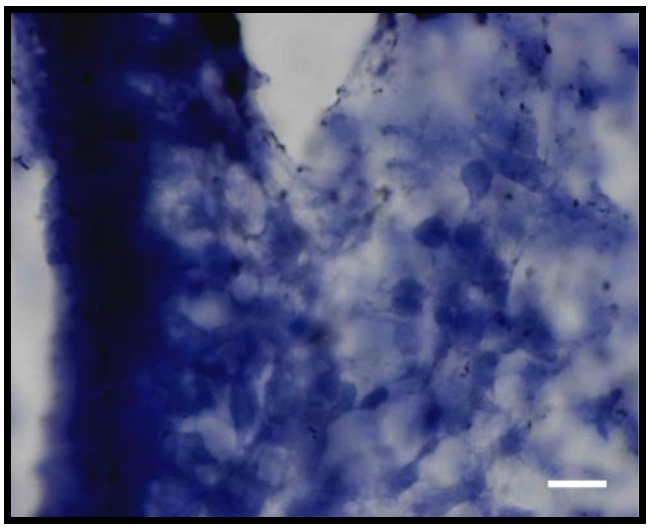


Figure 3.6 Gold Chloride - Toluidine Blue of the region between two telencephalic vesicles of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone. A series of magnifications were taken to investigate the effect of the glucocorticoid on neuronal development. Magnifications, Bar: (j) = 100μm, (k) = 50μm, (l) = 30μm and (m) = 10μm.

Chapter 4 : The effects of hydrocortisone on the ultrastructure of chick embryo neurons.

4.1 INTRODUCTION

Light microscopy offers an excellent way of visualizing different tissues and their organization within the body (Coetzee *et al.,* 1997). Therefore in this study light microscopy provided an overall view of structural changes that occurred following *in vivo* exposure of chick embryos to hydrocortisone. Additional information regarding fine, ultrastructural detail can be obtained by TEM (Burkitt *et al*., 1993). Advantages of TEM not shared by other morphological methods are that in properly prepared specimens, all cell types, intra- and extracellular structures including membranes such as the plasma and nuclear membranes are clearly visible (Peters *et al*., 1976).

Although the preparative procedures of TEM are time consuming, the improved discrimination between cells and conclusive damage seen to the various structures within the cell, makes this method an obvious choice for succeeding light microscopy in the investigation of neuron morphology, following hydrocortisone exposure. (Wuerker *et al*., 1983).

Programmed neuronal death occurs during normal development and is often regulated by steroid hormones (Kinch *et al*., 2003). It has been shown that glucocorticoids, and specifically hydrocortisone may be responsible for neuronal cell death (Lambroso *et al*., 1998) (Goodyer *et al*., 2001). Research undertaken by Kinch *et al*., (2003) on motorneurons indicated that certain steroid hormones activate cell death by means of caspase activation and loss of mitochondrial function. In the same study by Kinch *et al*., (2003), TEM was utilized to show that steroid hormones cause cellular damage, characterized either by aggregated ribosomes and breakages, or fragmentation of the nuclear envelope. Other TEM studies evaluated the effect of trauma that may cause the generation of ROS in neural tissue. Anoxic-ishaemic conditions in the cerebral cortex caused derangement of neuronal membranes and necrosis of cisterns of the rough ER (Castejon, 2004). As it is known that glucocorticoids, such as hydrocortisone, may induce ROS, it can be assumed that damage to neuronal membranes and membranous

structures may be observed in neural tissue exposed to hydrocortisone. (Lowe *et al*., 2000).

Cellular membranes are delicate structures that are vulnerable to the effects of toxins, drugs and chemicals (Wu and Wang, 2002) (Riedel and Davies 2005). Therefore samples for TEM need to be carefully prepared to ensure that these structures remain intact, especially as membrane structure is a specific focus of this study. Fixation is the most important step in preparing material for electron microscopy (Pillsbury, 1980). Therefore several fixatives should be evaluated since it is recommended that a structure e.g. the plasma membrane should have the same general appearance after fixation with a number of different fixatives (Glauert,1975). Fixatives that are often used include glutaraldehyde based fixations, a method utilizing a formaldehyde-glutaraldehyde combination and potassium permangenate.

Therefore, in this chapter the following research objectives were investigated:

- To determine the best procedure for tissue collection and method for fication to obtain optimal tissue integrity when evaluating chick embryo neural tissue by TEM.

- To apply the optimal fixation methods to the selected brain areas in the control sample and investigate neural tissue for any damage that might have occurred due to the processing.

- To evaluate samples exposed to hydrocortisone for any damage that may have occurred due to the action of the glucocorticoid.

4.2 MATERIALS

4.2.1 Reagents

Glutaraldehyde, sodium-dihydrogenphosphate-2-hydrate ($NaH_2PO_4 \cdot 2H_2O$), disodium hydrogen phosphate (Na_2HPO_4), sodium acetate (CH_3COONa) and uranyl acetate were all obtained from Merck, Johannesburg, South Africa. Potassium permangenate ($KMnO_4$) and formaldehyde were both from Saarchem Pty. Ltd. Muldersdrift, South Africa.

Magnesium chloride ($MgCl_2$) was supplied by Fluka, Sigma-Aldrich, Switzerland. Barbitone sodium was obtained from BDH Chemicals Ltd. Pode, UK. Osmium tetroxide (OsO_4) was supplied by Spi suppliers, West Chester, USA, and the resin (Quetol 651) was obtained from TAAB Laboratories, Reading AGAR Scientific Ltd. Essex, UK. Lead citrate was supplied by Polaron Equipment Ltd. Watford, UK.

4.3 METHODS

4.3.1 Dissection of chick embryo brain

The eggs were removed from the incubator on day 7.75 (Carnegie stage 21), the eggshell broken with a scalpel and the embryo removed. The embryo was placed in a Petri dish with the base lined with dental wax on its side with the prosencephalon and mesencephalon clearly visible. Dissecting on dental wax prevents additional mechanical damage done to the cells, due to the fact that the blade can easily slice through the tissue, and no extra force is encountered when it presses against the bare base of the Petri dish (Loots *et al*., 1993). A small insertion was made with a scalpel and the whole brain (method 1) or mesencephalon (method 2-4) was removed. In this study (for methods 2-4), only the areas that will later develop into the midbrain and cerebral aqueduct were removed.

In order to determine fixation procedures and stabilizing chemicals that would result in little or no disruption of cell membranes or membranous structures, 4 different fixation methods were evaluated and include two glutaraldehyde based fixations, a method utilizing a formaldehyde-glutaraldehyde combination and potassium permangenate

For method 1, the brain was placed in a glutaradehyde fixative and only dissected after fixation. Methods 2-4, on the other hand, required that the tissue was placed in the Petri dish lined with dental wax and covered in a small volume of fixative. Under a dissection microscope (Nikon SMZ800, Nikon Instech Co., Kanagawa, Japan), small 1mm X 3mm slices were cut from the mesencephalon, before the tissue samples were completely submerged in the fixative.

To ensure optimal conditions for these 4 fixation methods, $MgCl_2$ was added in some cases as a stabilizing chemical, to promote the stability of all membrane structures. (Peters *et al*., 1976)(Glauert, 1975)

4.3.2 Optimization of fixation procedures for chick embryo neuronal tissue

4.3.2.1Glutaraldehyde Method 1

The entire embryo brain was placed in a 2.5% glutaraldehyde solution for 3 days in the refrigerator. The brain was removed and while still submerged in fixative dissected into $1mm^3$ blocks. The tissue blocks were removed from the fixative and washed thrice in a 0.075M sodium phosphate buffer, pH 7.4. A 0.15M stock sodium phosphate buffer was prepared by dissolving 13.348g Na_2HPO_4 in 500ml ddH_2O. A volume of 100ml ddH_2O was also added to 2.34g NaH_2PO_4 and dissolved. The two solutions were added together in order to obtain a 600ml stock phosphate buffer solution. The working solution was obtained by dilution with ddH_2O.

Post fixation of the tissue in 1% osmium tetroxide was followed by three washes with sodium phosphate buffer. The tissue was dehydrated by increased concentrations of ethanol (30%, 50%, 70%, 90% and three changes 100%) before being embedded in resin.

4.3.2.2 Glutaraldehyde Method 2

The mesencephalon was dissected as described in Section 4.3.1 and the tissue fixed for a period of 1 hour in a 2.5% glutaraldehyde solution prepared in a 0.2M phosphate buffer, pH 7.4 with 2mM $MgCl_2$ included as a stabilizing chemical (Glauert, 1975). A 0.2M phosphate buffer was prepared by dissolving 17.799g Na_2HPO_4 in 500ml ddH_2O and 3.12g NaH_2PO_4 in 100ml ddH_2O. The two solutions were added together in order to

obtain a 600ml 0.2M phosphate buffer solution. After fixation the tissue was rinsed thrice with the phosphate buffer solution before being placed in 1% osmium tetroxide for 1 hour. The tissue was rinsed again and processing completed by serial dehydration and was embedded in resin.

4.3.2.3 Paraformaldehyde-Glutaraldehyde (Method 3)

The dissected mesencephalon was fixed for 1 hour in a combination of 2.5% formaldehyde and 2.5% glutaraldehyde with 0.2M phosphate buffer, pH 7.4 and 2mM $MgCl_2$. (Glauert, 1975) The tissue was rinsed thrice in a 0.075M sodium phosphate buffer, pH 7.4 and transferred to 1% osmium tetroxide for 1 hour, rinsed again, dehydrated and embedded in resin.

4.3.2.4 Potassium Permangenate (Method 4)

Dissected fragments of the mesencephalon were placed in a 1.2% potassium permangenate solution prepared in veronal acetate buffer, pH 7.2 for 2 hours. (Glauert, 1975) The veronal acetate stock buffer was prepared by dissolving 2.89g barbitone sodium and 1.15g sodium acetate in 100ml ddH_2O. The tissue was rinsed three times in a 0.075M sodium phosphate buffer, pH 7.4 before being dehydrated and embedded in resin.

4.3.3 Contrasting

For all samples, ultra-thin sections were prepared and stained with uranyl acetate for 15 minutes followed by 10 minutes of staining with lead citrate. The sections were then viewed and photographed by making use of TEM (Pillsbury, 1980). (Philips EM301. Eindhoven, Netherlands)

4.3.4 Inoculation of embryos with hydrocortisone

Embryos were randomly assigned to the control group and the treatment group. The treatment group received either 0.137μM or 0.685μM hydrocortisone as by the procedure discussed in Section 3.3.4

4.3.5 Fixation, processing and evaluation of embryos exposed to hydrocortisone

The glutaraldehyde (method 2) and potassium permangenate (method 4) were found to be optimal TEM fixation methods for chick embryo neural tissue and both fixation methods were used to determine the effect that hydrocortisone on the membranous

structures of chick embryo neural tissue. Changes in cell morphology and membrane structure were investigated by using TEM micrographs.

4.4 RESULTS AND DISCUSSION

In order to study the effects of hydrocortisone on the ultrastructure of the neuronal tissue of chick embryos four different fixation methods were evaluated using normal 8-day-old chick embryos.

4.4.1 Optimization of fixation procedures for chick embryo neuronal tissue

To answer the first research question of this chapter, namely: To determine which brain areas, fixation procedures and stabilizing agents would yield the best results when evaluating chick embryo neural tissue by TEM, four different fixation methods were evaluated.

It was therefore important to first determine which fixatives and additives did not cause disruption to the plasma and nuclear membrane, as well as other structures such as the mitochondria and ER, before the effect of hydrocortisone on neuron membrane morphology could be evaluated. (Glauert, 1975) (Sjostrand, 1997). Fixation is the most important step in preparing material for electron microscopy (Pillsbury, 1980). Multiple fixatives were evaluated, since, as already mentioned, it is recommended that a structure should have the same general appearance after fixation with a number of different fixatives (Glauert,1975). If any damage could be seen in a control sample due to the fixative, it would be futile to use in the experimental group, since it would be impossible to determine whether the damage to morphology observed may be a cause of the fixative or of the administration of the drug.

Electron microscopic investigations of the fine structure of the central nervous system require a well-preserved tissue ultrastructure (Morin *et al*., 1997). The choice of fixative for preservation of nervous tissue is governed by the type of study desired (Carson, 1980). The method itself should present optimal results, that is, cell membranes intact, cytoplasmic structures not displaced and not altered, such as mitochondria with intact cristae, ER intact and in place and nuclear material and membranes intact (Pillsbury, 1980). Some researchers even suggest that different fixatives should be used at

different stages of development, or for different structural elements within a tissue (Glauert, 1975). The end-point, however, as M.J Karnovsky (1985) stated, is the 'life-like' preservation of cells and tissues.

In this study, the first fixative evaluated was phosphate buffered glutaraldehyde (Glutaraldehyde method 1) solution (Kiernan, 2000) (Pillsbury, 1980). Glutaraldehyde forms excellent cross-linkages with protein chains and in this way stabilizes structures such as the ER, the ground substance of the cytoplasm and the extracellular matrices (Loots *et al*., 1993). Although the chemical reaction of glutaraldehyde with protein is rapid, penetration of the tissue is slow (Kiernan, 2000) (Pillsbury, 1980). Fixation with glutaraldehyde may result in low contrast and slight shrinkage may be seen. However, with a secondary fixative, such as osmium tetroxide, these obstacles are often corrected. Osmium tetroxide then also is particularly useful for preserving the continuity of membrane systems (Loots *et al*., 1993) (Glauert, 1975). It was found that glutaraldehyde contributes to the total effective osmolarity of the fixative and that fixation up to 4 hours had no effect on the cell volume (Lee *et al*., 1982). As a continuation of the previous histological investigation (Chapter 3) the entire brain was utilized and placed in fixative. In this method tissue fragments were only prepared following fixation and therefore a longer fixation time of three days was used.

Figure 4.1 is a micrograph showing two control neurons, labeled (1) and (2) of an 8-day-old chick embryo. The nuclear membranes of neurons (1) and (2) are respectively labeled (A) and (B). A nucleolus could be seen within the nucleus of cell (2), as indicated by the black arrows. The plasma membrane, separating these two neurons is labeled (C). Although it appeared as if the fixative caused no disruption to the membranes, the visual quality of the membranes and neurons were poor. The membrane structures were clearly visible at some areas, as can be seen within the oval drawn structure. Inside the oval structure, the double membrane structure of both the plasma membrane and nuclear membranes were easily distinguished, however, a hazy appearance with no clear distinction in structures, as indicated by the white arrows, could be seen throughout the other areas. In a control sample, such as this, clear and visible discrimination of all structures is needed, since it serves as the standard that treated samples would be measured against. A possible reason for reduced visual quality of membranes, is that the sample was fixed for a long period of time (3 days) and was only dissected after fixation. This may have lead to hardening of the tissue and postfixation dissection may

have caused some mechanical damage. It would also appear to be more advantageous to only use a part of the brain, since penetration of the tissue would be easier and comparison of the same areas would be possible.

To overcome some of these problems a phosphate buffered glutaraldehyde solution with 2mM $MgCl_2$ included as a stabilizing chemical (Glutaraldehyde method 2) was used.

Tissue fragments were fixed rather than the entire brain. The sample was dissected directly after termination, and only the midbrain area removed. Literature indicates that it would be advantageous to only keep the sample in glutaraldehyde fixative for a period no longer that 2 hours (Pillsbury, 1980), although some researchers found that up to 4 hours, no damage could be observed (Lee *et al*., 1982). The sample, however, only remained in the fixative for 1 hour and a stabilizing chemical, $MgCl_2$ was added (Glauert, 1975). This resulted in the quality of fixation of control neurons of an 8-day-old chick embryo, as shown in Figure 4.2 where two neurons (1) and (2) can be seen, separated by plasma membrane labeled (a). The nucleus (A) in cell (1) has a clear, easily distinguishable nuclear membrane (b) with a nuclear pore, indicated by the white arrow. Clear distinctions can be made between the double membrane structures of both the nuclear membrane and plasma membrane as indicated within the black oval and black rectangle respectively. The mitochondria (B) in cell (1) also appeared to be intact and the inner and outer mitochondrial membranes with cristae are clearly visible, as indicated by the black arrows. An axon (C) could also be seen passing across cell (1).

The adaptations done to the glutaraldehyde fixation procedure (shorter fixation time and added $MgCl_2$) appeared to have a great improvement on the visualization of the neurons. The double membrane structures of both the plasma and nuclear membranes, as seen within the oval and rectangle, were clear and easily distinguished. All membranous structures could be clearly seen throughout the micrograph and as a result this method was chosen for the evaluation of the effect of hydrocortisone on neural tissue.

An alternative method is the use of a combination of formaldehyde and glutaraldehyde in phosphate buffer containing $MgCl_2$ (Glauert, 1975). In this study the dissected mesencephalon was fixed for 1 hour. Karnovsky (1985) originally described this method in 1965 and later stated that the original buffered paraformaldehyde-glutaraldehyde

fixative, which contained 4% paraformaldehyde and 5% glutaraldehyde, was extremely hypertonic (Glauert, 1975). Compared to glutaraldehyde, formaldehyde does not form excellent cross-linkages with protein, and only mildly stabilizes the protein chains (Loots *et al*., 1993). However, when used in combination, glutaraldehyde is mainly responsible for cross-linking proteins whereas formaldehyde contributes to the cross-linking of protein and DNA (Sewell *et al*., 1984). Glutaraldehyde poorly penetrates tissue, in contrast formaldehyde penetrates tissue quite rapidly, thereby accelerating tissue fixation. A disadvantage is, however, that lipid droplets are extracted (Pillsbury, 1980).

Different authors use different formulations of the paraformaldehyde-glutaraldehyde fixative (Kiernan, 2000). For example, Loots *et al*., (1993) reported that commercial solutions contained methanol and should be avoided, whereas McDowell *et al*., (1976) recommended that a combination of 4% commercial formaldehyde and 1% glutaraldehyde should be used. Glauert (1975) reported that the concentrations of paraformaldehyde and glutaraldehyde in the original Karnovsky method should be reduced. For the evaluation of the rat nervous system, Somogyi *et al*., (1982) recommended that buffered picric acid paraformaldehyde supplemented with glutaraldehyde should be used as primary fixative. In contrast Morin *et al*., (1997) reported that for the best ultrastructural preservation of synaptic connections in the human cerebral cortex, 2% paraformaldehyde with 2.5% glutaraldehyde in a cacodylate buffer with 3mM calcium chloride ($CaCl_2$) and 2.5% Dimethyl Sulfoxide (DMSO) at 4°C for 4 hours should be used. Therefore, the fixation of neuronal tissue using paraformaldehyde-glutaraldehyde methods is a function of osmolarity, pH, temperature, time, concentration (Pillsbury, 1980) and the type of tissue evaluated e.g. rat or human, adult or embryo.

In this study the paraformaldehyde-glutaraldehyde method provided adequate tissue fixation as shown in Figure 4.3 where 5 neurons, labeled 1-5 that were fixed using the paraformaldehyde-glutaraldehyde method. Severe shrinkage of the cells could be seen which left wide open spaces in the cytoplasm, as indicated by the black arrows. Although the double nuclear membrane of cell 2, indicated by white arrows, was clearly visible and unaffected, other organelles, such as the mitochondria (in black ovals) were not easily identifiable. No membranous structures or cristae of the mitochondria could be distinguished. This could be due to the use of industrial produced formaldehyde, which may not be suitable for the use in electron microscopy, since it is known to contain high

methanol concentrations (Pillsbury, 1980). Another possibility is the ratio of paraformaldehyde to glutaraldehyde used. Different methods for different types of tissues use different ratios in combination with various additives such as DMSO and $MgCl_2$. Magnesium is preferred to calcium for the preservation of membrane lipids, because it is a smaller molecule and, at reasonable concentrations do not precipitate proteins. In addition, calcium may exert some physiological effects, such as stimulation of fibers or triggering of secretions. A 1-3mM magnesium chloride solution is preferred when using phosphate buffered fixatives, because precipitates only then form at high concentrations (Glauert, 1975). However, for this specific study, the paraformaldehyde-glutaraldehyde fixative used was not suitable for chick embryo neural tissue.

Potassium permangenate is not a method that is commonly used, but fixes membranes and membraneous structures excellently (Loots *et al*., 1993). Permangenates are known to destroy certain cell components (ribosomes, cytoplasmic fibrils etc) and extract RNA while preserving DNA. This fixative is excellent for studies of membrane systems, since comparative studies have shown that the continuity of membranes is better preserved by potassium permangenate than any other fixative. The membranes then also stand out with great clarity against the surrounding cytoplasm (Glauert, 1975). The membrane structure (denser lines) will even be more easily distinguished as the period of immersion in permangenate fixative are extended (Robertson, 1957). As hydrocortisone may have detrimental effects on the different membranous structures of neurons, a fixative that would ensure stability of these structures would be advantageous. Figure 4.4a and 4.4b are both control samples of neurons of an 8-day-old chick embryo fixed with potassium permangenate. Figure 4.4a shows 8 neurons, indicated by 1-8 with well-preserved, intact nuclear membranes. Neurons are known for their large nuclei and these excellent preserved nuclei just illustrates this better. The plasma membranes of the different cells, indicated by the black arrows, were also clearly visible and intact, which indicate that no damage due to the fixative could be observed. A well-preserved ER could also be seen, inside the black oval, next to nucleus 7. This was also an excellent example of the location of an ER, since a connection between the nuclear envelope and the ER could be seen (labeled (a)). Tubular extensions of the outer nuclear membrane link it with the cisternae of the ER. Another common feature of neurons is an invagination of the cytoplasm into the nucleus, as seen in this photograph, indicated by the white arrow (Johnson and Roots, 1972).

Figure 4.4b also a control sample of neurons of an 8-day-old chick embryo fixed with potassium permangenate, but taken at a higher magnification, show two neurons, labeled (1) and (2) with nuclear membranes (A) and (B). The plasma membrane (C) separates the two cells and could be seen as a clear, intact, continuous double membrane, as indicated by the black arrows. Both nuclear membranes also showed no disturbances and could be seen as double membrane structures (indicated by the white arrows). It would appear that all the characteristics of the ultrastructure of neurons were made visible by this fixative and it would therefore be a suitable method to use in further studies with hydrocortisone.

Therefore the phosphate buffered glutaraldehyde solution containing $MgCl_2$ (Glutaraldehyde, Method 2) and the potassium permangenate (Potassium Permangenate Method 4) was used to evaluate the effect of hydrocortisone on embryo neuronal structure.

4.4.2 Effect of hydrocortisone on embryonic neuronal ultrastructure

In order to answer the last research questions of this chapter, namely: To apply the optimal fixation methods to the selected brain areas in the control sample and investigate neural tissue for any damage that might have occurred due to the processing, AND to evaluate samples exposed to hydrocortisone for any damage that might have occurred due to the action of hydrocortisone, it is first important to give a brief review on how cell death can be visualized using TEM.

Research has shown that glucocorticoids can act at three different avenues, namely by ROS generation, by binding to FAS, or by activating p53 (Lowe *et al*., 2000) (Konopleva *et al*., 1999) (Burns *et al*., 1999). All three pathways are associated with mitochondrial damage, where the mitochondria releases factors that can either lead to apoptosis and necrosis (See diagram 2.1). Because apoptosis and necrosis are associated with changes to membranes and organelles, morphologically these changes should be visible when studying exposed neurons with TEM.

Zhang *et al*., (2003) indicated that apoptotic death of neurons could be visualized by TEM. They found that a disrupted nuclear envelope with condensed chromatin was a clear indicator of apoptotic cell death via p53. p53 in turn have an effect on Bcl-2. Bcl-2

is widely expressed in the nervous system and is localized in the outer mitochondrial membrane, ER and nuclear membrane, all structures involved in the apoptotic pathway (Yang *et al*., 1998). Hossman *et al*., (2001) then also found that ultrastructural alterations associated with cell death in the hippocampus include swelling of the mitochondria and ER which finally had an effect on neuronal volume. Swelling of the mitochondria is a downstream effect of the opening of the MPT, which in turn is induced by both the p53 and FAS apoptotic pathways.

Hydrocortisone was found to cause severe alterations in the membranous structure and cristae organization of the mitochondria (Mashanskii *et al*., 1986). That being the case, it would be possible for hydrocortisone to have and effect on MPT formation and therefore mitochondrial and finally cellular swelling.

The mitochondria remain an important organelle in the homeostasis of neural functioning. In fact, neurotoxic agents have shown to exacerbate mitochondrial dysfunction, which is associated with neurodegeneration (Moreira *et al*., 2003). The MPT also play a central role in neurodegeneration. MPT is responsible for mitochondrial swelling by means of the disruption of calcium ion movements. Brain mitochondria specifically demonstrated a higher capacity to accumulate calcium ions and thereby increasing mitochondrial and cellular swelling (Moreira *et al*., 2002). Bcl-2, which is located in the outer mitochondrial membrane should protect the mitochondria by interacting with the permeability transition pore complex, however, hydrocortisone have been shown to inhibit Bcl-2 via various pathways thereby compromising the protective properties of Bcl-2 (Vieira *et al*., 1999).

From all these studies it is obvious that it would be necessary to evaluate the morphology of the mitochondria, ER, nuclear and plasma membranes for any disruptions, to determine the neurotoxicity of hydrocortisone.

Figure 4.5a and figure 4.5b both illustrate mitochondria in control neural tissue from 8-day-old chick embryos fixed with potassium permangenate. In Figure 4.5a a mitochondria (A) and ER (B) can be seen. The cristae of the mitochondria (indicated by white arrows) and double membrane (a) with inner and outer surfaces (black parallels) could be clearly distinguished. The vesicles or cisternae of the endoplasmic reticulum

(indicated by black arrows) all appeared to be intact and could be seen as fine membranous structures.

Two different neurons of a control 8-day-old chick embryo, fixed with potassium permangenate could be seen in figure 4.5b and is indicated by (1) and (2). The plasma membrane separating these two cells is indicated by a series of black arrows and could be seen as an intact structure. The mitochondria (A) appeared to be well-preserved, with no disruptions and its double membrane (a) was clearly visible. Various cristae are indicated by white arrows.

Figure 4.6a and figure 4.6b are micrographs showing nuclear and plasma membranes of control tissue fixed with glutaraldehyde method 2. Figure 4.6a shows two cells, (1) and (2), separated by the double plasma membrane, indicated by the white arrows. The double membrane could be clearly distinguished and had a smooth intact surface. A mitochondrion (A) with double membrane (a) was visible and located just outside the nucleus (B) of cell 2. The nuclear membrane indicated by black arrows could be seen as a double membrane with no disruptions and a smooth appearance. A nuclear pore (b) was also visible in the nuclear membrane.

The high magnification of Figure 4.6b allowed great visibility of the membranes in two cells (labeled 1 and 2, respectively). The double membrane, indicated by black arrows, of the plasma membrane (C) was clearly defined and showed no disruptions. Two nuclei (A) and (B) of cells 1 and 2 could be seen on either side of the plasma membrane. A nuclear pore (a) was visible in the nuclear membrane of nucleus (B). The inner membranes (small white arrows) of the nuclear membranes as well as the outer membranes (large white arrows) showed intact undisrupted surfaces. The nuclear material in both nuclei appeared to be evenly distributed.

All these TEM micrographs of the control samples illustrated that the fixation methods indeed did cause no disruption to the tissue and that any further damage observed in these preserved structures would be due of the action of hydrocortisone.

The effect of hydrocortisone exposure to 0.137μM at day 3.75 (Carnegie stage 16) and day 5.5 (Carnegie stage 18)) on chick embryo neural tissue at day 8 of development

was then studied. The effect on the mitochondria, ER, nuclear and plasma membranes were evaluated.

Figure 4.7a and figure 4.7b are both samples of 8-day-old chick embryos exposed to 137μM hydrocortisone on day day 3.75 (Carnegie stage 16) and day 5.5 (Carnegie stage 18) of development, and fixed in glutaraldehyde method 2. Figure 4.7a shows two cells, (1) and (2), separated by a plasma membrane, which can be seen between the two parallel lines on the micrograph. However, there appeared to be gaps in the plasma membrane, indicated by the black oval and could seen as a discontinuation of the double membrane of cell (1). Within cell (1) and cell (2), various mitochondria (A) were visible. A nucleus (B) could also be seen in cell (1) with the double nuclear membrane, indicated in the black square. It was, however, difficult to distinguish between the outer and inner membranes, due to the fact that the smooth appearance seemed to be compromised. Despite this, a nuclear pore (a) was visible in the membrane and the nuclear material seemed to have congregated at the inner membrane of the nucleus (b). This thick condensation of nuclear material could be a sign of possible chromatin margination. Condensed chromatin near the nuclear envelope is an indication of apoptosis (Zhang *et al*., 2003). In all three mitochondria (A), vague cristae (c) were present. The double membrane of the mitochondria was intact at some places (d), however, breakages in the membrane occurred and these are indicated by white arrows. Breakages in the mitochondrial membrane may be a cause of the formation of MPTs, indicating that hydrocortisone is a neurotoxic agent (Moreira *et al*., 2003).

The internal organelle structure in Figure 4.7b appeared to be disturbed. The ER (A) showed wide open gaps between the vesicles or cisternae. The ER was swollen, this resulted in the vesicles or cisternae being pulled from each other, creating open spaces. Some ribosomes were still attached to the outer membranous structures (a), however, there were some areas that the membranous vesicles appear thin, smooth, without ribosomes and slightly disrupted, indicated by (b) and (c). Openings in the ER membrane, indicated by black arrows, could be seen in some of the vesicles. The nucleus (B), also showed disruptions, indicated by the black square and black oval, and congregation of the nuclear material was visible just within the limits of the inner nuclear membrane, indicated by the white arrows. Since the anti-apoptotic gene Bcl-2 is also known to be located in the ER, any damage to the ER may result in the inhibition of Bcl-2 and the stimulation of apoptosis (Yang *et al*., 1998). The rupture of the vesicles of the

ER may be a cause of ROS induced by hydrocortisone and will result in cellular swelling, culminating in cell death (Van Cruchten *et al*., 2002).

Figure 4.8 is another sample of neurons of 8-day-old chick embryos exposed to 0.137μM hydrocortisone, but in this case, was fixed with potassium permangenate. Figure 4.8 shows two cells (1) and (2). The nuclear membrane (A) of cell (1) and the nuclear membrane (B) of cell 2 are separated by a plasma membrane (C). It was impossible to distinguish the two plasma membranes of the adjacent cells, and only a vague single line could be seen, as indicated within the black square. At some locations (within the black ovals), breakages and discontinuation of the adjacent plasma membranes could be seen. Although the double nuclear membrane (A) of cell (1) was clearly visible, breakages within the membrane were also present, as indicated by the white arrows. The nuclear membrane (B) of cell 2 appeared to be more severely affected. The membrane bulged out at certain parts and even appeared to discontinue after the bulge (as indicated by black arrows). Lyses of the nucleus (karyorhexis) is a common structural change that occur during apoptosis (Majno *et al*., 1995). All neurons exposed to the 0.137μM hydrocortisone concentration appeared to have damage to plasma membranes, double nuclear membranes as well as mitochondrial membranes.

The effect of hydrocortisone exposure to 0.685μM at day 3.75 (Carnegie stage 16) and day 5.5 (Carnegie stage 18)) on chick embryo neural tissue at day 8 of development was then studied. The effect on the mitochondria, ER, nuclear and plasma membranes were evaluated. Severe damage due to this high concentration of hydrocortisone can be seen in Figure 4.9, Figure 4.10a and Figure 4.10b. All structures appear to be damaged beyond repair and cell death would seem the only fate for these neurons.

Figure 4.9 shows a neuron exposed to 0.685μM hydrocortisone and fixed with glutaraldehyde method 2. The nucleus (A) and mitochondria (B) appeared to have suffered severe damage. At some parts, the double membrane structure of the nucleus (a) and mitochondria (b) were clearly visible, however, as indicated by the black square, disruptions in the nuclear membrane were present. Some breakages, indicated by the white arrows, could also be seen. Only a few vague mitochondrial cristae (c) were visible. The mitochondrial membrane appeared to have pulled away from the structure (within black oval), leaving the contents of the mitochondria exposed (black arrows). This surely is accompanied by the release of cytochrome *c* from the mitochondria and the cell

will most definitely have undergone apoptosis (Van Cruchten *et al*., 2002) (Pretorius *et al*., 2005). Severe swelling and rupture of the mitochondrial membrane, however, does not only occur in apoptosis but also in necrosis. This cell death mechanism should therefore also be mentioned (Michea *et al*., 2002).

Potassium permangenate was used as a fixative for the embryonic tissue exposed to 0.685μM hydrocortisone in Figure 4.10a and Figure 4.10b. Figure 4.10a were taken at a relatively low magnification to illustrate the overall damage caused by this high concentration of hydrocortisone. Five nuclei of five neurons (1-5) could be seen. Huge breakages and discontinuation of the nuclear membranes were visible, as indicated by the black arrows. Where the membranes still appeared to be intact, no distinction could be made between the inner and outer membranes (white arrows). Whatever remained of the cells also appeared to have clumped next to the nuclear membranes. No plasma membranes could be distinguished and the tissue appeared to be in a horrific state. At a higher magnification, as indicated in Figure 4.10b, the nucleus (A) and cytoplasm (B) of a severely damaged neuron could be seen. The nuclear membrane was visible, although it appeared hazy and it was impossible to distinguish a double membrane structure, indicated within the black square. A great break in the membrane could be seen (black arrows), and the condensed nuclear material were visible as clumps within the cytoplasm (within black ovals). A thin, damaged plasma membrane could be seen as a single thin line, indicated by the white arrows. This severe damage to neuronal membranes, clumping and margination of chromatin are typical indications of apoptosis (Majno *et al*., 1995). Cell death in these samples is not only a possibility, but a probability.

4.5 CONCLUSION

Glutaraldehyde method 2 and potassium permangenate were two fixatives that caused little to no disruption to neural tissue, and with an added stabilizing chemical, such as $MgCl_2$ proved to be excellent fixatives. These two fixation methods were applied to control neural tissue as well as tissues exposed to 0.137μM and 0.685μM hydrocortisone. When evaluated by TEM the control tissue appeared to be in excellent condition and all structures were intact with no displacement. Exposure of neurons to 0.137μM hydrocortisone appeared to have severe effects on the morphology of neural tissue and disruptions could be detected in the mitochondria, ER, nuclear and plasma

membranes. More extensive damage was noted with 0.685μM hydrocortisone, leaving almost no cellular structure. Cell death mechanisms could be either apoptosis or necrosis, since evidence of both could be detected. Yang *et al*., (1998) found that some neurotoxins at an acute dosing regimen induce necrotic cell death, and at a more chronic dosing regimen apoptotic cell damage would be more likely. Here we hypothesize that a combined mechanism, aponecrosis is responsible for cell death. This hypothesis will be discussed in chapter 6.

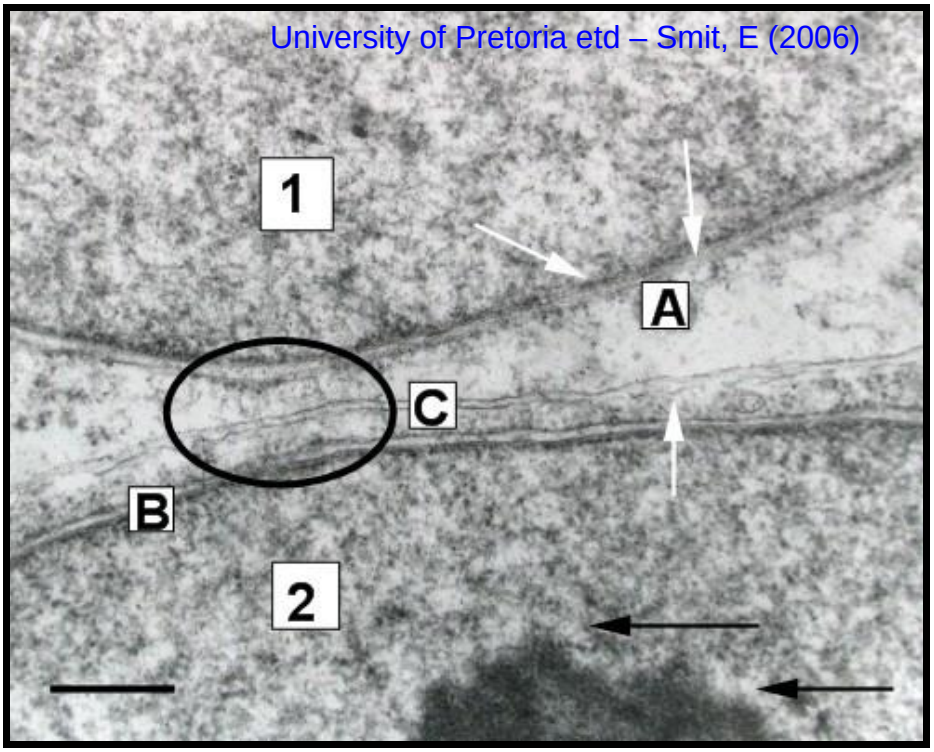


Figure 4.1 TEM micrograph showing two neurons (1) and (2), of a control 8-day-old chick embryo, fixed with glutaraldehyde method 1. The two nuclear membranes (A) and (B) are separated by plasma membrane (C). The nucleolus (black arrows) in cell (2) can be clearly distinguished. Clear membrane structures are visible in some areas of the micrograph (within black oval), although poor visual quality of membranes were noted in other regions (indicated by white arrows). (Bar = 0.5μm)

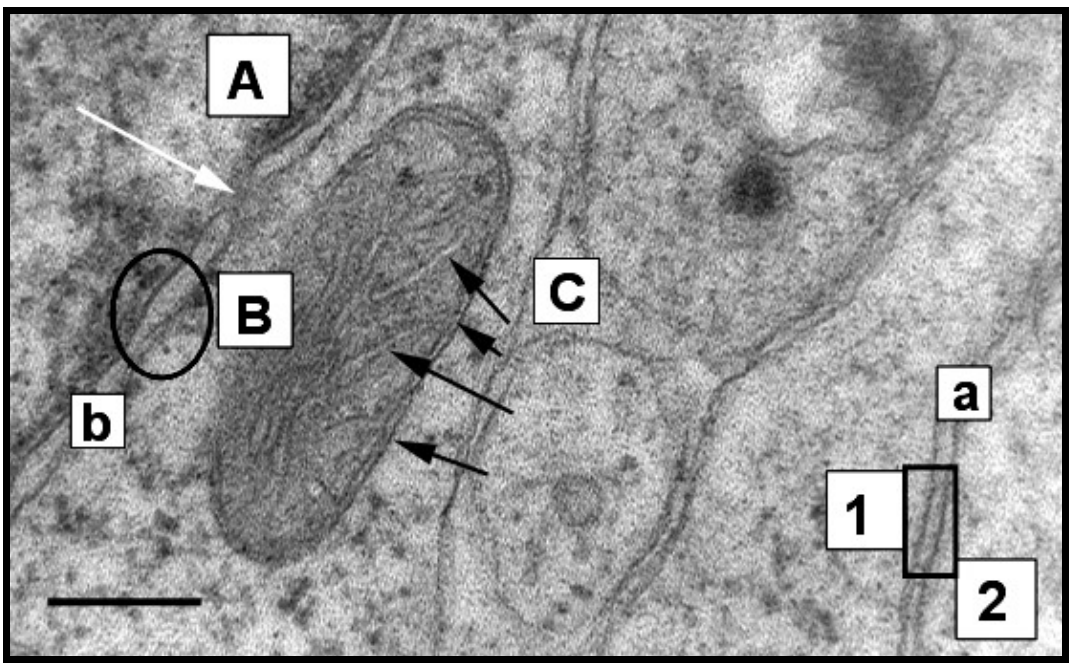


Figure 4.2 TEM micrograph of neurons of an 8-day-old chick embryo, indicating an improvement on the glutaraldehyde method, namely glutaraldehyde method 2 with added $MgCl_2$. Two neurons (1) and (2) are separated by a plasma membrane (a). In cell (1) various structures can be identified; a nucleus (A), mitochondria (B) with cristae (black arrows), axon (C) and a nuclear membrane (b) with a nuclear pore (white arrow). Clear distinctions can be made between the double membrane structures of both the nuclear membrane and plasma membrane as indicated within the black oval and black rectangle respectively. (Bar = 0.25μm)

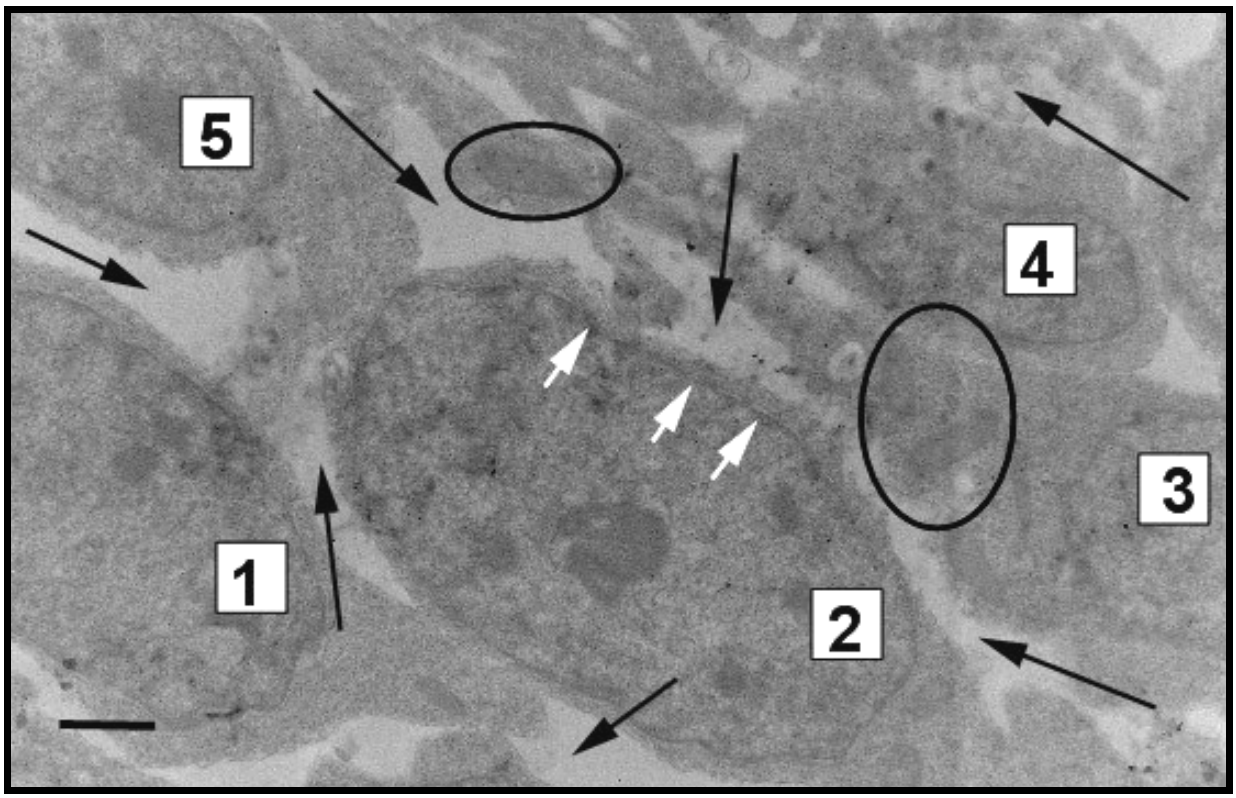


Figure 4.3 TEM micrograph showing five neurons (1-5) of an 8-day-old chick embryo fixed with paraformaldehyde-glutaraldehyde. This fixative caused severe shrinkage of the tissue, as indicated by the black arrows. The double nuclear membrane of cell 2, indicated by white arrows, was clearly visible and unaffected, whilst other organelles, such as the mitochondria (in black ovals) were not easily identifiable. (Bar = 1μm)

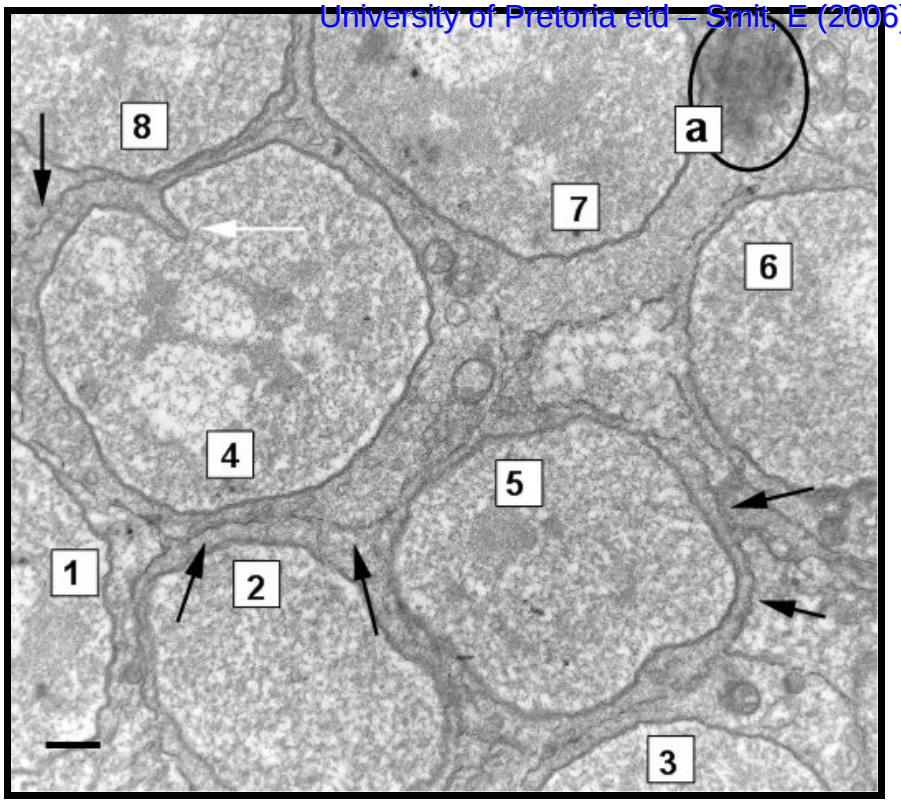


Figure 4.4a TEM micrograph showing nuclei of eight neurons (1-8) of a control 8-day-old chick embryo fixed with potassium permangenate. All membraneous structures were excellently preserved (black arrows). An intact ER (black oval) can be seen next to nucleus 7 with a connection to the nuclear envelope (a). An invagination, typical to neuron nuclei could be seen in nucleus 4 (white arrow). (Bar = 1μm)

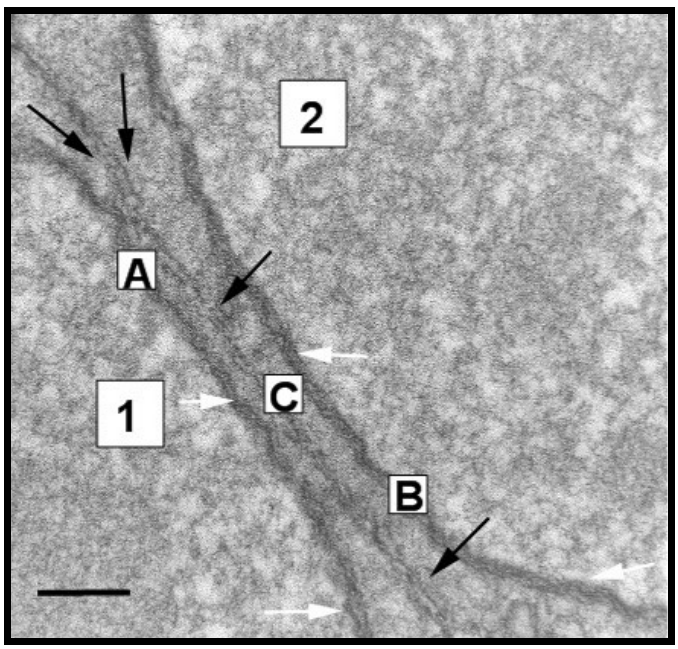


Figure 4.4b TEM micrograph with two neurons (1) and (2) of a control 8-day-old chick embryo fixed with potassium permangenate. Two nuclear membranes (A) and (B) with plasma membrane (C) are clearly visible. Double membrane structures of both the plama membrane and nuclear membranes could be distinguished, as indicated by the black and white arrows respectively. (Bar = 0.5μm)

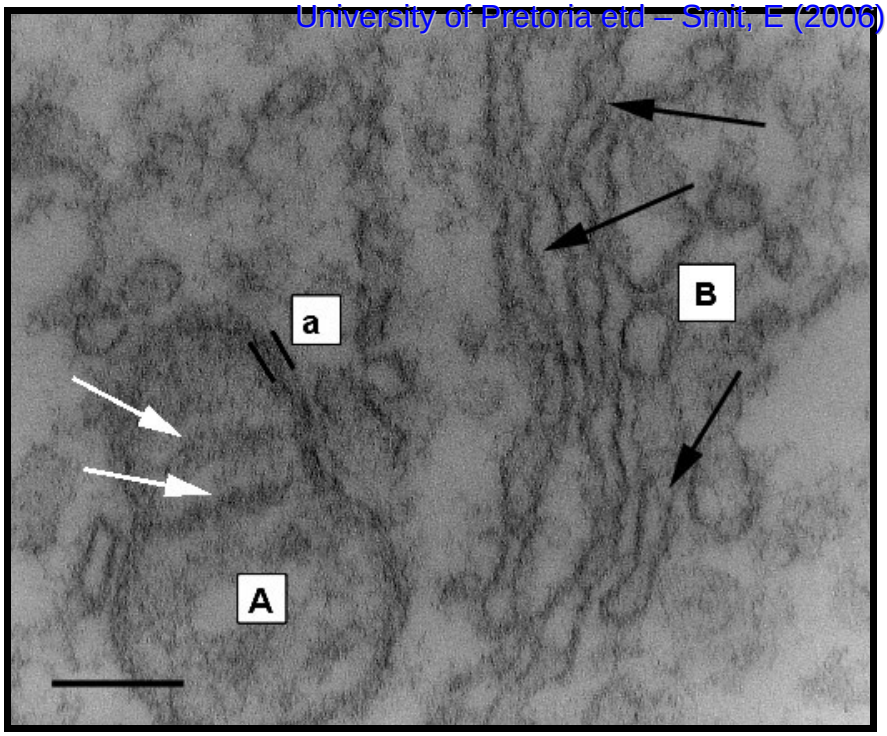


Figure 4.5a TEM micrograph of control 8-day-old chick embryo neural tissue fixed in potassium permangenate. The mitochondria (A) and ER (B) appears as intact, undisrupted organelles. The cristae of the mitochondria (white arrows) and double membrane (a) with inner and outer surfaces (black parallels) could be clearly distinguished. The cisternae of the ER (black arrows) all appeared to be intact. (Bar = 0.25μm)

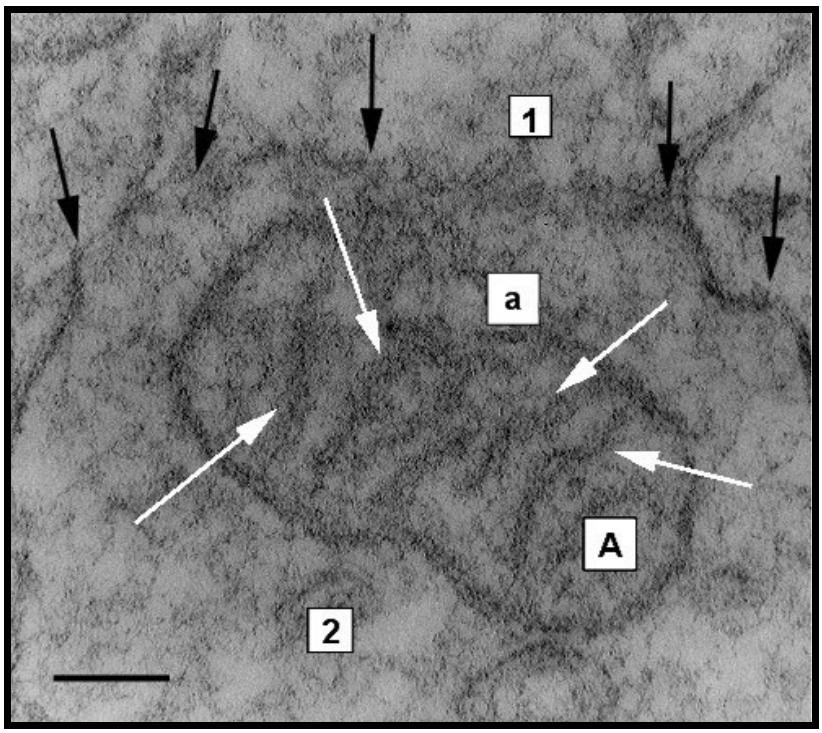


Figure 4.5b TEM micrograph at a higher magnification, showing a mitochondria (A) with clear cristae (white arrows) and double membrane (a) of control 8-day-old chick embryo neural tissue also fixed in potassium permangenate. Two neurons (1) and (2) can be distinguished, separated by a plasma membrane (black arrows). (Bar = 0.25μm)

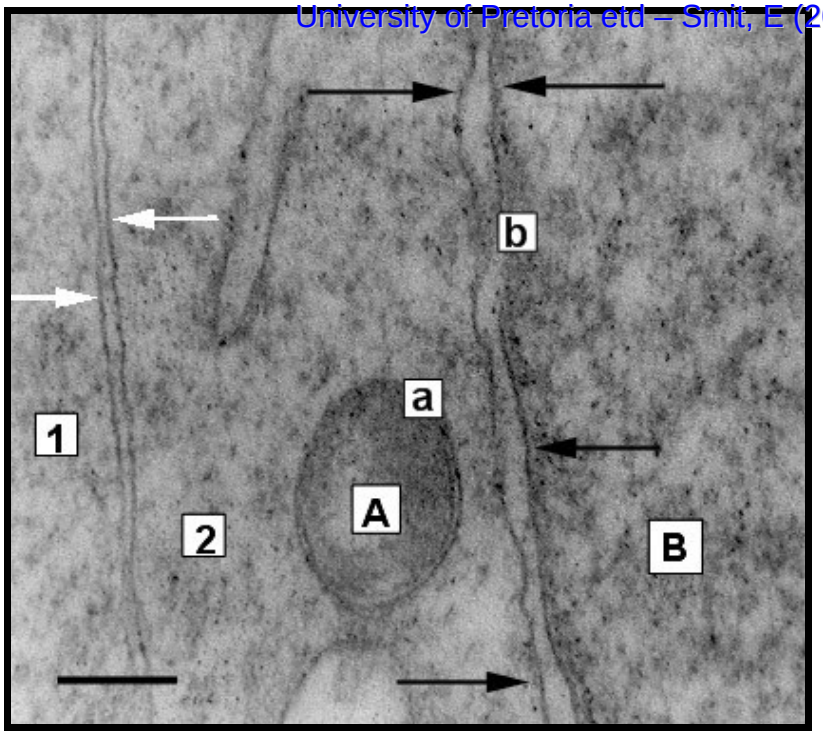


Figure 4.6a TEM micrograph of control neural tissue of an 8-day-old chick embryo fixed in glutaraldehyde method 2. Two neurons (1) and (2) can be seen, separated by a clear plasma membrane (white arrows). The nucleus (B) in neuron 2 had a smooth undisrupted appearance (black arrows) with a nuclear pore (b). A mitochondria (A) with its membrane (a) could be easily distinguished. (Bar = 0.25μm)

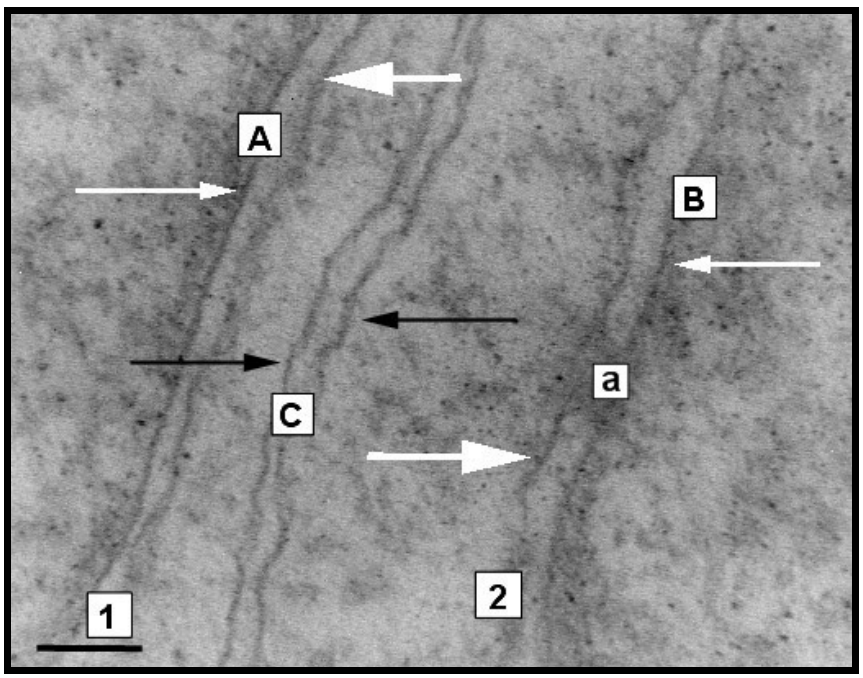


Figure 4.6b Very high magnification TEM micrograph, showing two neurons (1) and (2) of control 8-day-old chick embryo neural tissue, fixed with glutaraldehyde method 2. Two nuclear membranes (A) and (B) with nuclear pore (a) can be detected. Both the nuclear membranes and the plasma membrane (C) had a smooth undisrupted appearance (white and black arrows). (Bar = 0.125μm)

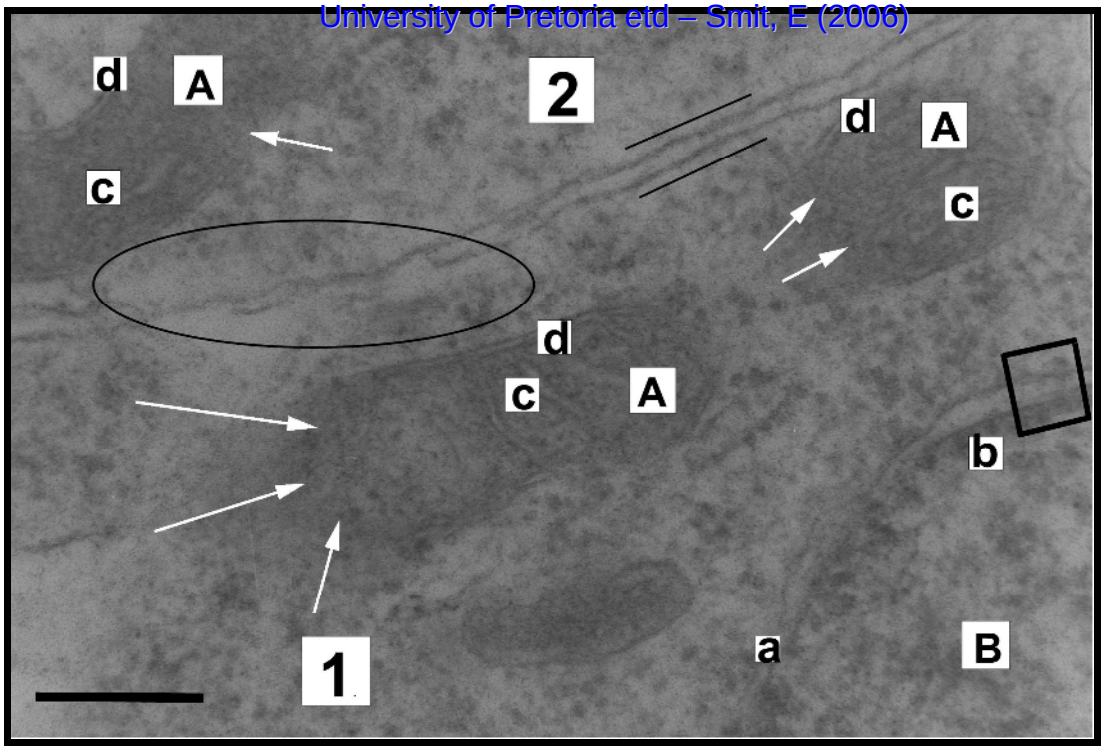


Figure 4.7a TEM micrograph of 8-day-old chick embryo neural tissue exposed to 0.137μM hydrocortisone and fixed in glutaraldehyde method 2. Two neurons (1) and (2) can be distinguished separated by a plasma membrane (between two parallel lines), that appeared to have suffered severe damage (black oval). A nucleus (B) with a nuclear pore (a) and disrupted nuclear membrane (black square) could be identified. Chromatin condensation at the inner membrane of the nuclear envelope was also noted (b).Various mitochondria (A) could be seen with cristae (c) and double membranes (d). Disruption in the mitochondrial structure were observed throughout the micrograph (white arrows). (Bar = 0.25μm)

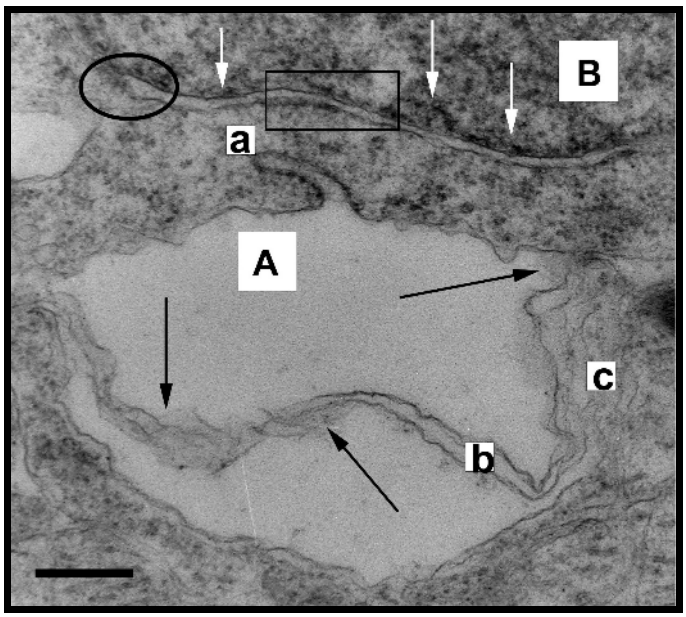


Figure 4.7b TEM micrograph illustrating an ER (A) in neural tissue of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone and fixed in glutaraldehyde method 2. At some areas ribosomes (a) could be seen still attached to the ER, while at other areas the vesicles appeared thin and smooth (b) and (c). Disruptions to the vesicles could also be seen (black arrows). The nucleus (B) with disrupted membranes (black oval and square) and chromatin condensation (white arrows) were also noted. (Bar = 0.25μm)

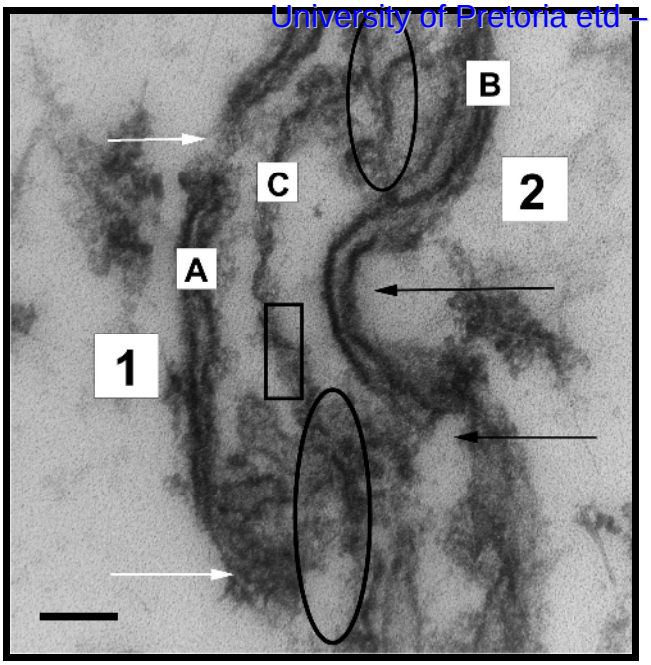


Figure 4.8 TEM micrograph showing two nuclear membranes (A) and (B) of two neurons (1) and (2) of an 8-day-old chick embryo exposed to 0.137μM hydrocortisone and fixed in potassium permangenate. No distinction could be made between the two plasma membranes (C) of the adjacent cells, and only a vague single line could be seen (black square). At some locations (black ovals), breakages and discontinuation of the plasma membranes could be seen. Breakages within the nuclear membranes were also present (white arrows). Nuclear membrane B, bulged out at certain parts and even appeared to discontinue after the bulge (black arrows). (Bar = 0.25μm)

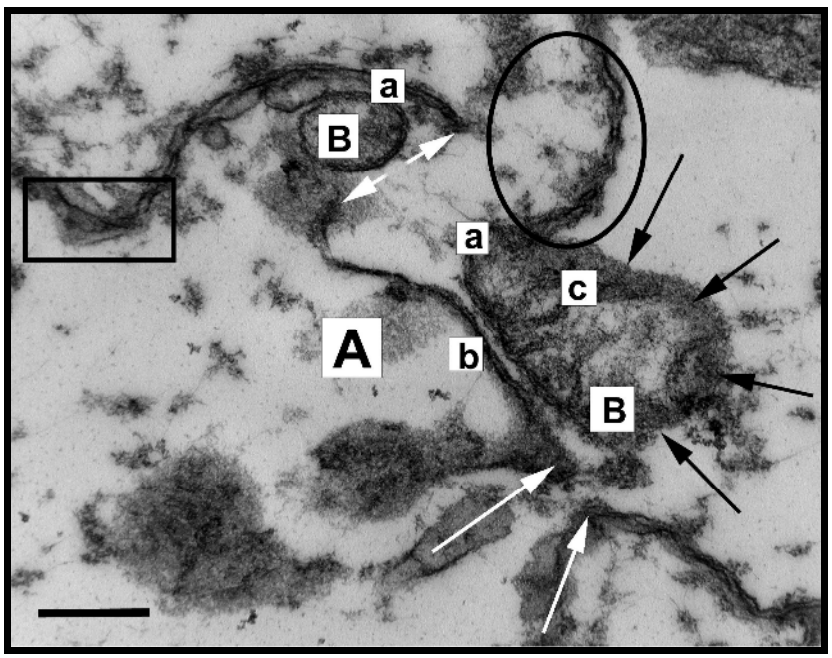


Figure 4.9 TEM micrograph of 8-day-old chick embryo neural tissue exposed to 0.685μM hydrocortisone and fixed with glutaraldehyde method 2. The nucleus (A) and mitochondria (B) appeared to have suffered severe damage. In some areas, the double membrane structure of the nucleus (a) and mitochondria (b) were clearly visible, however, disruptions in the nuclear membrane were present (black square) with breakages in the membrane (white arrows). Only a few vague mitochondrial cristae (c) were visible. The mitochondrial membrane appeared to have pulled away from the structure (black oval), leaving the contents exposed (black arrows). (Bar = 0.5μm)

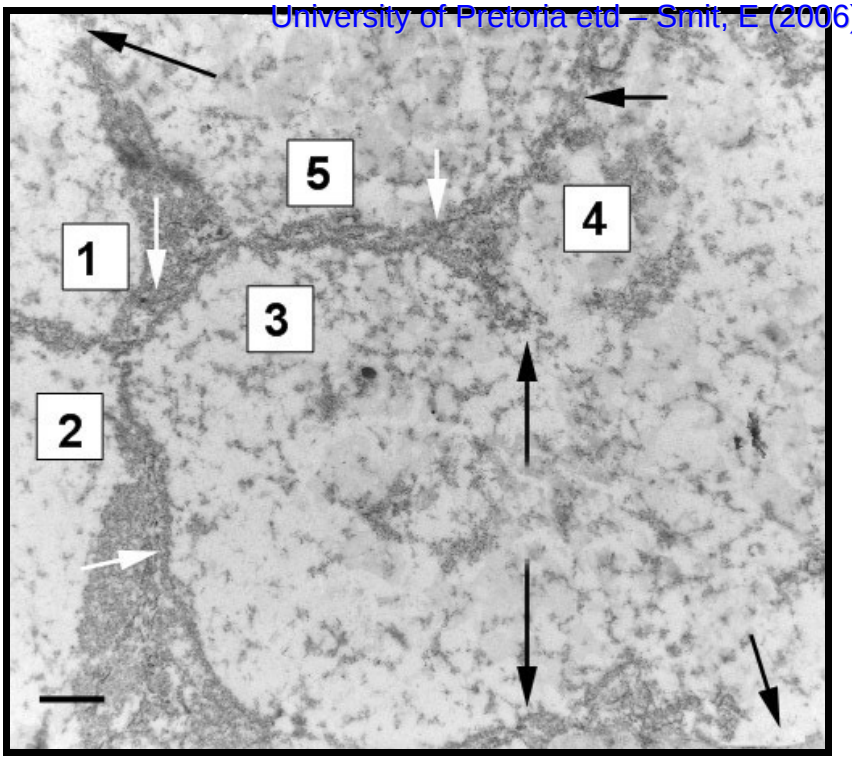


Figure 4.10a TEM micrograph of five neurons (1-5) of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone and fixed with potassium permangenate. Severe damage to nuclear membranes (black arrows) can be detected. Where the membranes still appeared to be intact, no distinction could be made between the inner and outer membranes (white arrows). (Bar = 1μm)

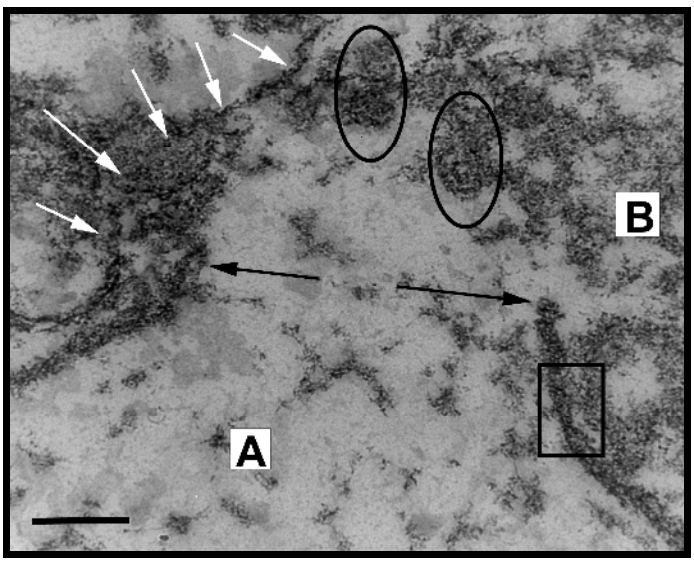


Figure 4.10b High magnification TEM micrograph showing the nucleus (A) and cytoplasm (B) of a severely damaged neuron of an 8-day-old chick embryo exposed to 0.685μM hydrocortisone and fixed with potassium permangenate. The nuclear membrane with indistinguishable double membrane structure (black square) and great breakages (black arrows) could also be seen. Condensed nuclear material were visible as clumps within the cytoplasm (black ovals). A thin, damaged plasma membrane could be seen as a single thin line (white arrows).(Bar = 0.5μm)

Chapter 5 : Fluorescence Microscopy of primary chick embryo neuronal cultures exposed to hydrocortisone to determine cell viability and ROS generation

5.1 INTRODUCTION

Determination of cellular viability is important in neurotoxic research, where neurotoxic agents cause mitochondrial dysfunction, often resulting in a loss of cell viability and ultimately neurodegeneration (Bell *et al*., 1988) (Moreira *et al*., 2003). Primary neuronal cultures are experimental models that closely reflect the function of neurons in living organisms and therefore primary neuronal cultures are considered to be the best experimental models for the evaluation of the neurotoxicity of a drug (Los *et al*., 2001). Most of the cell viability assays are based on the premise that cell death is an unequivocal indication of toxicity (Kemp, 1992). Decreased cell viability is often associated with morphological changes, changes in membrane permeability and/or physiological state as deducted from the exclusion of certain dyes or the uptake and retention of others (Coder, 1997). Examples of such dyes are; propidium iodide (PI), acridine orange (AO) and fluorescein diacetate (FDA).

Glucocorticoids can induce apoptosis and necrosis through activation of p53, Fas or by ROS generation. Several different methods can be used to detect ROS production in neuronal cells. Boldyrev *et al*., (2004) utilized 2'7'-diclorodihydrofluorescein diacetate (DCH_2FDA) for the detection of hydrogen peroxide (H_2O_2) in neuronal cells when evaluating compounds such as carnosine, that might protect neurons from degeneration by ROS. H_2O_2 induced apoptosis was investigated, using the fluorescent probe, DCH_2FDA by Kazey *et al*., (2003), Koudinov *et al*., (2004) and Popescu *et al*., (2002).

In this study it has been shown that in the *in vivo* chick model, hydrocortisone causes a decrease in neuron density, altered neuron morphology and increased neuron death. *In vitro* studies in primary neuron culture can be used to investigate the effect of hydrocortisone on cell viability in greater detail. In this chapter, the effect of hydrocortisone on cell viability will be determined using the combined FDA and PI assay. The mechanism whereby cell death occurs will be studied using DCH_2FDA, a specific

indicator of ROS production. Cell morphology will be investigated by fluorescence microscopy to determine whether increased ROS production leads to necrosis or apoptosis.

In this chapter the following research objectives were investigated:

- To establish primary cultures from chick embryonic neural tissue that will be suitable for fluorescence microscopy studies.

- To determine the effect of increasing concentrations of hydrocortisone (0, 26.3nM, 0.16μM, 0.63μM, 3.8μM, and 22.8μM) on cell viability by utilizing the FDA, PI double fluorescence staining method.

- Furthermore to use primary neuron cultures to determine the effect of hydrocortisone on ROS production and to determine the mechanism of cell death using the fluorescent probe DCH_2FDA.

5.2 MATERIALS

5.2.1 Plasticware and other disposable items

The 5ml and 10ml pipettes, the 15ml and 50ml centrifuge tubes, the 75cm^2 cell culture flask and the 24 well plates were all from NUNC^{TM} supplied by AEC - Amersham, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters (0.22μm) were from National Separations, Johannesburg, South Africa.

5.2.2 Media, supplements and reagents

Hanks Balanced Salt Solution (HBSS), Dulbecco's Modified Eagles Medium (DMEM) and Foetal Calf Serum (FCS) were purchased from Highveld Biological Company, Johannesburg, South Africa. All antibiotics (Streptomycin sulphate, penicillin G and Amphotericim B) and Trypsin were from Life Technologies Laboratory supplied by Gibco BRL Products, Johannesburg, South Africa.

Bovine Serum Albumin (BSA), Poly-L-lysine, fluorescein diacetate (FDA), propidium iodide (PI) and 2'7'-diclorodihydrofluorescein diacetate (DCH_2FDA) were all obtained from Sigma-Aldrich, Johannesburg South Africa.

Acetone, Ethylene diamine tetra acetate (EDTA), sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium hydrogen carbonate ($NaHCO_3$) and Trypan blue were from Merck, Johannesburg, South Africa.

All glassware was sterilized at –140^{0}C in a Prestige Medical Autoclave (Series 2100). The HBSS/BSA and DMEM/FCS media was sterilized by filtration through 0.22µm Sartorius cellulose acetate membrane filters under aseptic conditions in a laminar flow cabinet. The number of cells were determined by a haemocytometer from Brand supplied by Merck, Johannesburg, South Africa. All primary cultures were maintained at 37°C and 5% CO_2 in a CO_2 -water-jacketed incubator from Forma Scientific.

5.3 METHODS

5.3.1 Cultivation and maintenance of neurons in primary cultures

The incubated eggs were removed on day 7 (Carnegie stage 21) and placed in a laminar flow hood. All procedures were done using aseptic technique. The shells were thoroughly wiped with 70% ethanol, the shell of each egg was cracked, opened and the shell above the air sac was peeled off. The chorioallantoic membrane was opened and the embryo removed with a spoon spatula. The embryo was immediately placed in a sterile petri-dish containing HBSS and BSA. HBSS was prepared by dissolving 9.86g/L of the balanced salt solution with 0.35g/L $NaHCO_3$ in ddH_2O. BSA at a concentration of 3g/L, and 2% antibiotic solution was added to 1L HBSS/BSA. The antibiotic stock solution was prepared by dissolving 10mg/ml Penicillin G (sodium salt), 6mg/ml Streptomycin sulphate and 25µg/ml Amphotericim B in 0.85% saline. The antibiotic solution was kept at –10^{o}C and thawed when needed. The solutions were filtered through a 0.22µm membrane filter under aseptic conditions. Aliquots of 100ml HBSS/BSA with antibiotics were prepared, stored at 4^{o}C and warmed to 37^{o}C before use.

The embryo was decapitated and the midbrain area was removed. The tissue fragments were transferred to a clean sterile petri-dish, containing HBSS/BSA, and were cut into small fragments, using scalpels. The HBSS/BSA containing finely chopped tissue fragments was then transferred by pipette to a sterile 50ml test tube and the tissue fragments were allowed to settle. The tissue was washed again by resuspension in HBSS/BSA, allowing the tissue to settle, and the supernatant was removed. This step was repeated twice.

Most of the supernatant was removed after the third wash, and 10ml of a 0.025% trypsin solution added. The trypsin was prepared as a 10x Trypsin/EDTA (5g/L Trypsin, 2g/L EDTA.Na_4) stock solution, by mixing 0.25g Trypsin, 0.1g EDTA and 0.425g NaCl in 50ml HBSS/BSA. The solution was stored at -10^{o}C and warmed up at 37^{0}C before use. The tube containing the cells with the trypsin solution was placed in the CO_2 incubator at 37ºC for 20 minutes.

After incubation, the trypsin solution was removed and DMEM medium containing 10% FCS and 2% antibiotics was added to inhibit enzyme activity. DMEM medium was prepared by dissolving 13.53g/L of the medium powder with 3.7g/L $NaHCO_3$ in ddH_2O, and was supplemented with 10% FCS and 2% antibiotics. The DMEM/FCS with antibiotics were filtered through a 0.22μm membrane filter under aseptic conditions. Aliquots of 100ml DMEM/FCS with antibiotics were prepared and the medium was stored at 4^{o}C and warmed to 37^{o}C before use.

The isolated cells were allowed to settle, the supernantant was removed and the cells were resuspended in DMEM/FCS medium. The washing step with DMEM/FCS medium was repeated twice. Finally, single cell suspensions were prepared by mechanical trituration, this was accomplished by pipetting the suspension several times through a 5ml pipette until it would appear as if the cells were evenly dispersed. The suspension was then left for 1-2 minutes to allow any large fragments of cells to settle to the bottom of the test tube. The supernatant was removed, transferred to a 75cm^2 cell culture flask, and an additional 10ml DMEM/FCS medium was added, and was placed in the incubator at 37°C for 20-30 minutes, to allow the attachment of fibroblasts and non-neuronal cells.

A 0.01% solution of poly-L-lysine was prepared, 500µl was added to each well of a 24-well plate and left for 30 minutes. Poly-L-lysine growth matrix contains many amide groups, which promote cell adhesion. The poly-L-lysine solution was removed and the wells were washed with DMEM medium. The plates were left to dry in the laminar flow hood at room temperature.

After this incubation period, the flask was removed from the incubator and the unattached cells were transferred to a sterile tube. The cell number was determined by the Trypan Blue (0.4% Trypan Blue solution in dH_20) assay with a haemocytomemter.

The cells were plated at a concentration of $20x10^4$ cells per milliliter in the poly-L-lysine coated 24-well plates with a culture area of $1.9cm^2$/well in a final volume of 500µl. The cells were maintained at 37ºC and 5% CO_2 content for 48 hrs to allow optimal dendrite and axon development. The medium was not changed, as this causes dendrite and axon detachment.

5.3.2 Exposure of primary neuron cultures to hydrocortisone

Primary neuron cultures were exposed after 24 hours, when well-established neuronal networks were observed by phase contrast microscopy. The primary neuron cultures were exposed to hydrocortisone (SOLU-CORTEF™ 100mg Act-O-Vial consist of a two compartment vial containing per 2 ml when mixed, hydrocortisone sodium succinate equivalent to 100 mg hydrocortisone, 0.9% benzyl alcohol and water for injection, final concentration 50mg/ml, 0.138M) at concentrations of 0M (control), 26.3nM , 0.16µM, 0.63µM, 3.8µM and 22.8µM hydrocortisone. The exposed cells were again placed in the incubator at 37ºC and 5% CO_2 for another 24 hours before esterase activity and cell viability were determined using FDA and PI.

To determine the presence of ROS, cells were kept unexposed in the incubator at 37ºC and 5% CO_2 for 48 hours. The cells were only exposed (at above mentioned concentrations), 30 minutes before the fluorescent stain DCH_2FDA was added to indicate ROS formation.

5.3.3 Fluorescein Diacetate (FDA)

FDA is a fluorescent probe that easily diffuses across the plasma membrane and once inside the cells it is cleaved by non-specific esterases into fluorescein, which exhibits a green fluorescence when viewed by UV light (Prudêncio *et al*., 2002). The medium was removed from each well, and the attached neurons were washed with DPBS. A 10x DPBS stock solution was prepared by dissolving 2g/L KCl, 2g/L KH_2PO_4, 80g/L Na_2HPO_4 and 80g/L NaCl in 1L ddH_2O.

A volume of 200µl DPBS (1:10 dilution) was then added to each well, together with 10µl of FDA (0.0084g FDA powder in 1.5 ml acetone). After 5 minutes in the dark, the FDA/DPBS solution was removed and DPBS was added to each well (just enough to cover the bottom of each well). The neurons were then evaluated by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss Werke, Göttingen, Germany). Photographs were taken by a digital camera attached to the microscope (Zeiss Axiocam MRc5, Carl Zeiss Werke, Göttingen, Germany) and evaluated to investigate the effect of hydrocortisone on the esterase activity, and thus the viability of the cells.

5.3.4 Propidium Iodide (PI)

PI diffuses across plasma membranes that have lost their integrity, enters the cell and binds DNA. It is therefore an indicator of non-viable cells, when it exhibits a red fluorescence (Verhaegen *et al*., 1998). The exact same procedure were followed as described in section 5.3.4, except that instead of FDA, 5µl PI (1mg PI in 1ml dd H_2O) in 200µl DPBS was used.

Both FDA and PI fluorescence were evaluated simultaneously. For every well the number of dead and alive cells were evaluated. Experiments were repeated six times with different batches of cells. The results of one representative experiment are illustrated.

5.3.5 Diclorodihydrofluorescein diacetate (DCH_2FDA)

DCH_2FDA is a fluorescent probe that detects the formation of ROS, specifically the ROS, H_2O_2. This probe can easily enter the cell, where it is also cleaved by non-specific esterase into diclorodihydrofluorescein (DCFH). DCFH is oxidised by H_2O_2 to form

dichlorofluorescein (DCF), which is fluorescent and the cell exhibits a green fluorescence (Hipler *et al*., 2002).

After 30 minutes of exposure to hydrocortisone, the medium from each well was removed, and the attached neurons were washed with DPBS. A volume of 200μl DPBS was then added to each well, together with 10μl DCH_2FDA (5μg DCH_2FDA in 1ml ddH_2O). After 7 minutes in the dark, the solution was removed and DPBS was added to each well (just enough to cover the bottom of each well). The neurons were then evaluated by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss Werke, Göttingen, Germany). Photographs were taken by a digital camera attached to the microscope (Zeiss Axiocam MRc5, Carl Zeiss Werke, Göttingen, Germany) and evaluated to illustrate the areas where ROS generation occurred.

All experiments were repeated six times, although only one representative experiment is illustrated.

5.3.6 Statistical Analysis

The percentage viability (cytotoxicity) that hydrocortisone may have on primary neuronal cultures can be determined as the number of FDA positive cells divided by the total number of the cells. The total number of cells were calculated by adding the number of green fluorescent cells (FDA) and red fluorescent cells (PI) at each hydrocortisone concentration, using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu). The percentage viability was then expressed at each concentration by making use of a column graph.

5.4 RESULTS AND DISCUSSION

The effects of hydrocortisone after 0M (control), 26.3nM , 0.16μM, 0.63μM, 3.8μM and 22.8μM exposure on neuronal cell viability and ROS production was determined using the combined FDA /PI and DCH_2FDA fluorescent dyes respectively.

5.4.1 Evaluation of neuronal cell viability after hydrocortisone exposure using the fluorescent probes, fluorescein diacetate (FDA) and propidium iodide (PI)

FDA is a rapid and sensitive method when evaluating the number of living cells and can therefore be used to quantify toxic effects (Didier *et al*., 1990). FDA is a non-polar compound, which readily diffuses into the viable cells (Kemp, 1992). It is metabolized by esterases resulting in the formation of the negatively charged and highly polar fluorescein that accumulates in viable cells (Nikolova *et al*., 2002) (Coder, 1997) (Kemp, 1992). Cellular toxicity causes increased permeability of the cell membrane and increased diffusion of fluorescein from the cell and subsequently a decrease in fluorescence associated with loss in cell viability (Nikolova *et al*., 2002). The cell membrane of viable cells is impermeable to PI. However, if the cell membrane is damaged, PI can cross the cell membrane and once inside the cell, PI enters the nucleus and binds DNA to exhibit a red fluorescence, indicating dead cells (Spahr-Schopfer *et al*., 2000) (Chin *et al*., 2001). PI and FDA are usually used in combination to evaluate cytotoxicity. In combination this is a rapid, convenient and reliable method to determine the number of viable and dead cells in a specific cell population. (Jones *et al*., 1985). Ktsube *et al*., (1999) utilized FDA and PI to evaluate cell viability in neurons after exposure to ONO-1603, an anti-dementia drug. Vaudry *et al*., (2000) investigated the effect of pituitary adenylate cyclase-activating polypeptide on caspase activity in cultured cerebellar granule cells also by using FDA and PI. These assays were then also used by Jordán *et al*., (2003) to study the role of p53 in neuronal death.

Cell viability was determined using the FDA/PI combined bioassay following exposure of neurons to 0M (control), 26.3nM , 0.16μM, 0.63μM, 3.8μM and 22.8μM hydrocortisone. Neurons not exposed to hydrocortisone showed cell bodies with axons and dendritic processes, and several synaptic connections could be seen between cells. (Figure 5.1 - 5.2). Phase contrast microscopy was used to evaluate cell morphology (Figure 5.3a-5.8a and 5.9), fluorescence microscopy was used to determine the number of viable (green) cells (Figure 5.3b-5.7b) and dead (red) cells (Figure 5.3c-5.7c and 5.8b). A computer generated overlay provided information regarding the number of viable relative to dead cells (Figure 5.3d-5.7d). In the control (not exposed to hydrocortisone) several dead cells were observed and this was due to stress experienced during the staining procedure (Figure 5.3c). With increasing concentrations an increase in the number of dead cells were observed with no FDA staining and strong PI staining at 22.8μM. (Figure 5.8b)

Microscopy is useful in evaluating morphology, however, the quantification of the viability requires the use of a flourometric bioassay. The number of stained cells in each micrograph can also be counted, although this can be very time-consuming. Using the Image Tool program the total number of red and green stained cells was determined and the percentage viability was then calculated as the number of FDA positive cells (green staining) divided by the total number of the cells counted and these results are presented in Figure 5.10.

For the control cellular viability was 90%, for neurons exposed to 26.3nM and 0.16μM hydrocortisone, viability was at 88.2% and 86.4% respectively with no significant decrease in cell viability compared to the control. At 0.63μM, 3.8μM and 22.8μM neuron viability was reduced to 80%, 75% and 0% respectively, which were significantly reduced when compared to the control.

The effects of glucocorticoids appear to be contradictive where some researchers have shown protective and mitogenic effects (Chang *et al*., 2002) (Basu *et al*., 1978). Other researchers, such as Longui *et al*., (2005) have shown that glucocorticoids reduce cellular proliferation and induce apoptosis in *in vivo* and *in vitro* systems. The conclusion drawn by these researchers was that glucocorticoids are cytotoxic at high concentrations. It was also found that elevated glucocorticoid levels in the brain endangered neuronal survival and that hydrocortisone exposure caused an inhibitory effect on cellular proliferation in granulocyte and monocyte colonies (Patel *et al*., 2002) (Van Merris *et al*., 2004). Schmidt *et al.*, (1987) and Chang *et al*., (2002) then also both stated that the cytotoxicity of hydrocortisone depends on the dosage and the experimental system studied.

Many studies have shown that at low concentrations, hydrocortisone has a mitogenic effect and is even used in some cases as a supplement in cell culture (Basu *et al*., 1978) (Briand *et al*., 1987). Low concentrations of glucocorticoids as an additive in the culture medium causes changes in protein synthesis and an increase in enzyme activity, such as the enzyme tyrosine-3-monooxygenase (Quarto *et al*., 1992) (Tischler *et al*., 1983). At high concentrations, however, this glucocorticoid has indicated a toxic effect (Asanuma et al., 2001) (Longui *et al*., 2005).

5.4.2 The effect of hydrocortisone on reactive oxygen species (ROS) generation in primary cultures of neuronal cells using the fluorescent probe, 2'7'-dichlorodihydrofluorescein diacetate (DCH_2FDA)

Ito *et al.*, (1996) stated that glucocorticoids were proven to be cytotoxic through the induction of apoptosis. The induction of apoptosis can occur via either the intrinsic or extrinsic pathway. Glucocorticoids can induce apoptosis and necrosis through activation of p53, Fas or by ROS generation. As described in chapter 2 (Diagram 2.1), glucocorticoids induce the phosphorylation of p53, and thereby cause it to become stable and active, leading to increased transcription of Bax and activation of the rest of the apoptotic cascade (Lowe *et al*., 2000). Glucocorticoids can also activate the Fas receptor with downstream activation of Bid, also resulting in apoptosis (Konopleva *et al*., 1999). Glucocorticoids are also known to generate ROS. ROS can either cause activation of p53 culminating in apoptosis, or cause severe damage to the ER disturbing calcium homeostasis resulting in necrosis (Jordán *et al*., 2003) (Van Cruchten *et al*., 2002).

In this study, DCH_2FDA was used to determine whether ROS occurred and cell morphology was evaluated to identify characteristics associated with apoptosis, such as apoptotic body formation.

Iuchi *et al*., (2003) found that excess glucocorticoids caused the overproduction of ROS in vascular endothelial cells. Long-term exposure of neurons to glucocorticoids results in MPT integrity being compromised causing a decrease in ATP production, a subsequent increase in calcium ion leakage and mitochondrial ROS production (Patel *et al*., 2002). All cellular components are susceptible to the action of ROS, though membranes are the most affected due to lipid peroxidation, which causes changes in cell membrane structure and permeability. Consequently there is a loss in the selectivity of lysosomal enzymes and the formation of cytotoxic products that ultimately culminates in cell death (Cerqueira *et al*., 2005). The generation of ROS *in vitro* can be assessed by using the fluorescent probe DCH_2FDA (Biswal *et al*., 2000). DCH_2FDA specifically measures the intracellular ROS, H_2O_2 (Lee *et al*., 2002).

DCH_2FDA is a nonpolar, nonfluorescent probe that easily crosses the cell membrane due to two esterified acetate functional groups (Chini *et al*., 1997). On entering the cell,

the diacetate group is cleaved enzymatically by esterases to form polar, dichlordihydrofluorescein that accumulates intracellularly (Scivittaro *et al*., 2000) (Kim *et al*., 2000). In the presence of the ROS, H_2O_2, dichlordihydrofluorescein is oxidized to the highly fluorescent 2'7'-dichlorofluoresein. Consequently the fluorescent signal produced by 2'7'-dichlorofluoresein is an index of oxidative stress in the biological system (Grishko *et al*., 2001).

To answer the last research question, namely: The effect of hydrocortisone on ROS generation in primary neuronal cultures by making use of the fluorescent probe DCH_2FDA, primary chick embryo neuronal cultures were exposed to increasing concentrations of hydrocortisone.

Neurons were also exposed to 0M (control), 26.3nM, 0.16μM, 0.63μM, 3.8μM and 22.8μM hydrocortisone and the effect of hydrocortisone was evaluated by phase contrast and fluorescence microscopy with DCH_2FDA staining. No DCH_2FDA fluorescence and therefore no ROS generation could be observed in the control sample as well as neurons exposed to 26.3nM, 0.16μM and 0.63μM hydrocortisone (Figure 5.11 and 5.12, *only control and 26.3nM shown*). Staining was observed for neurons exposed to 3.8μM and 22.8μM hydrocortisone. (Figure 5.13 and 5.14) At 3.8μM and 22.8μM, the majority of neurons had a rounded appearance with clumping, rather than the typical morphology associated with axon- or dendrite-like extensions, and strong green fluorescence associated with ROS generation was seen. (Figure 5.13 and 5.14) At close investigation it was found that green stained cells formed blebs that are morphological characteristics associated with apoptosis (Figure 5.15 and 5.16, white arrows). Not all neurons had this typical morphology and this is probably due to cell death being a function of necrosis. As mentioned already, ROS has been associated with both apoptotic and necrotic death, and can activate these pathways either by phosphorylation of p53, severe damage to the ER or loss of MPT integrity (Jordán *et al*., 2003) (Van Cruchten *et al*., 2002) (Patel *et al*., 2002). These results therefore clearly indicate that cell death (apoptotic, necrotic or both) did occur and is probably due to ROS generation.

5.5 CONCLUSION

In conclusion, in *in vitro* studies using primary cultures of chick neurons it is shown that hydrocortisone is non-toxic at low concentrations (26.3nM – 3.8μM) but is toxic at high concentrations (22.8μM), when cell viability is determined using the combined PI/FDA assay. This process occurs due to ROS production which leads either to cellular apoptosis or necrosis.

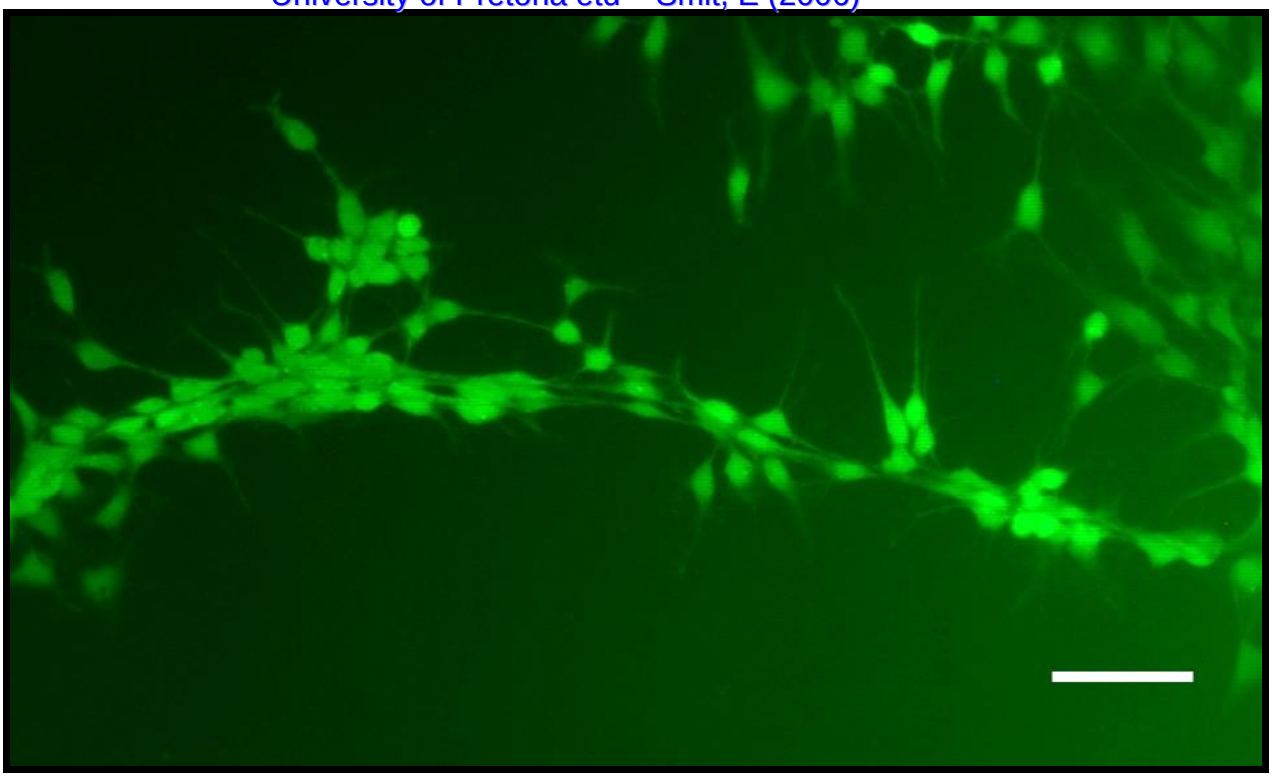


Figure 5.1 FDA staining of primary neuronal culture in the absence of hydrocortisone to illustrate neuronal connections. (Bar = 50μm)

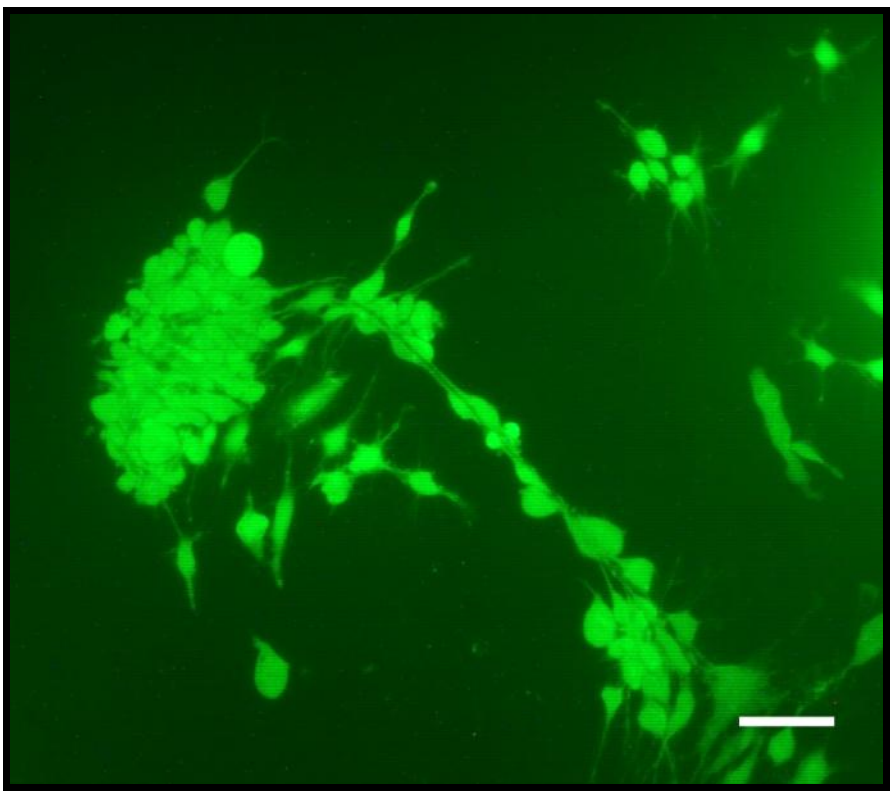
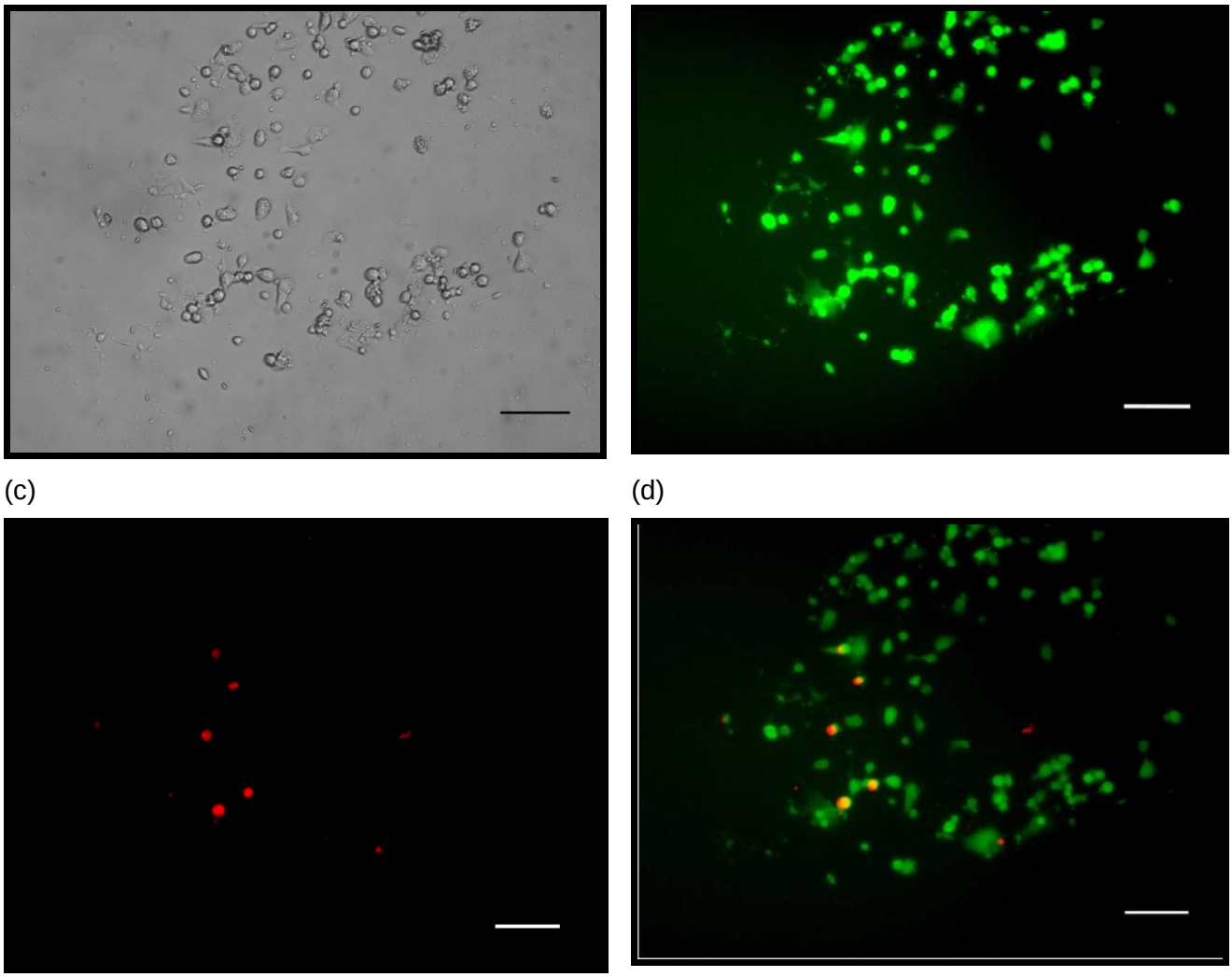


Figure 5.2 FDA staining of primary neuronal culture in the absence of hydrocortisone to illustrate neuronal connections. (Magnification: Bar = 50μm)

(a) (b)



(c) (d)

Figure 5.3 FDA and PI staining of primary neuronal cultures in the absence of hydrocortisone. (a) Phase contrast microscopy to illustrate all the cells present, (b) FDA, (c) PI staining and (d) computer generated overlay of FDA and PI staining. (All photos magnification: Bar = 50 μm)

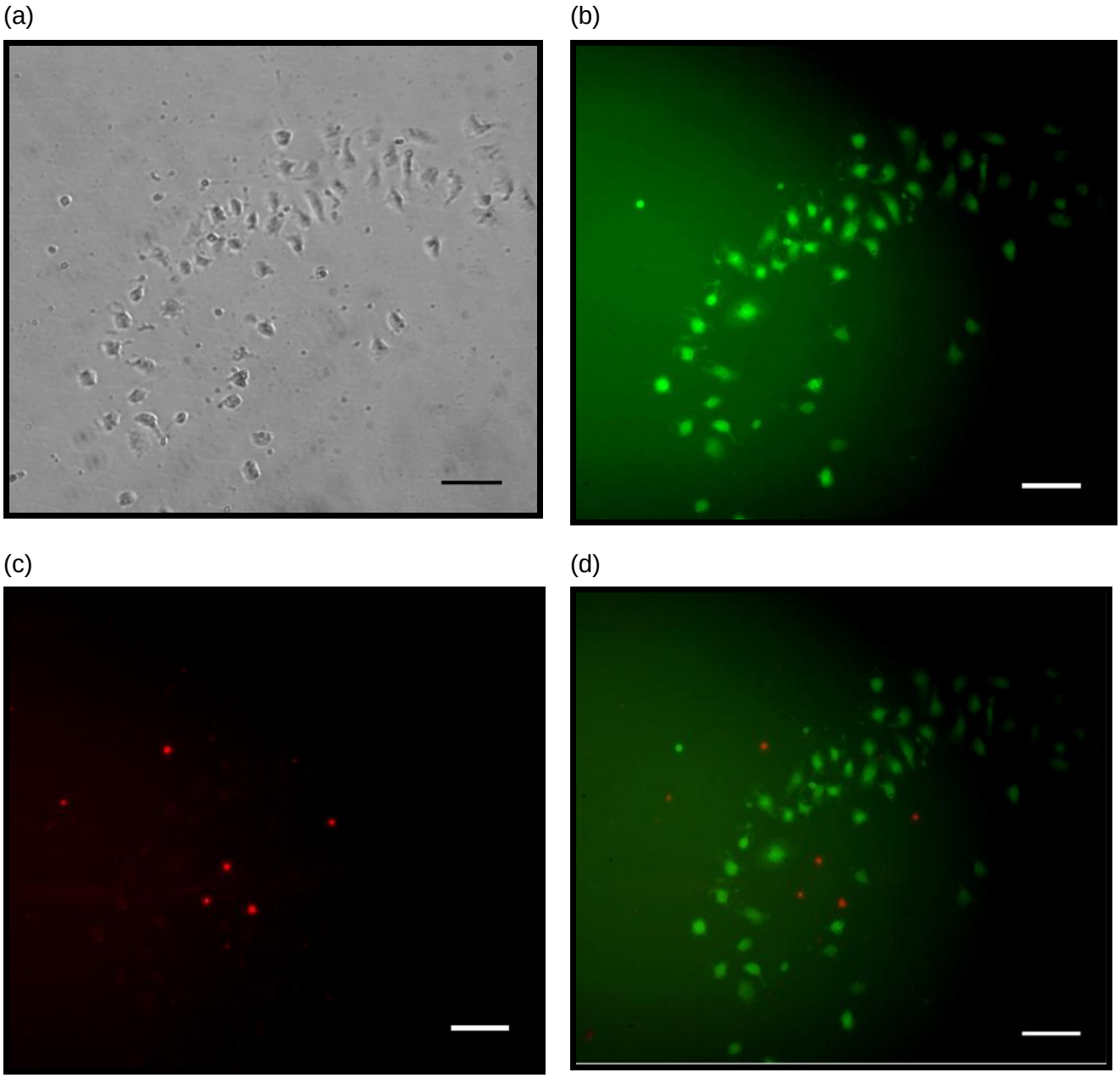


Figure 5.4 FDA and PI staining of primary neuronal cultures exposed to 26.3nM hydrocortisone. (a) Phase contrast microscopy, (b) FDA, (c) PI staining and (d) computer generated overlay of FDA and PI staining (All photos magnification: Bar = 50μm)

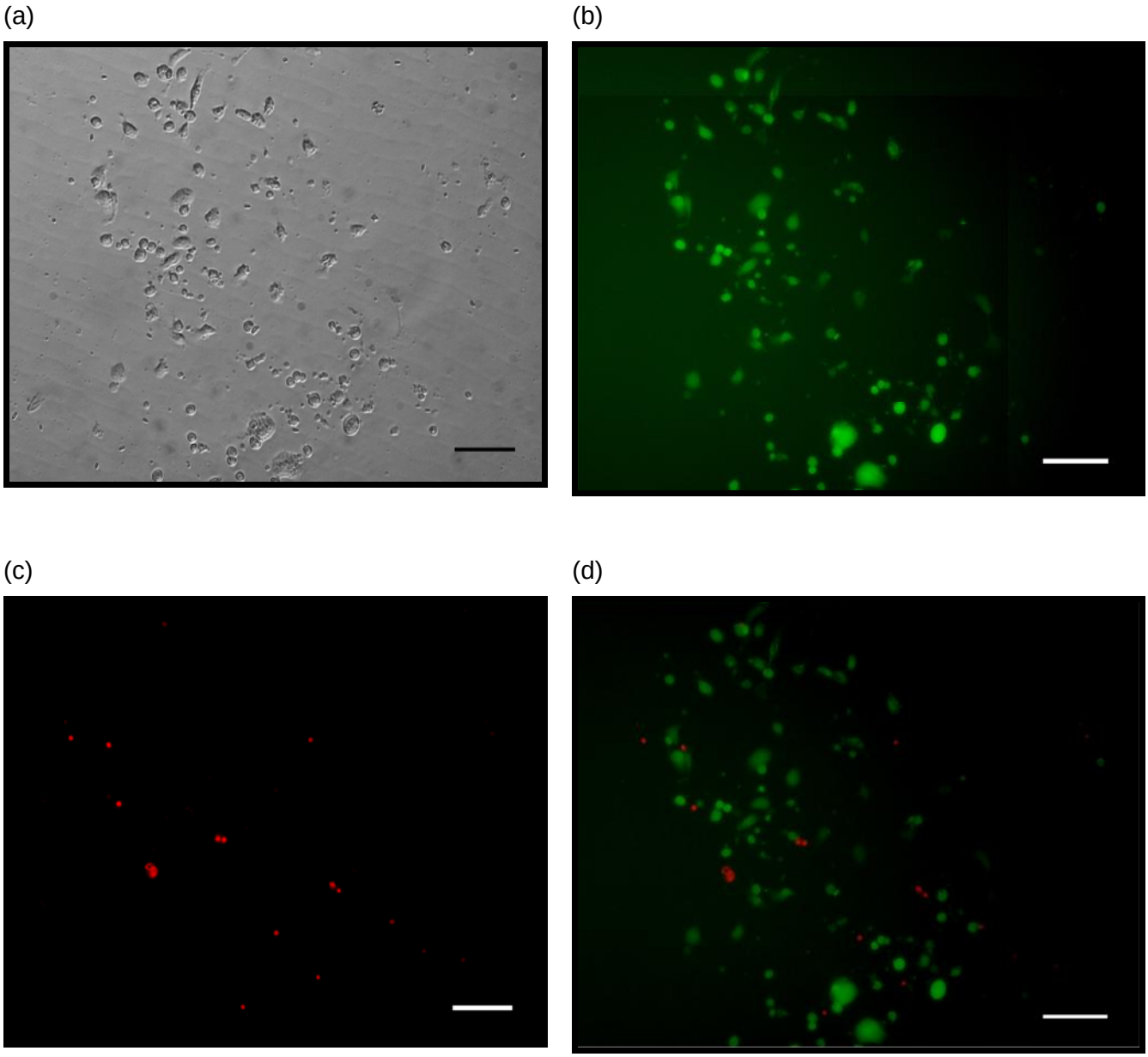


Figure 5.5 FDA and PI staining of primary neuronal cultures exposed to 0.16μM hydrocortisone. (a) Phase contrast microscopy, (b) FDA, (c) PI staining and (d) computer generated overlay of FDA and PI staining (All photos magnification: Bar = 50μm)

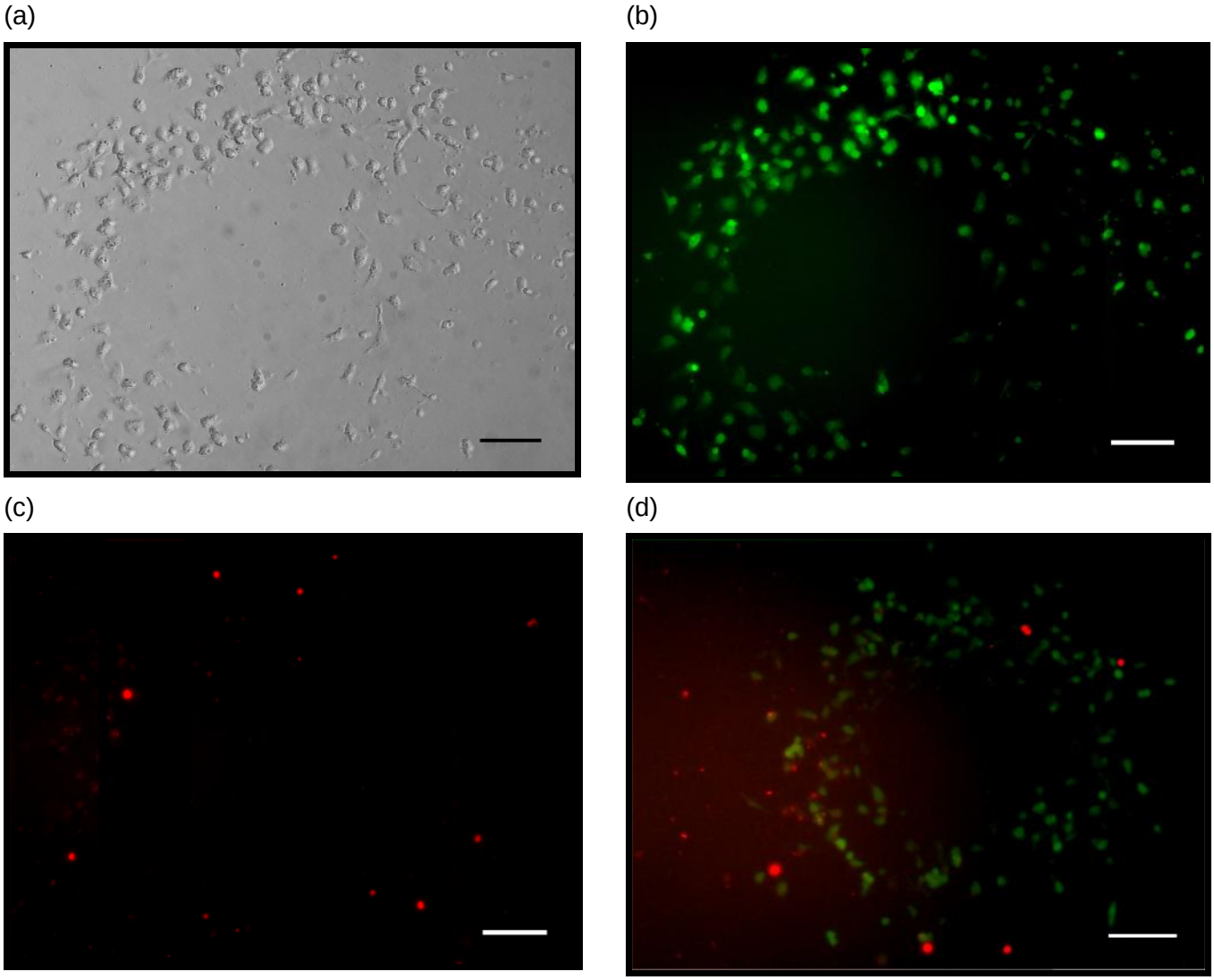
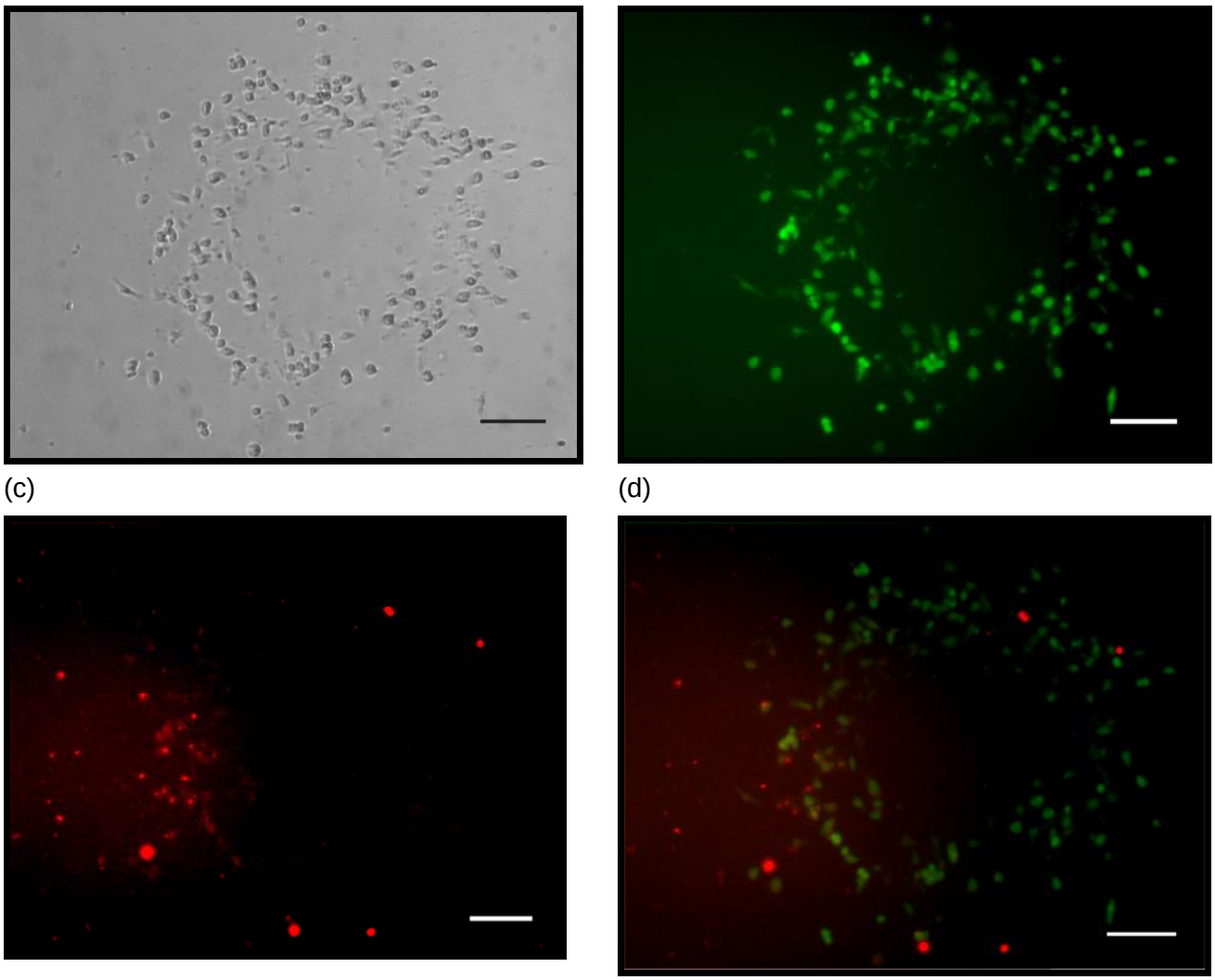


Figure 5.6 FDA and PI staining of primary neuronal cultures exposed to 0.63μM hydrocortisone. (a) Phase contrast microscopy, (b) FDA, (c) PI staining and (d) computer generated overlay of FDA and PI staining (All photos magnification: Bar = 50μm)

(a) (b)



(c) (d)

Figure 5.7 FDA and PI staining of primary neuronal cultures exposed to 3.8μM hydrocortisone. (a) Phase contrast microscopy, (b) FDA, (c) PI staining and (d) computer generated overlay of FDA and PI staining. (All photos magnification: Bar = 50μm)

(a) (b)

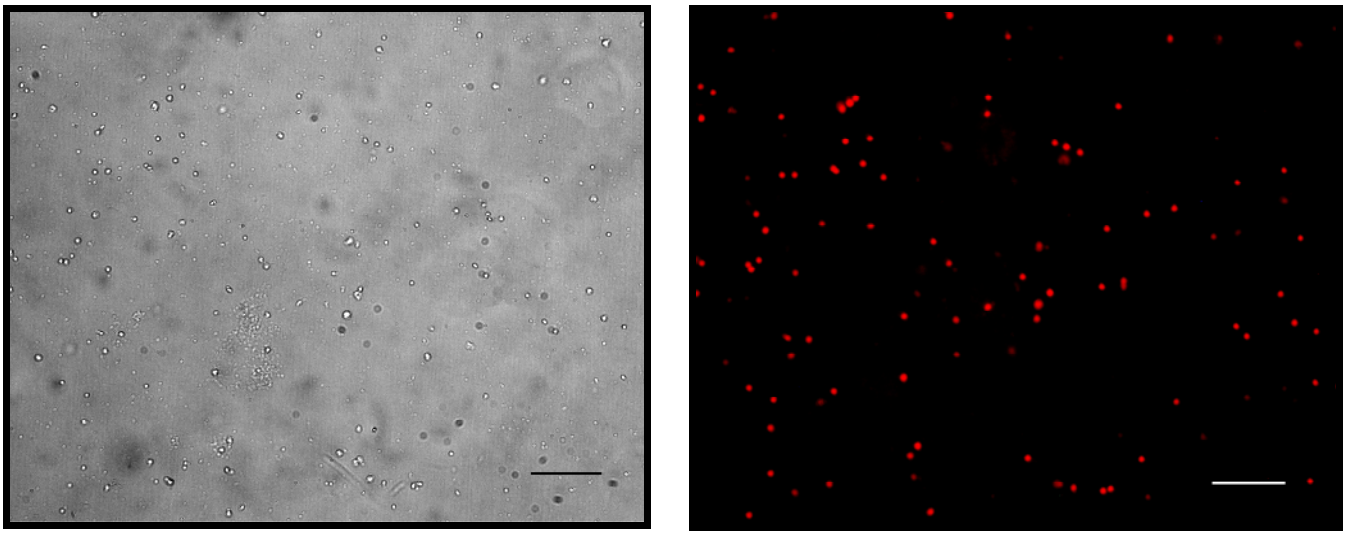


Figure 5.8 PI staining of primary neuronal cultures exposed to 22.8μM hydrocortisone. (a) Phase contrast microscopy and (b) PI staining. *No FDA fluorescence were seen* (not shown)(Both photos magnification: Bar = 50μm)

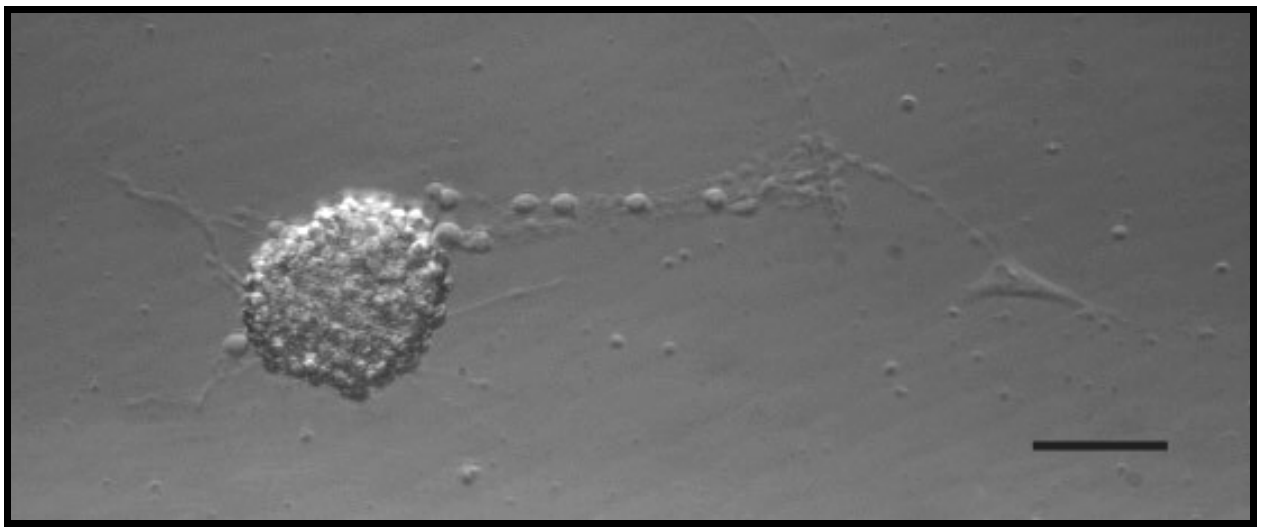


Figure 5.9 Phase contrast microscopy photograph of neuronal connections in primary neuronal cultures exposed to 26.3 nM hydrocortisone. (Bar = 50μm)

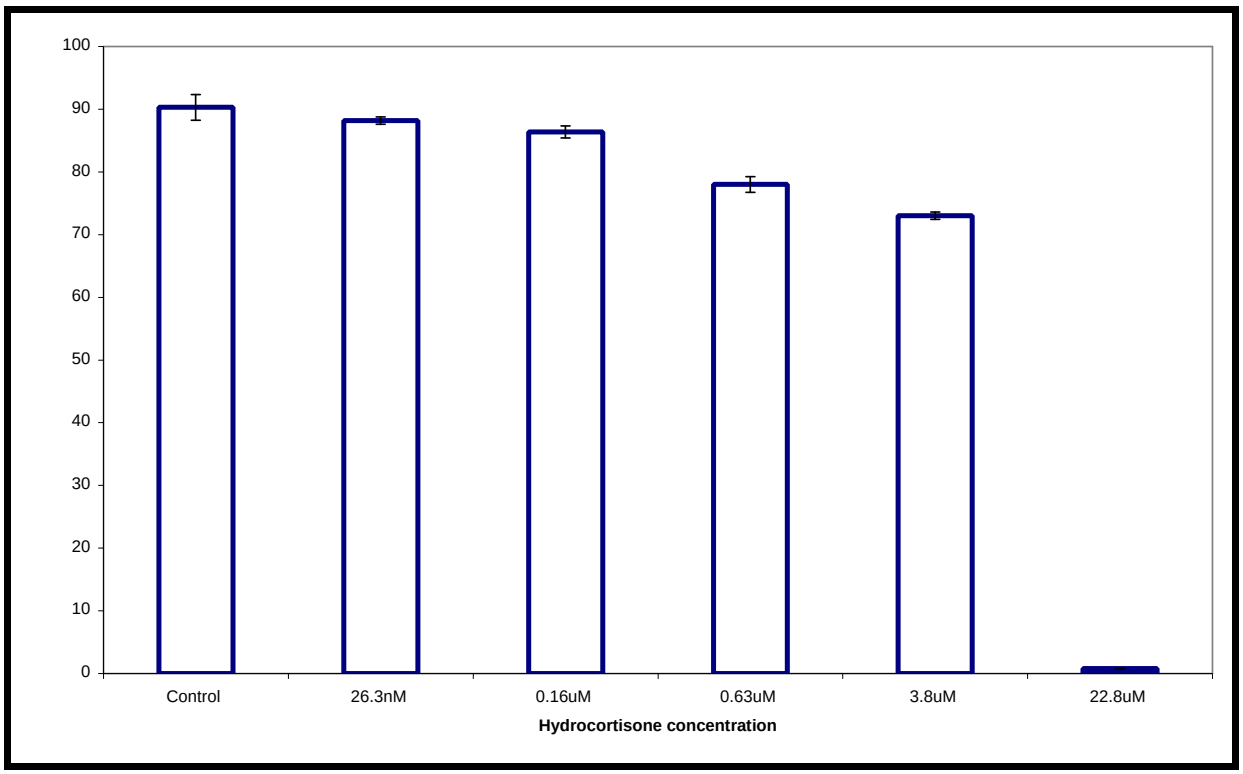


Figure 5.10 The effect of hydrocortisone on the percentage viability of neurons exposed to different concentrations of hydrocortisone.

(a) (b)

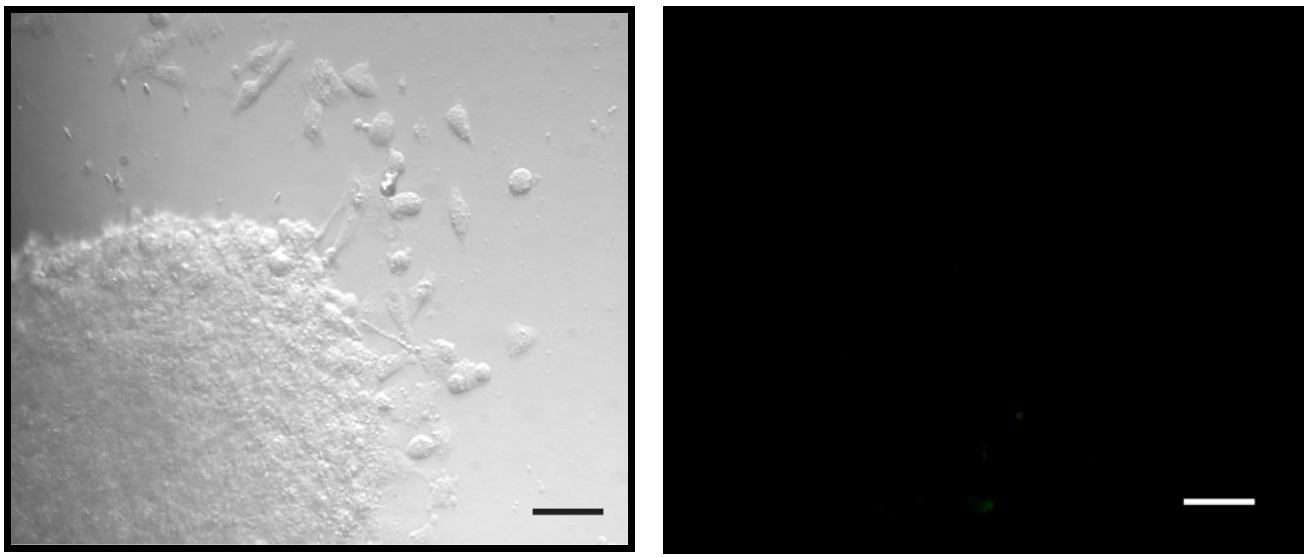


Figure 5.11 DCH_2FDA staining of primary neuronal cultures not exposed to hydrocortisone – Control Sample. (a) Phase contrast microscopy and (b) DCH_2FDA staining. (Both photos magnification: Bar = 50μm)

(a) (b)

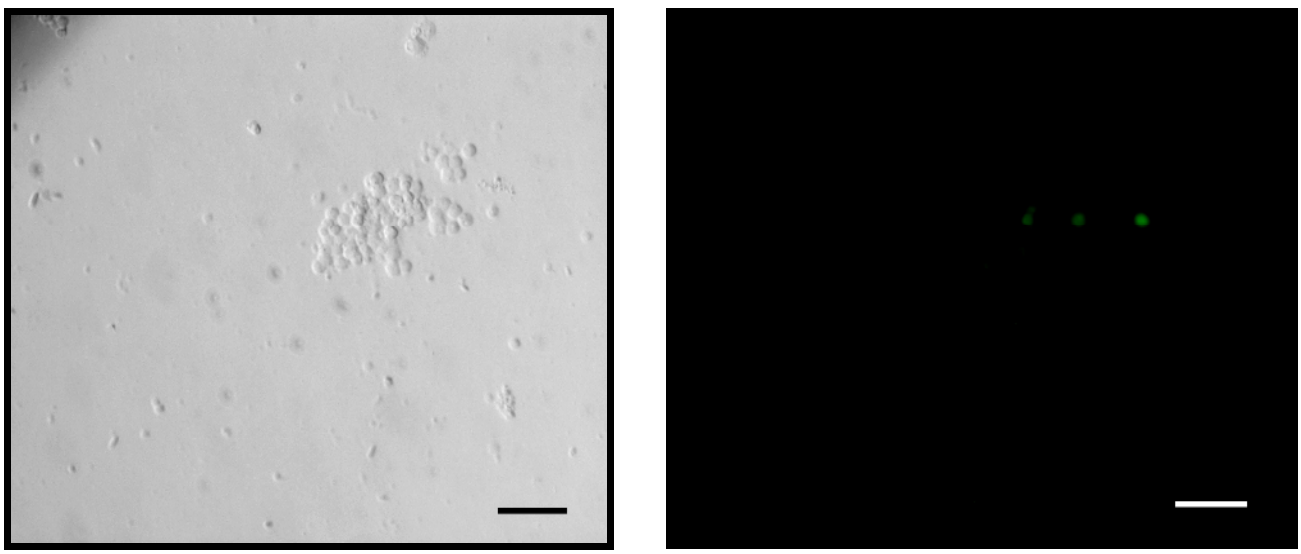


Figure 5.12 DCH_2FDA staining of primary neuronal cultures exposed to 26.3 nM hydrocortisone. (a) Phase contrast microscopy (b) DCH_2FDA staining of the same region. (Both photographs magnification: Bar = 50μm)

(a) (b)

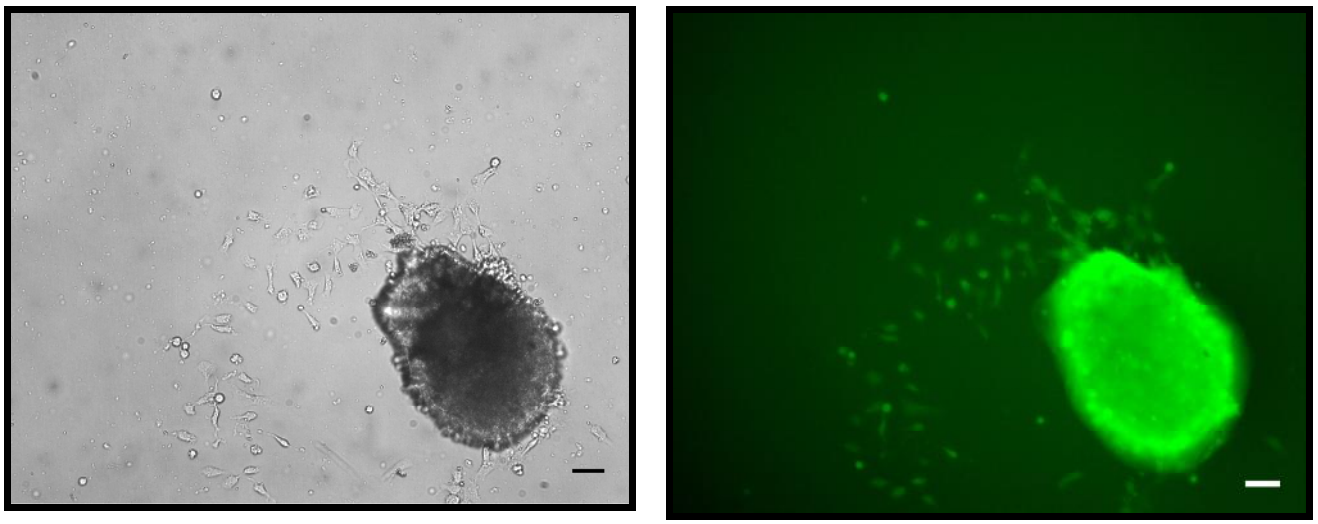


Figure 5.13 DCH_2FDA staining of primary neuronal cultures exposed to 3.8μM hydrocortisone. (a) Phase contrast microscopy and (b) DCH_2FDA staining. (Both photos magnification: Bar = 50μm)

(a) (b)

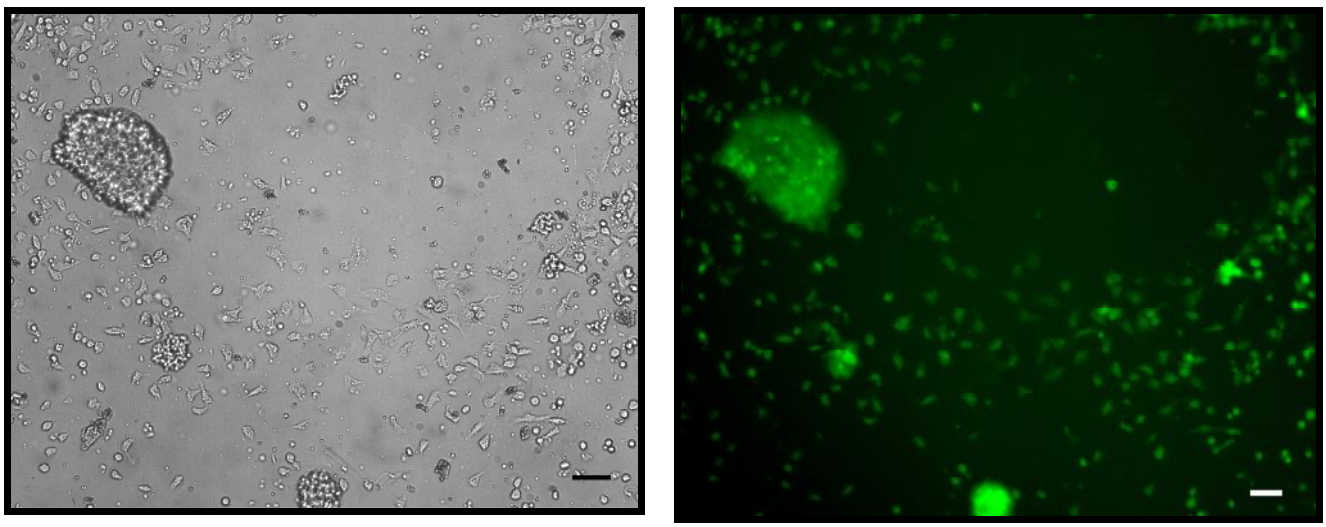


Figure 5.14 DCH_2FDA staining of primary neuronal cultures exposed to 22.8μM hydrocortisone. (a) Phase contrast microscopy (b) DCH_2FDA staining of the same region. (Both photographs magnification: Bar = 50μm)

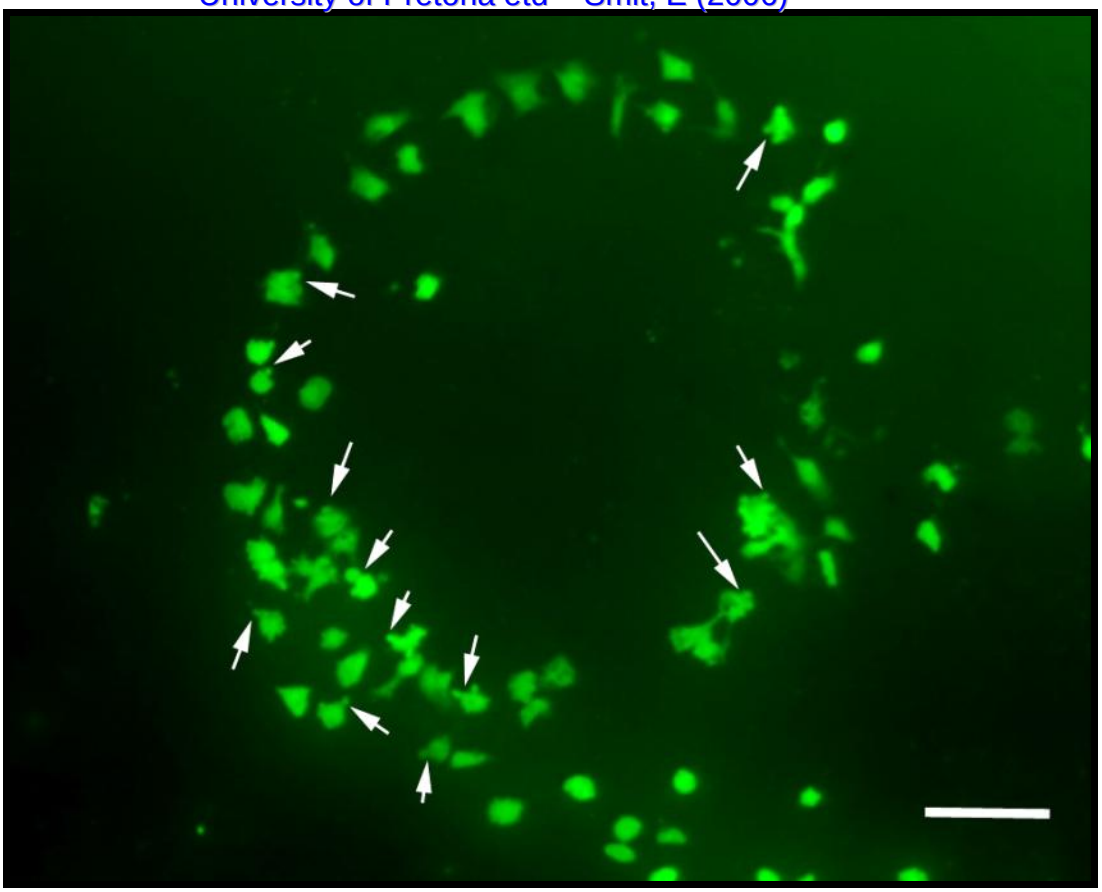


Figure 5.15 DCH_2FDA staining of primary neuronal cultures exposed to 22.8μM to illustrate apoptotic bodies (white arrows) in cells also showing ROS generation. (Bar = 100μm)

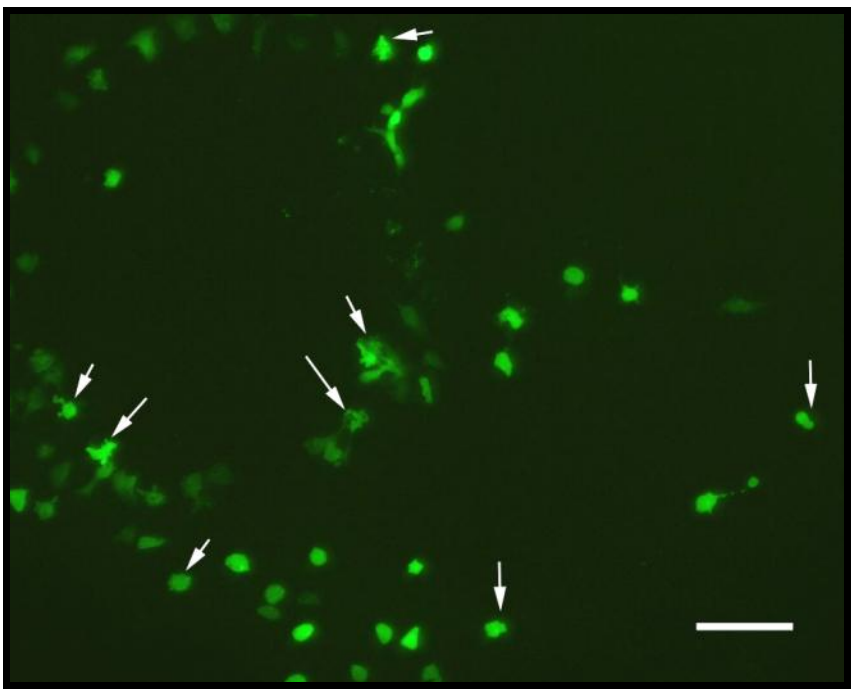


Figure 5.16 DCH_2FDA staining of primary neuronal cultures exposed to 22.8μM to illustrate apoptotic body formation(white arrows) (Bar = 100μm)

Chapter 6 : Concluding discussion

Elevated levels of glucocorticoids may cause degenerative changes in the central nervous system. These changes occur both as a result of hypersecretion of endogenous glucocorticoids or exogenous glucocorticoids administered in high doses for therapeutic processes (Sekita-Krzak *et al*., 2003). Since neurons in the developing brain are very susceptible to any chemical or mechanical insult, glucocorticoids may have diverse effects on neuropeptide and neurotransmitter systems, thus affecting the functional brain system (Smith *et al*., 2000) (Erickson *et al*., 2003). Glucocorticoid induced modification of neurons with changes in morphology and cell number were also noted (Carlos *et al*., 1992) (Cochemore *et al*., 2002). Yet, despite the evidence that glucocorticoids have potent effects on the developing nervous system, this medication is still administered to pregnant mothers at risk for preterm delivery and children suffering from diseases, such as asthma.

Therefore, to evaluate the neurotoxicity of the glucocorticoid hydrocortisone, the following aspects were investigated.

- Changes in the general neuron morphology of the region between the two telencephalic vesicles, the floor of the myelencephalon and the region that later develops into the pineal gland of the chick embryo after exposure to hydrocortisone.
- The effect of hydrocortisone on the ultrastructure of the plasma and nuclear membrane structure as well as the other membranous organelles, such as the ER and mitochondria.
- The effect of hydrocortisone on cell number and viability on chick embryo neurons established in primary culture.
- Finally to identify the mechanism whereby hydrocortisone mediates cell death in primary cultures of the chick embryo neuron.

Histology was used to evaluate general morphological changes in the neurons exposed to hydrocortisone and different staining procedures were used to study the effect of hydrocortisone on neuronal structure and cell number. Serial sectioning of chick embryo heads allowed comparison of similar brain areas and specific staining procedures enhanced visual quality. To better illustrate the damage observed by light microscopy,

TEM was utilized to enable visualization of the different organelles. Specifically the membranes and membranous structures were investigated for evidence of apoptosis or necrosis. Fluorescence microscopy with fluorescent dyes FDA and PI were used to evaluate cell viability after exposure of primary cultures to hydrocortisone. The fluorescent probe DCH_2FDA was applied to detect ROS generation after hydrocortisone administration. All the experimental procedures (histology, TEM and fluorescence microscopy) contributed to the evaluation of the mechanism of cell death.

In order to evaluate samples by histology, conditions for chick embryo head processing needed to be optimized. Parameters such as the stage of embryonic development, the fixation period, the pre-embedding processing and the section thickness were optimized. At Carnegie stage 20 (day 8 of development) of embryological development, the size of the head was easily manageable during fixation, processing, embedding and sectioning. The time needed for optimal fixation was determined to be 5 days. Little was altered to the pre-embedding processing as described by Sheehan *et al.,* (1980). It was however found that a time period of 5 hours in 70% ethanol and 2 hours in each of the three changes of absolute ethanol were sufficient for dehydration. For this study the optimal tissue thickness for sectioning was found to be 7μm and was used throughout this study.

Once all parameters were optimized the effect of hydrocortisone on neuronal tissue was studied. Chick embryos were exposed to two dosages (0.137μM or 0.685μM) of hydrocortisone at day 3.75 (Carnegie stage 16) and day 5.5 (Carnegie stage 18) of development. At Carnegie stage 20 (day 8) the embryos were removed, the tissue was processed and histological sections were prepared. Slides were prepared and stained with H&E, Cresyl Fast Violet, Silver impregnation or a combination of Gold Chloride and Toluidine Blue. Following exposure to 0.137μM hydrocortisone, the tissue was more fragile and tore easily during the preparation of sections when compared to the control samples. This increased fragility of the tissue could be a result of the action of the glucocorticoid, since acute disturbances in the neuronal cytoskeleton was found by Schwab *et al*., (2001). Neurons also appeared to be smaller with fewer processes and had a more intensive staining than was observed for control sections. Banuelos *et al*., (2005) found that this type of staining is associated with neuronal damage. Wall *et al*., (2000) utilized the ‘dark’ neuron characteristic as evidence of neuronal degeneration and cell loss. Exposure of neurons to 0.137μM hydrocortisone therefore caused a decreased

cell number and an increased neuronal staining while some other neurons stained lighter, with nuclei that appear to be fragmented, a morphological feature commonly associated with neuronal injury and DNA degradation according to Murakami *et al*., (1997). Turner *et al*., (2004) found that the degree of dark cell change is a histological indicator of apoptosis and is usually accompanied by an increase in caspase 3 expression.

Neuronal tissue exposed to 0.685μM hydrocortisone appeared to be more intensely stained compared to the control samples. At a higher magnification the neurons did not have clearly distinguishable plasma membranes and very few processes could be identified. Disrupted cell membranes, increased intercellular spaces and fragmentation of nuclei were also noted. Fix *et al*., (1996) found that loss of morphological integrity of neurons in the central nervous system may be a result of necrosis. It would therefore appear as if glucocorticoids caused apoptosis or necrosis or a combination, apopnecrosis in the developing nervous system, when evaluated by light microscopy.

To further investigate apoptotic and necrotic evidence found using light microscopy, the TEM was utilized. Fixatives and additives that did not cause disruption to membrane structures were optimized before the effects of hydrocortisone on neuron membrane morphology were evaluated. Glutaraldehyde based fixation with $MgCl_2$ as stabilizing chemical and potassium permangenate proved to be suitable fixatives for chick embryo neuronal tissue, especially for the evaluation of plasma and nuclear membranes. Because apoptosis and necrosis are associated with changes to membranes, the ER and mitochondria, morphologically these changes should be visible when studying exposed neurons with TEM. Zhang *et al*., (2003) confirmed this by indicating that apoptotic death of neurons could be visualized by TEM.

Samples of 8-day-old chick embryos exposed to 137μM hydrocortisone on day day 3.75 and day 5.5 of development were fixed in glutaraldehyde with added $MgCl_2$ and potassium permangenate. The nuclear material of these samples seemed to have accumulated at the inner membrane of the nucleus. This condensation of nuclear material could be a sign of possible chromatin margination. Condensed chromatin near the nuclear envelope is an indication of apoptosis (Zhang *et al*., 2003). Breakages in the

mitochondrial membrane were also observed and may be a cause of the formation of MPTs, indicating that hydrocortisone is a neurotoxic agent (Moreira *et al*., 2003).

The ER also appeared to be swollen, resulting in the vesicles or cisternae being pulled from each other. Since the anti-apoptotic gene Bcl-2 is also known to be located in the ER, any damage to the ER may result in the inhibition of Bcl-2 and the stimulation of apoptosis (Yang *et al*., 1998). The rupture of the vesicles of the ER may also be a cause of ROS induced by hydrocortisone and may result in cellular swelling, culminating in cell death (Van Cruchten *et al*., 2002).

The effect of 0.685μM hydrocortisone exposure to chick embryo neural tissue at day 3.75 and day 5.5 was then studied. The effect on the mitochondria, ER, nuclear and plasma membranes were again evaluated. Severe damage due to this high concentration of hydrocortisone was seen. All structures appeared to be damaged beyond repair and cell death would seem the only fate for these neurons. It was also found that the mitochondrial membranes appeared to have pulled away from the structure, leaving the contents of the mitochondria exposed. This was possibly accompanied by the release of cytochrome *c* from the mitochondria and subsequently may have resulted in apoptosis (Van Cruchten *et al*., 2002) (Pretorius *et al*., 2005). Severe swelling and rupture of the mitochondrial membrane, however, do not only occur in apoptosis but also in necrosis (Michea *et al*., 2002).

Again evidence of both apoptosis and necrosis could be detected. However, more substantial evidence for death induced by ROS, as seen by severe damage to the mitochondria and ER, were found. Fluorescence microscopy was applied next, not only to confirm loss of cell viability as seen in light microscopy, but also to confirm the generation of ROS in neurons exposed to hydrocortisone.

The effect of hydrocortisone on cell viability was determined using the combined FDA and PI assay. The mechanism whereby cell death occurred was studied using DCH_2FDA, a specific indicator of ROS production. Cell morphology was investigated by fluorescence microscopy to determine whether increased ROS production lead to necrosis or apoptosis.

FDA is a rapid and sensitive method for the evaluation of living cells and can therefore be used to quantify toxic effects (Didier *et al*., 1990). FDA readily diffuses into viable cells and is metabolized by esterases to form the fluorescent compound fluorescein (Kemp, 1992) (Nikolova *et al*., 2002). The cell membrane of viable cells is impermeable to PI. However, if the cell membrane is damaged, PI can cross the cell membrane enter the nucleus and bind DNA to exhibit a red fluorescence, indicating dead cells (Spahr-Schopfer *et al*., 2000) (Chin *et al*., 2001). PI and FDA are usually used in combination to evaluate cytotoxicity. These assays were used by Jordán *et al*., (2003) to study the role of p53 in neuronal death.

Cell viability *in vitro* was determined using the FDA/PI combined bioassay following exposure of primary chick embryo neurons to 0M (control), 26.3nM , 0.16μM, 0.63μM, 3.8μM and 22.8μM hydrocortisone. Neurons not exposed to hydrocortisone showed cell bodies with axons, dendritic processes and several synaptic connections. With increasing concentrations an increase in the number of dead cells were observed with no FDA staining and strong PI staining at 22.8μM. The total number of red and green stained cells was determined and the percentage viability was then calculated as the number of FDA positive cells divided by the total number of the cells counted. For the control cellular viability was 90%, for neurons exposed to 26.3nM and 0.16μM hydrocortisone, viability was at 88.2% and 86.4% respectively with no significant decrease in cell viability compared to the control. At 0.63μM, 3.8μM and 22.8μM neuron viability was reduced to 80%, 75% and 0% respectively, which were significantly reduced when compared to the control.

The effects of glucocorticoids appear to be contradictive where some researchers have shown protective and mitogenic effects (Chang *et al*., 2002) (Basu *et al*., 1978). Other researchers, such as Longui *et al*., (2005) have shown that glucocorticoids reduce cellular proliferation and induce apoptosis in *in vivo* and *in vitro* systems. The conclusion drawn by these researchers was that glucocorticoids are cytotoxic at high concentrations. Schmidt *et al.*, (1987) and Chang *et al*., (2002) then also both stated that the cytotoxicity of hydrocortisone depends on the dosage and the experimental system studied.

Glucocorticoids can induce apoptosis and necrosis through activation of p53, Fas or by ROS generation, as described in chapter 2. In this study, DCH_2FDA was used to determine whether ROS occurred and cell morphology was evaluated to identify characteristics associated with apoptosis, such as apoptotic body formation.

DCH_2FDA easily crosses the cell membrane and once inside the cell is cleaved by esterases to form dichlordihydrofluorescein. (Scivittaro *et al*., 2000) (Kim *et al*., 2000). In the presence of the ROS, H_2O_2, dichlordihydrofluorescein is oxidized to the fluorescent 2'7'-dichlorofluoresein. Consequently the fluorescent signal produced is an index of oxidative stress (Grishko *et al*., 2001). No DCH_2FDA fluorescence and therefore no ROS generation could be observed in the control sample as well as neurons exposed to 26.3nM, 0.16μM and 0.63μM hydrocortisone. Staining was observed for neurons exposed to 3.8μM and 22.8μM hydrocortisone. At 3.8μM and 22.8μM, the majority of neurons had a rounded appearance with clumping, rather than the typical morphology associated with axon- or dendrite-like extensions, and strong green fluorescence associated with ROS generation was seen. At close investigation it was found that green stained cells formed blebs that are morphological characteristics associated with apoptosis. Not all neurons had this typical morphology and this is probably due to cell death being a function of necrosis. These results therefore clearly indicate that cell death (apoptotic, necrotic or both) did occur and is probably due to ROS generation.

Futhermore, to evaluate the comparison between the chick embryo model and the human embryo, the following factors should be taken into consideration. According to "The Tietz Textbook of Clinical Chemistry", which is used internationally by pathologists, blood cortisol levels in a normal individual, range between 138 and 635nM (Burtis and Ashwood, 1998). Since it is known that when pregnant individuals undergo prolonged stress, an increase in glucocorticoid levels can be detected in mother and fetus (Buitelaar *et al*., 2003), and that exogenous glucocorticoids readily cross the placenta to influence the developing fetus (Seckl, 2004), it can be deduced that the developing fetus will be exposed to the same concentration as the mother. In mothers suffering from diseases such as Cushing's plasma cortisol levels can reach a level of 1612 nM (Gorges *et al*., 1999). Preterm infants who has been exposed to glucocorticoids *in utero* (mother administered glucocorticoid due to risk of preterm delivery) may have cortisol levels that reach 1282nM after the first postnatal week (Glover *et al*., 2005). Keeping this in mind,

the chick embryo *in ovo* study, where chick embryos were exposed to 137nM and 685nM would therefore give a very close representation of what would happen in a human embryo. The chick embryo neuronal cultures which were exposed to 26.3nM,160nM, 630nM, 3.8μM and 22.8μM hydrocortisone gave quite a range of exposure. Table 6.1 gives a comparison of the normal values and the experimental values of the hydrocortisone levels per kilogram.

The chick embryo was terminated at Carnegie stage 21. At this stage of development the weight of the chick embryo was determined at 0.6g (results not shown). At the same stage of development, a human embryo is known to weigh 10g. When these factors are taken into consideration, it would appear as if the chick embryos were exposed to a higher concentration of hydrocortisone. The blood cortisol levels of newborn infants, which range between 17-550nM (Thomas, 1998), may therefore give a more accurate estimation when comparing the chick embryo and the human embryo.

Table 6.1 Hydrocortisone levels for the human fetus, chick embryo and primary neuronal cultures

MODEL	**VALUES**
HUMAN EMBRYO	
Normal human adult blood cortisol levels	138-635nM
Mass of human fetus at stage 21 of development	0.01kg
Normal range	**0.0135-0.0635mM/kg**
*Mothers suffering from Cushing's syndrome may have higher blood cortisol levels	1612nM
*Mothers at risk of preterm delivery may be administered hydrocortisone that increases blood cortisol levels in newborns	1282nM
CHICK EMBRYO	
Mass of chick embryo at stage 21 of development	0.0006kg
Exposure concentrations	**0.228 and 1.14mM/kg**
PRIMARY NEURONAL CULTURES	
Wet mass of cells	$1g = 1x10^{9}$
Total number of cells plated	$2x10^{5}$
Total mass of cells plated	0.0000002kg
Exposure concentrations	**131.5, 800, 3150, 19000, 14000mM/kg**

The results obtained from the *in vitro* study indicated quite high levels when compared to the *in ovo* study. This may be due to the fact that the neuronal cultures are a non-dividing population and more comparable results may be obtained when a diving cell population is used. From the table it also becomes evident that the lowest concentration given to the chick embryo (*in ovo*) is four times the highest point of the normal range. Treatment of patients with hydrocortisone for disorders such as Cushing's causes the range to increase to half that given to the chick embryos.

All the results obtained in this study indicate that evidence of cell death due to apoptosis and necrosis could be found. It was therefore hypothesized that a combined mechanism of cell death, aponecrosis would be more applicable. And since Edwards *et al*., (2001) and Cortez *et al*., (2003) stated that neurotoxic changes that include alterations of neuronal differentiation, growth and migration may have possible underlying mechanisms such as exitotoxicity and disturbed calcium homeostasis, these events and their relation to aponecrosis should also be considered.

6.1 HYPOTHESIS OF EVIDENCE OF EXCITOTOXICTY AND ITS CONTRIBUTION IN APONECROSIS

Apoptosis is a form of cell death that occurs as an integral part of tissue homeostasis and in association with various diseases and disorders. Apoptosis can be triggered after receiving any one of a variety of death signals. These can include stimulation of cell surface receptors or cellular stressors (Biswal *et al*., 2000). Glucocorticoids have been shown to trigger apoptosis through a variety of mechanisms. Goel and Khanduja (1998) found that glucocorticoids can induce apoptosis through the generation of ROS and by Fas receptor activation. Cochemore *et al.,* (2002) then also found that glucocorticoids increased translocation of p53 to nucleus, thereby activating apoptosis.

During apoptosis, several morphological changes occur in the cell. The cell shrinks and becomes denser, the chromatin becomes pyknotic (margination of chromatin), the nucleus undergo lysis (karyorhexis) and the cell emits processes that often contain nuclear material resulting in apoptotic bodies (Majno *et al*., 1995). The mitochondria may also undergo swelling and changes to the ER may occur (Jaeschke *et al*., 2003). Patel

and Finch (2002) proved that chronic treatment with the glucocorticoid cortisone caused shrinkage of cell bodies and pyknosis of nuclei in neurons.

Physiologically, the cell undergoes a certain cascade of events (Huppertz *et al*., 1999). Apoptosis is initiated through activation of p53, Fas or by ROS generation, all ultimately affecting the mitochondria or ER. The p53 tumor suppressor gene must be activated by phosphorylation. Active p53 leads to an increased transcription of the pro-apoptotic protein Bax, which is moved to the mitochondria. Activation of the Fas receptor causes caspase-8 and eventually Bid, another pro-apoptotic protein to become activated and translocated to the mitochondria. ROS generation, another apoptosis activator, could either stabilize p53, thereby affecting the mitochondria, or could directly cause damage to the ER.

In the mitochondria Bax and Bid induce the opening or formation of the MPT. As soon as this megachannel is formed or opened, there is an influx of calcium ions. A damaged ER may also result in disruptions to the calcium pool and an increase in mitochondrial volume (Sukocheva *et al.*, 1997). This swelling of the mitochondria may lead to the rupture of the outer membrane and release of cytochrome *c*. In the cytosol, cytochrome *c* complex with Apaf-1 and dATP to activate caspase 9, which in turn activates caspase 3 and the cell enters apoptosis (Van Cruchten *et al*., 2002) (Pretorius *et al*., 2005).

Necrosis is signaled by irreversible changes in the nucleus and cytoplasm. The formation of a MPT also occurs in necrosis and swollen mitochondria are present. The swollen mitochondria lead to cellular swelling, a very typical feature of necrosis. This is usually accompanied by chromatin condensation and eventually lysis of the nuclei and the cell itself. Plasma membrane permeabilization causes release of all cellular enzymes and other contents, culminating in the destruction of the cell by necrosis (Jaeschke *et al*., 2003).

This study revealed histological, ultrastructural and fluorescent evidence of cell death by means of apoptosis and necrosis as could be seen in the apoptotic body formation, nuclear membrane damage, swollen and damaged mitochondria and ER, plasma membrane damage and ROS generation. These results then also supported similar

findings by Hu *et al*., (1996); Vaudry *et al*., (2000); Smith *et al*., (2000); Edwards *et al*., (2001); Seckl, (2005); Rademaker *et al.*, (2006) and many other researchers mentioned.

Although much evidence of damage to nuclear and plasma membranes were found, the effect that hydrocortisone had on the mitochondria and calcium will be focussed on as the hypothesis of excitotoxicity is evaluated.

Excitotoxicity is thought to be a major mechanism contributing to neurodegeneration. Synaptic overreactivity leads to excessive release of glutamate, a major excitatory neurotransmitter. Glutamate then activates a number of cell membrane receptors which open their ion channel pores to produce an influx or efflux of ions such as calcium. An excess of calcium intracellularly activates a calcium dependent signaling cascade that will cause MPTs to open. Since MPTs are associated with both apoptosis and necrosis a combination of the two cell death mechanisms aponecrosis will result (Sattler and Tymianski, 2000). Excess calcium intracellularly due to glutamate receptor activation as a cause of excitotoxicity has also been associated with an increase in ROS production by the mitochondria (Greenamyre and MacKenzie, 1999). ROS generation has also been tied with MPT formation by the mitochondria again giving evidence of death by apoptosis, necrosis or a combination aponecrosis. A schematic representation of the mechanism of excitotoxicity is shown in Diagram 6.1 (compiled from various literature sources used).

A neurotoxin is capable of inducing severe excitotoxic damage (Los et al., 2001). It would therefore appear as if hydrocortisone as neurotoxin may have severe effects on excitotoxic cell death. There is, however, much disagreement how excitotoxic and apoptotic cell death processes relate to one another. Some authors believe that an excitotoxic stimulus directly triggers apoptotic cell death although this interpretation is largely speculative (Young *et al*., 2004). Whether glucocorticoids are responsible for excitotoxicity in the developing nervous system still needs to be investigated more extensively. The fact however remain that neurons exposed to pharmacological doses of glucocorticoids appear to die. Although aponecrosis would seem to be the most possible explanation of cell death, some researchers believe that glucocorticoid exposure lead to cells being arrested in the G1 phase of the cell cycle. Cochemore *et al.*, (2002) who

evaluated this phenomenon found that either cell cycle arrest or apoptosis depend on the cell type and cell environment.

From all the results found, it would therefore be advantegeous to further investigate whether in fact only apoptosis or necrosis caused cell death. Immunolabelling of certain proteins associated with either apoptosis or necrosis could be utilized in immunohistochemistry and immunocytochemistry. This same application could be applied in immunogold labelling of TEM samples to evaluate the precise location of apoptotic proteins. Fluorescent labelling of caspases specific to apoptosis such as caspase 3 could be applied in vitro. TUNEL assay could also be performed to detect any apoptosis induced DNA fragmentation. For further differentiation of glucocorticoid induced apoptosis or necrosis a new apo/necro-COMET assay with the use of Annexin-V staining and dye exclusion has been developed by Morley *et al.*, (2006). To quantify the effects of hydrocortisone exposure, Western blotting where protein extracts derived form the entire chick embryo brain may be used. Antibodies raised against specific proteins of apoptosis, such as p53 can be measured. Furthermore, increased expression of RNA can be measured using the RT-PCR with either gel electrophoresis or real time PCR (Der *et al*., 1997).

6.2 FUTURE IMPLICATION OF GLUCOCORTICOIDS

The current opinions of glucocorticoid therapy in developing individuals result in great controversy. It was found that although high levels of glucocorticoids have been shown to induce apoptosis of neurons, they are also crucial for a healthy neuronal circuitry (Vreugdenhill, 2001). It is also interesting that although some researchers found that glucocorticoids had no effect on neuronal development and that the glucocorticoid hydrocortisone is a suitable medication for treatment of chronic lung disease in the neonatal period (Lodygensky *et al*., 2005), it would appear as if medical professionals question the effectiveness of this drug. Cole and Schumacker (2005), implied that further controlled high-quality studies of glucocorticoids are needed. Yet glucocorticoids have been used for 30 years to accelerate fetal lung maturation in pregnancies at risk of preterm delivery and are currently the initial long-term control therapy in young children (Antonow-Schhlorke, 2003) (Liu and Szefler, 2003). Various authors, however appeared to have realized the consequences of excessive glucocorticoid exposure since short-

duration high glucocorticoid therapy is discouraged and advise is given to avoid high-dose inhaled glucocorticoids (Thompson, 2003) (Crowley, 2003). The best treatment strategy would be the discovery of new or alternative medications that still possessed all the positive characteristics of glucocorticoids, but eliminated its neurotoxic properties. Hydrocortisone, however do remain a life saving medication, but care should be taken when administering this drug to pregnant mothers and children.

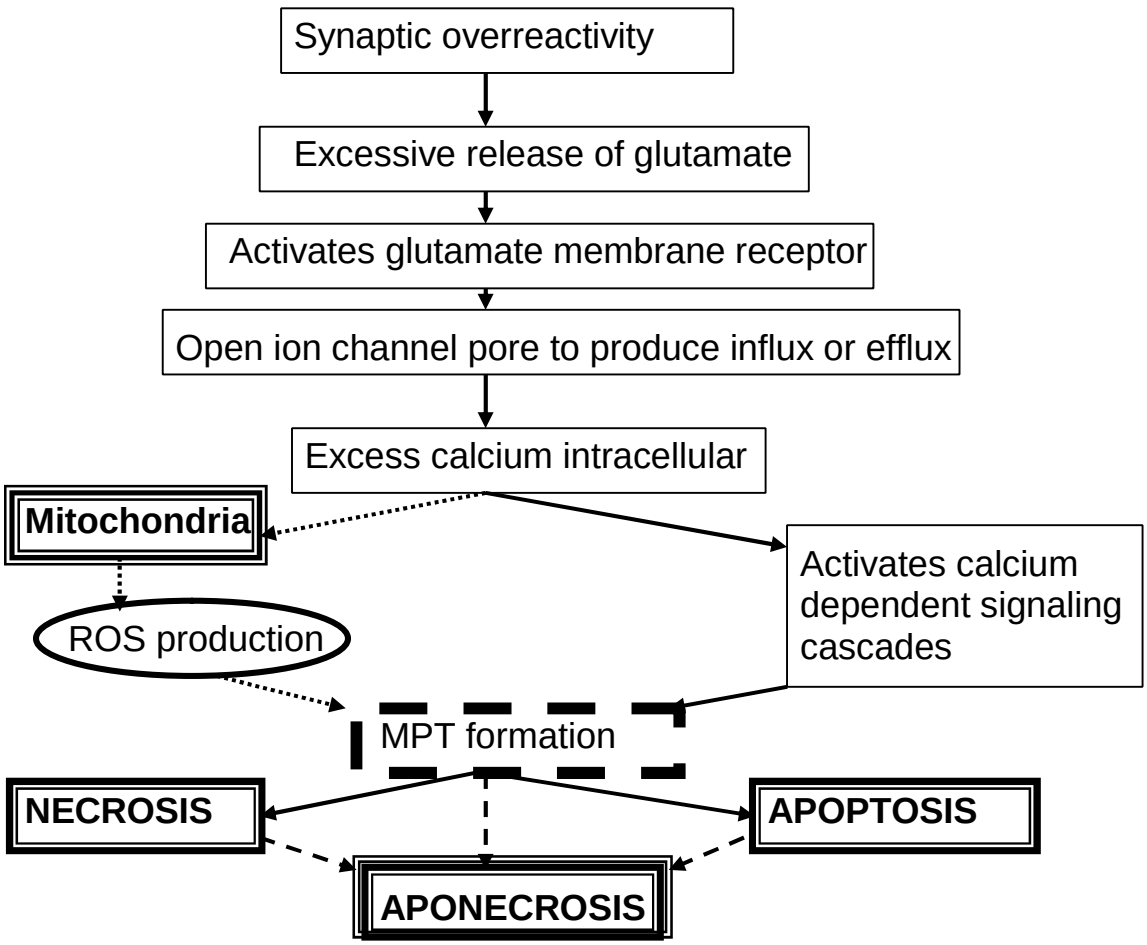


Diagram 6.1 A schematic representation of the mechanism of excitotoxicity (compiled from various literature sources used)

Chapter 7 : References

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