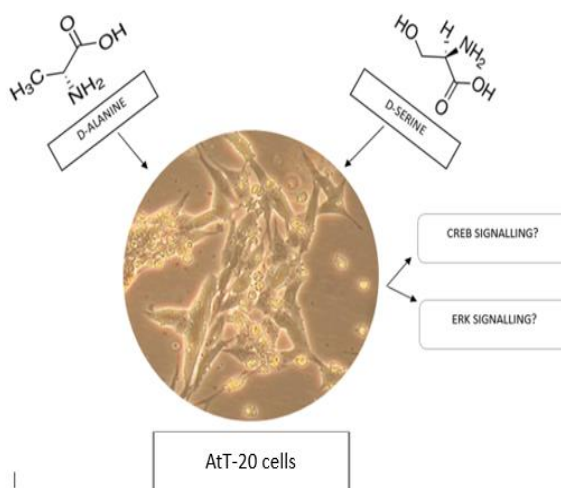


Investigating the effects of D-alanine on pituitary intracellular signalling: a comparison with D-serine



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DECLARATION

I, Miss Kalina Naidoo, declare as follows:

1. That the work described in this thesis has not been submitted to the University of Oxford, or any other tertiary institution, for purposes of obtaining an academic qualification, whether by myself or any other party.
2. That my contribution to the project was as follows:
 - I did the entirety of the laboratory work to procure the results for this dissertation
 - I had written the entire first draft of the dissertation based on the literature surrounding the topics and the results I derived from my experimentation. This draft was then reviewed by my supervisor, Prof Phil Burnet, who suggested changes and I made these amendments accordingly for the final dissertation.



Signature

Date: 5 October 2016

ABSTRACT

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The D-amino acid, D-alanine, is a co-agonist of the N-methyl-D-Aspartate receptor (NMDAR), and an agonist of the glycine receptor (GlyR). In the rat, D-alanine is highly concentrated in the pituitary gland, where it is localized to adrenocorticotrophic hormone (ACTH) containing cells. This suggests that D-alanine may influence the release of ACTH, and ultimately the secretion of the stress hormone, corticosterone. Previous studies have shown that ACTH secretion from pituitary cells involves CREB and ERK1/2 signalling. We have, therefore, investigated the influence of D-alanine on the levels of these signalling proteins (western blot) and ACTH (ELISA) in cultures of the AtT-20 mouse pituitary tumour cell-line. For comparison, the effect of D-serine, a co-agonist of central NMDARs, but not GlyRs, was also examined.

AtT-20 cells were incubated with D-alanine or D-serine, CRH, dexamethasone (DEX) and MK-801 (NMDAR antagonist) in different combinations. Data was expressed as ratios of total/phosphorylated protein, where tCREB is CREB/ β -actin and pERK1/2 is pERK/ERK, and subjected to one-way ANOVA statistics.

Compared to controls, the levels of tCREB and ACTH expressed by AtT20 cells were significantly greater when incubated with 500 μ M of D-alanine (+35%, $p=0.011$ and +12%, $p=0.028$ respectively) or D-serine (+29%, $p=0.028$ and +13%, $p=0.043$ respectively) for 24 hours. The level of tCREB and ACTH in AtT20 cells was significantly lower when incubated with D-alanine and DEX for 24 hours (-30%, $p=0.019$ and -44%, $p=0.030$ respectively), but significantly greater for pERK/ERK and ACTH in cells treated with D-serine and DEX for 24 hours compared to controls (+38%, $p=0.039$ and +16%, $p=0.038$ respectively).

The current data show that D-alanine and D-serine differentially modulate stress hormone-mediated negative feedback signalling in AtT-20 cells. Based on our findings, the changes suggest that D-alanine might augment the inhibition of ACTH, whereas D-serine may attenuate negative feedback, and release ACTH. The elevation of both ERK (with DEX and D-serine), and CREB (with both D-amino acids alone) implies NMDAR activation. The combination of DEX and D-alanine, suggests the possible involvement of GlyRs.

Keywords: D-alanine, D-serine, CREB, ERK, ACTH

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AMP	adenosine monophosphate
AP1	Activator Protein 1
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
CaM	Calmodulin
CaMK	Ca ²⁺ /calmodulin-dependent kinase
CaMKK	Ca ²⁺ /calmodulin-dependent kinase kinase
cAMP	cyclic AMP
CaRE	Ca ²⁺ -responsive element
CART	Cocaine and amphetamine regulated transcript
CBP	CREB binding protein
CD	common docking
CRE	cAMP response element
CREB	CAMP response element binding protein

CRH	Corticotropin Releasing Hormone
CRH-BP	CRH binding protein
CRHR1	CRH receptor type 1
CRHR2	CRH receptor type 2
CRS	cytosolic retention sequence
DEF	docking site for ERK
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Media
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated Kinase
FBS	Fetal Bovine Serum
GEF	guanine-nucleotide exchange factors
Glu184	Glutamine184
Glu203	Glutamine203
GluN2A	Glutamate (NMDA) receptor subunit epsilon-2A
GluN2B	Glutamate (NMDA) receptor subunit epsilon-2B
GlyR	glycine receptor
GPCRs	G protein-coupled receptors

GR	glucocorticoid receptor
GRE	glucocorticoid response elements
GTP	Guanine triphosphate
HPA	hypothalamic pituitary adrenal axis
HSP	heat shock protein
KIM	kinase interaction motif
KSR	Kinase suppressor of Ras
LVSCCs	Ligand gated V-SCCs
MAPK	Mitogen Activated Protein kinases
MAPKK	Mitogen activated protein kinase kinase kinase
MC2R	melanocortin type 2 receptor
MKP3	MAP Kinase phosphatase 3
MNKs	MAPK-interacting kinases
MSK	mitogen-and stress activated kinase
NGF	Nerve Growth Factor
NMDAR	N-Methyl-D-aspartate receptor
Nur	Nuclear receptor subfamily 4
PAK1	P21 Protein-activated Kinase 1

PBS	Phosphate Buffered Saline
PC12	pheochromocytoma -12
PKA	Protein kinase A
PKC	Protein Kinase C
POMC	proopiomelanocortin
PP1	protein phosphatase-1
PP2A	protein phosphatase-2A
CaN	Calcineurin,
PVDF	Polyvinylidene difluoride
PVN	paraventricular nucleus
Rap1	Ras related protein 1
RIPA	Radioimmunoprecipitation assay buffer
RTKs	receptor tyrosine kinases
Ser218	Serine218
Ser222	Serine222
SGK1	serum-and glucocorticoid-inducible kinase-1
SRE	serum response element
SRF	serum response factor

Thr183	Threonine183
Thr202	Threonine202
Tyr185	Tyrosine185
Tyr204	Tyrosine204
VSCCs	voltage-gated Ca^{2+} channels

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CHAPTER 1: INTRODUCTION

1.1. The role of D-amino acids in the central nervous system

Amino acids are the building blocks of functional proteins, and so are the most important molecules in nature (Wolosker et al., 2008; Fuchs et al., 2005). They exist as two stereoisomers, the *laevus* (L-) and *dexter* (D-) forms. Mammalian proteins comprise of L-amino acids, whereas microbes predominantly contain D-amino acids. However, D-serine and D-aspartate have been detected in the mammalian central nervous system and are considered to play an integral role in brain function. These D-amino acids are synthesized from their L-isomers by amino acid racemases (Fuchs et al., 2005; Morikawa et al., 2001), though small amounts can arise from the indigenous gut flora and diet (Fuchs et al., 2005). In contrast, D-alanine, which is found at high concentrations in the pituitary gland and pancreas, stems principally from the gut bacteria, and cannot be synthesised by mammals. Foods and dairy produce that contained fermenting bacteria are also a rich source of D-alanine. However, whilst D-serine and D-aspartate have been extensively researched, particularly the former since it is an intrinsic co-agonist of the N-Methyl-D-aspartate receptor (NMDAR), a key mediator of neurodevelopment, learning and memory, the properties of D-alanine remain to be comprehensively researched. This is important because, D-alanine is also a co-agonist of the NMDAR, and may have similar actions to mammalian D-serine.

1.1.1. D-serine

The primary source of D-serine is through the conversion of L-serine by serine racemase (Fuchs et al., 2005, **Fig 1.1**). Differential levels of brain D-serine has been associated with neuropsychiatric disorders such as schizophrenia, where decreased D-serine levels results in dysfunction of NMDA receptor, and amyotrophic lateral sclerosis, caused by the death

of motor neurons as a result of heightened levels of D-serine, which supplements glutamate toxicity via NMDA receptors, (Sasabe et al., 2007; Hashimoto et al., 2003). Free D-serine has been localized principally in the forebrain, with highest quantities detected in the cerebral cortex, hippocampus and striatum. The anatomical distribution of D-serine in the CNS mimics the distribution of the GluN1, GluN2A and GluN2B subtypes of the NMDA receptor (Fuchs et al., 2005).

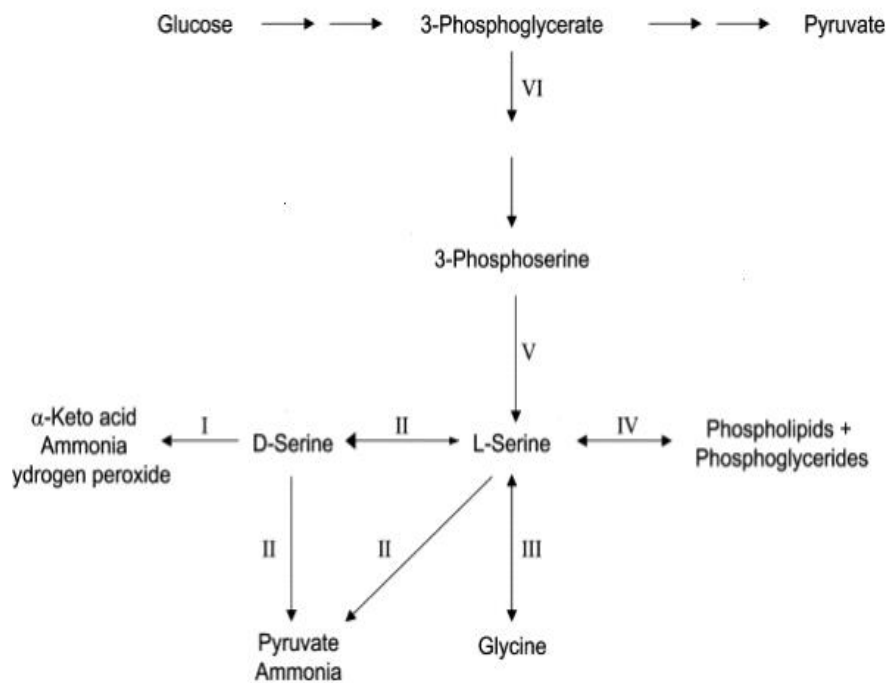


Figure 1.1. The interdependency between D-serine, L-serine and glycine via the pathways involved in biosynthesis and catabolism. Numerals represent the enzymes catalysing the reaction:

- I) serine racemase
- II) serine hydroxymethyltransferase,

- III) Represents the synthesis of phospholipids and phosphoglycerides from cytidine diphosphodiacylglycerol and palmitoyl-coenzyme A
- IV) 3-phosphoserine phosphatase
- V) 3-phosphoglycerate dehydrogenase (Fuchs et al., 2005).

1.1.2 D-alanine

In addition to being a co-agonist of the NMDAR, D-alanine is also a full agonist of the strychnine-sensitive glycine receptor (GlyR) (Dutertre et al., 2012). Fisher et al (1991) showed that free D-alanine is present in the white matter of the brain at an average concentration of 12.3 nmol/g of wet tissue, whilst it is present at an average of 9.5nmol/g wet tissue in grey matter. By comparison, D-serine is present at 108 ± 6 nmol/g wet tissue in the grey matter of prefrontal cortex of normal brain tissue, and 128 ± 12 nmol/g wet tissue in white matter of prefrontal cortex of normal brain tissue (Kumashiro et al., 1995). The major source of D-alanine is the natural breakdown of the D-alanine-rich cell wall of the microbiota in the intestines, where it is absorbed into the circulation and transported to various tissues, (Fuchs et al., 2005). In the rat, D-alanine is highly concentrated in the adrenocorticotrophic hormone (ACTH) containing cells of the pituitary. Tissue distribution of D-amino acids provide insight into their function, e.g. the prevalence of D-serine in the cortex and hippocampus of the brain, suggests that it is involved in complex central processes such as memory, and this has been confirmed. Therefore, D-alanine's localization in the ACTH releasing cells in the anterior pituitary gland may reflect a role in the modulation of ACTH secretion and/or metabolism, and therefore the activity of the hypothalamo-pituitary-adrenal axis (Etoh et al., 2009).

1.2 The Hypothalamic-Pituitary-Adrenal Axis

The stress response in animals is triggered by a perceived threat to its health and normal functioning, and involves the nervous, endocrine and immune systems, that subsequently modify behaviours (improved cognition, heightened awareness and euphoria) and physiology (respiratory rate, cardiovascular tone etc.) to allow adaption to stressful challenges. The hypothalamic pituitary adrenal axis (HPA) consists of the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary and the adrenal glands, which mediate and modulate the secretion of stress hormones (Smith and Vale, 2006) (**Figure 1.2**).

1.2.1. CRH

The hypothalamus secretes two hormones, Corticotropin Releasing Hormone (CRH) and arginine vasopressin (AVP) (Daban et al., 2005). The principle regulator of the HPA axis is CRH, a 41 amino acid peptide that is synthesized and released from the hypophysiotropic neurons in the parvocellular subdivision of the PVN. The binding of CRH to receptors on the pituitary corticotropes stimulates the release of ACTH into the systemic circulation, which then increases the synthesis and release of glucocorticoids in the adrenal cortex (Smith and Vale, 2006).

The actions of CRH are orchestrated through its binding to its receptors, CRH receptor type 1 (CRHR1) (**Figure 1.2**) and CRH receptor type 2 (CRHR2). The CRHR1 is expressed in greater quantities in the brain and pituitary than peripheral tissue, whilst the opposite occurs for CRHR2, where there are high expression levels of CRHR2 in the peripheral tissue in comparison to its presence in brain tissue (Kishimoto et al., 1995). CRHR1 and CRHR2 exhibit different pharmacological properties, and CRH binds to CRHR1 with a higher affinity than CRHR2. The neuroendocrine properties in the

anterior lobe of the pituitary are exerted by the binding of CRH to CRHR1. CRH binding protein (CRH-BP) assists in the binding of CRH, and is greatly expressed in the pituitary. CRH-BP assists with the sequestration of CRH in the pituitary, which then inhibits the CRHR activity. CRH binding to its receptor on the anterior pituitary cells results in stimulation of adenylate cyclase, which then activates the cyclic AMP (cAMP) pathway, resulting in the release of ACTH from pituitary corticotropes. (Smith and Vale, 2006; Chang et al., 1980). CRH stimulates the transcription factors AP1, CREB and Nur, and these in turn stimulate ACTH biosynthesis and the promotion of proopiomelanocortin (POMC). Therefore, CRH is a potent stimulator of ACTH production in the pituitary gland (Vila et al., 2004).

1.2.2. ACTH

The POMC gene is prevalent in both the hypothalamus and pituitary gland, and is the prohormone to ACTH. POMC can be processed into various biologically active proteins such as melanocortins, β -endorphins, β -lipotropic hormone and ACTH. ACTH release is stimulated by the binding of CRH, and ACTH then binds to receptors on the adrenal cortex. Specifically, ACTH binds to the melanocortin type 2 receptor (MC2R) in the parenchymal cells of the adrenal zona fasciculata, which then trigger cAMP pathway events, that leads to the steroidogenesis and the secretion of the adrenal hormones, glucocorticoids, mineralocorticoids and androgenic steroids. ACTH facilitates the conversion of cholesterol into 5-5 pregnenolone in the first step of the synthesis of glucocorticoids (Smith and Vale, 2006; Raffin-Sanson et al., 2003).

1.2.3. GLUCOCORTICIDS

Glucocorticoids influence the regulation of neuronal survival, neurogenesis, the size of anatomical structures such as the hippocampus, the attainment of new memories and

emotional judgement of events (Pariante and Lightman, 2008). In response to acute stress, glucocorticoids stimulate adaptive changes, such as the mobilisation of energy reserves, whilst long- term adaptive changes involve shaping and managing psychological processes such as immune responsiveness, and activation of the sympathetic nervous system (Daban et al., 2005).

Cortisol (glucocorticoid in humans) and corticosterone (glucocorticoid in rodents) are responsible for the regulation of various cardiovascular, immune, metabolic and behavioural processes, via the binding of glucocorticoids to its 94 kDa glucocorticoid receptor (GR), found in both the CNS and peripheral tissue. There are notably high levels of GRs in the hypophysiotrophic neurons of the PVN, as well as the hippocampus. When in a state of inactivation, GR is associated with the heat shock protein (HSP) complex which undergoes multiple cycles of disassociation and ATP-dependent re-association. When a ligand binds to GR, a conformational change is triggered, resulting in its disassociation from HSP, and the subsequent translocation to the nucleus where it then binds to glucocorticoid response elements (GRE) in the promotor region of glucocorticoid responsive genes. Gene expression is then regulated via interaction with transcription factors (Smith and Vale, 2006).

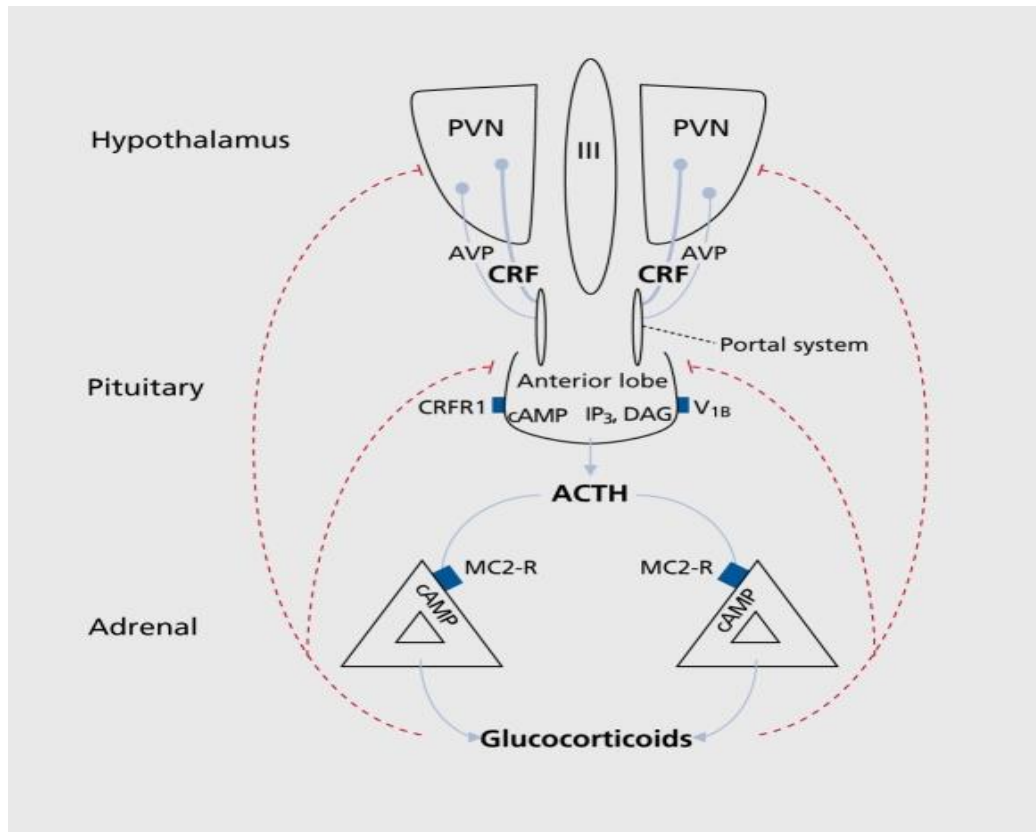


Figure 1.2. Diagrammatic representation of the HPA axis, showing the hormones and their receptors. PVN-paraventricular nucleus, AVP-arginine vasopressin, CRF-corticotropin releasing factor, CRFR1- CRF receptor 1, cAMP- cyclic AMP, MC2-R – melanocortin 2 receptor, ACTH- adrenocorticotrophic hormone (Smith and Vale, 2006).

Animal studies have demonstrated the importance of corticosteroids in influencing neurotransmitter receptor expression and function, long-term potentiation and cell survival. Therefore, when corticosteroids are manipulated, it is likely to influence the behavioural indices of neurotransmitter function, such as mood and cognition. Chronic administration of glucocorticoids impairs learning and memory in animals and humans, along with atrophy of hippocampal neurons (Daban et al., 2005).

An exacerbated response of the HPA axis to stress is a common feature of major psychiatric disorders (Dieleman et al., 2015; de Kloet et al., 2006). This exacerbated response is inferred from high levels of cortisol secretion (Corcoran et al., 2012). For

instance, salivary cortisol levels were measured in 61 depressed patients and 40 healthy control subjects, at different time points in the day. There was a consistently higher level of cortisol in depressed patients than healthy controls (23.5nmol/litre vs. 19.5nmol/litre at 8am, 12.8 nmol/litre vs 6.6nmol/litre at noon, 8.3nmol/litre vs. 5.9nmol/litre at 4pm and 7.6nmol/litre vs. 3.5nmol/litre at 8pm, respectively) (O'Brien et al., 2004). There is also a higher baseline level of cortisol in schizophrenic patients than healthy controls ($12.48 \pm 3.2 \mu\text{g/dl}$ vs $10.31 \pm 3.1 \mu\text{g/dl}$) (Yilmaz et al., 2007).

Therefore, a comprehensive understanding of the modulation of stress hormone release is of clinical importance, since the characterization of pathways that suppress an exaggerated stress responses and/or enhance glucocorticoid negative feedback, will help identify and develop new therapeutic targets for neuropsychiatric disorders associated with HPA axis over-activity. Thus, there is an urgent need to determine the function of D-alanine in the pituitary as it may be involved in hitherto unknown mechanisms that modulate ACTH synthesis and/or secretion. This can be directly addressed by measuring ACTH secretion from pituitary cells in the presence or absence of D-alanine. A preliminary analysis of the molecular mechanisms that may underlie the influence of D-alanine on ACTH, is likely to be based on an examination of intra-cellular signalling cascades, given that it can stimulate NMDARs and GlyRs that are linked to these pathways, and that they influence ACTH secretion.

1.3 Signalling Pathways

Extracellular stimuli prompt long-term phenotypic alterations in cells, such as differentiation of precursor cells, or synaptic strengthening of a mature neuron, that provoke alterations in gene expression (Shaywitz and Greenberg, 1999). Cells react to these external stimuli via signalling pathways, that are activated by plasma membrane

receptors, such as G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). These pathways transmit, process, encode, and integrate both internal and external signals. The RTKs play a role in the modulation of embryogenesis, cell survival, motility, proliferation, differentiation, glucose metabolism and apoptosis, and malfunction to this receptor can result in developmental defects, diabetes and chronic inflammatory syndromes (Kholodenko, 2006). For any specific receptor pathway, there is no single protein or gene that is accountable for signalling specificity. Specificity is determined by the temporal and spatial dynamics of downstream signalling components. These consist of a long line of phosphorylation of kinases, that result in the phosphorylation of specific signalling proteins responsible for various processes. Two key signalling proteins in the intracellular signalling pathways of the HPA axis are CREB and ERK 1/2.

1.3.1 CREB

1.3.1.1. Form and Function of CREB

Phosphorylation is the predominant mechanism by which transcription factors are activated in response to cellular signals (**Figure 1.3**). CAMP response element binding protein (CREB) was the very first transcription factor to be studied, and its phosphorylation is mediated by protein kinases under the control of cAMP and/or calcium. Changes in CREB function can lead to long term synaptic alterations and affect long term memory (Bito et al., 1996). All signalling pathways that stimulate CREB, result in the phosphorylation of the serine 133 residue (Ser133) of the CREB molecules, though CREB activity can be further regulated through phosphorylation at additional sites (Shaywitz and Greenberg, 1999).

1.3.1.2 CREB and cAMP

Phosphorylation of CREB stimulates gene expression in response to cAMP, but supplementary promoter-bound factors are necessary for target gene activation by CREB in response to mitogen or stress signals, which implies that there are cellular components in the CREB pathway that differentiate between different inputs. The cAMP molecule is a classic intracellular second messenger, and its accumulation in response to the activation of neurotransmitter and hormone guanine-nucleotide (G)-protein-coupled receptors, stimulates most cellular responses via the cAMP-dependent protein kinase A (PKA). When activated by cAMP, the PKA translocates from the cytoplasm to the nucleus and phosphorylate CREB and other targets (Mayr and Montminy, 2001; Montminy, 1997; Gonzalez and Montminy, 1989).

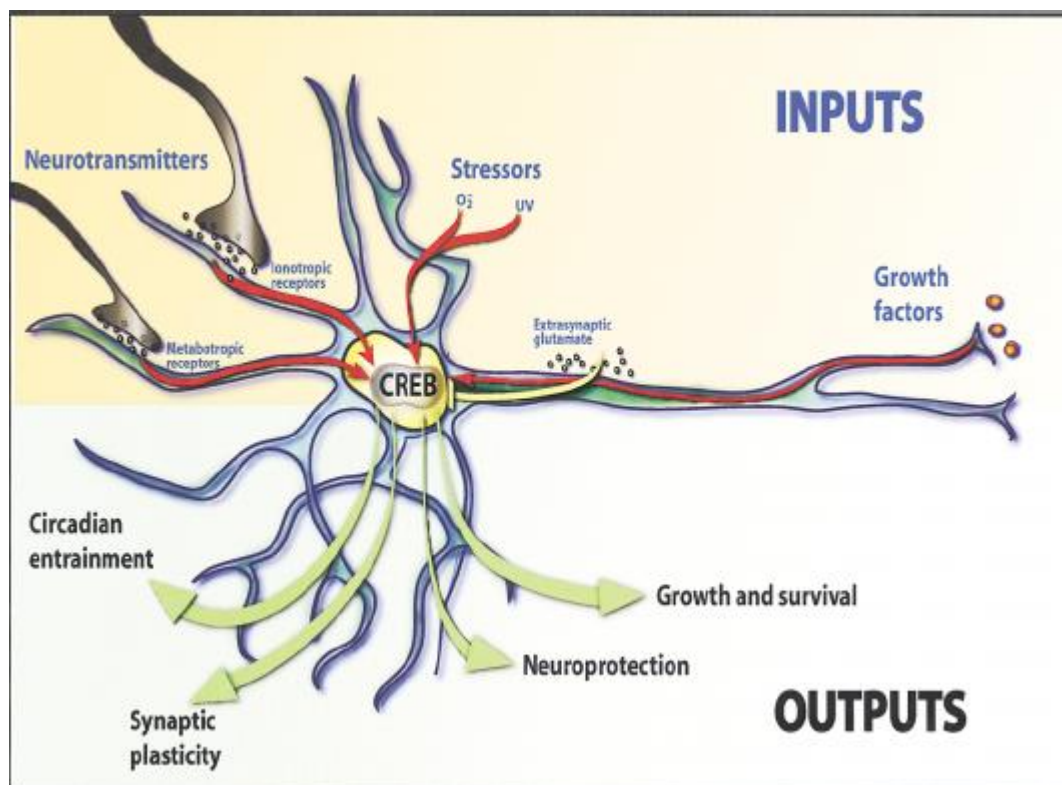


Figure 1.3. CREB dependent gene expression is vital for function in both the developing and mature nervous system. In the top half, stimulants and conditions that promote CREB phosphorylation and CREB mediated gene expression, are shown, whilst the lower half of the diagram represent physiology and pathological consequences of CREB activation (Lonze and Ginty, 2002).

Phosphorylation of CREB at Ser133 endorses recruitment of the transcriptional co-activator CREB binding protein (CBP). Mayr and Montminy (2001) discovered a peak in the transcription of cellular genes after 30 minutes of stimulation with cAMP, which overlaps with the time needed for C-subunit levels to become maximal in the nucleus. Under continuous stimulation, CREB activity attenuated over the next two to four hours, due to the dephosphorylation at Ser133 by serine/threonine phosphatases, protein phosphatase-1 (PP-1) and protein phosphatase-2A (PP-2A).

The duration of the phosphorylation of Ser133 determines which genes are targeted. In neurons, where the phosphorylation of Ser133 is maintained, *c-fos* is stimulated, whereas when Ser133 is transiently phosphorylated, then *c-fos* is not induced (Shaywitz and Greenberg, 1999). In pheochromocytoma -12 (PC12) cells, it was found that mutating the serum response element (SRE) in the *c-fos* promoter abolishes growth factor induction, and to activate *c-fos* transcription in response to growth factors, CREB needs the serum response factor (SRF) to bind to SRE. Because CREB becomes phosphorylated at Ser133 in growth factor-stimulated cells, phosphorylation at Ser133 is not sufficient for CREB activation in the absence of CREB interaction with other promoter-bound factors (Shaywitz and Greenberg, 1999).

1.3.1.3 Calcium second messenger and CREB

Similar to cAMP, Ca^{2+} is a pleiotropic second messenger, and in the nervous system, alteration in membrane potential can result in enhanced intracellular Ca^{2+} levels. Calcium is responsible for proliferation, differentiation, and synaptic plasticity in the adult mammalian nervous system (Berridge, 1993).

Changes in cytoplasmic calcium affects the SRE, whilst alterations in intracellular calcium levels involves the cAMP response element (CRE) and this involves gene expression that may be critical for long-term information storage in the nervous system (Hardingham et al., 1999). There is a 60 base pair Ca^{2+} -responsive element (CaRE), which also serves as a CRE. There is similarity between *c-fos* CaRE and somatostatin CRE, which suggests that CREB or CREB-like factors might be the targets of Ca^{2+} -activated signalling pathways, and CREB has been identified as the CaRE binding factor by various studies (Shaywitz and Greenberg, 1999; Sheng et al., 1990; Greenberg et al., 1986).

The release of neurotransmitters from synapse activate ligand-gated ion channels, such as the NMDA receptor, a major glutamate receptor subtype, allows Ca^{2+} entry. As depicted in **Figure 1.4**, changes in membrane depolarization can open voltage-gated Ca^{2+} channels (VSCCs), inducing influx of extracellular Ca^{2+} , whilst other neurotransmitter receptors that are G-protein coupled receptors activate enzymes that incite a rise in intracellular Ca^{2+} by inducing release of Ca^{2+} from internal stores (Shawywitz and Greenberg, 1999).

Using phospho-peptide mapping *in vitro*, researchers found that CaMKI, CaMKII and CaMKIV all phosphorylate CREB at Ser133. There is sufficient evidence that indicate that CaMKIV mediates membrane depolarization/ Ca^{2+} -dependent activation of CREB, such as the kinetics of CaMKIV activation within minutes after membrane depolarization

correlates with induction of Ser133 phosphorylation (Bito et al., 1996), CaMKIV is also located in the nucleus, where CREB is localized (Nakamura et al., 1995), and co-transfection of constitutively active CaMKIV drives CREB and CRE-dependent gene expression (Matthews et al., 1994). It is less likely that CaMKI and CaMKII activate CREB-dependent transcription in response to depolarization of the membrane, as CaMKI is not localised to the nucleus in neurons, and CaMKII has not been shown to activate CREB/CRE-dependent transcription in transcription assays (Shaywitz and Greenberg, 1999).

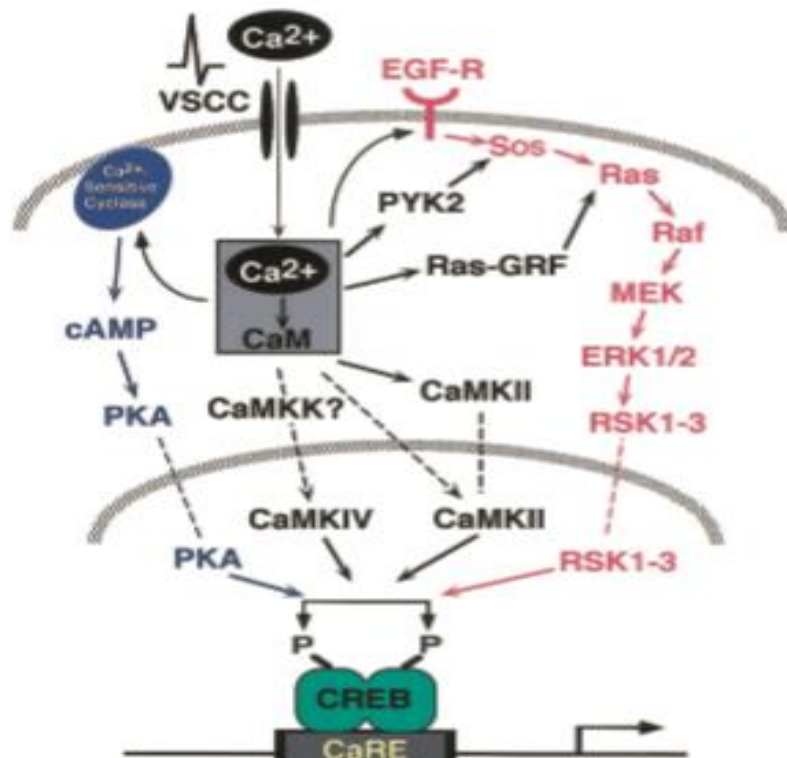


Figure 1.4. In the above diagram, dashed lines represent translocation from the cytoplasm to the nucleus. In neuronal cells, electrical activity results in membrane depolarization, opening VSCCs in the plasma membrane, leading to an influx of extracellular Ca^{2+} and the eventual activation of CREB (Shaywitz and Greenberg, 1999).

As depicted in **Figure 1.4**, when Ca^{2+} enters the cell, it binds to calmodulin (CaM), the Ca^{2+} /CaM complex can then activate the PKA pathway by directly stimulating calcium-sensitive adenylyl cyclases, resulting in the generation of cAMP and the activation of PKA. PKA can then translocate to the nucleus where it phosphorylates CREB at Ser133. Ca^{2+} /CaM also activates members of the CaMK family, all of which have the ability to phosphorylate CREB at Ser133. Ca^{2+} /CaM can activate CaMKK, which can activate CaMKIV and CaMKI. Ca^{2+} /CaM also has the ability to activate the Ras/MAPK pathway, as Ca^{2+} activation of Ras may occur via multiple mechanisms. Ca^{2+} influx can result in the activation of Epidermal Growth Factor Receptor (EGFR), which results in the activation of guanine-nucleotide exchange factors (GEF) like Sos and Ras. The activation of Ras induces Raf, MEK and extracellular signal-regulated kinase (ERK) 1/2 kinase cascade. The Mitogen Activated Protein kinases (MAPK) ERK 1/2 can directly activate members of the pp90^{RSK} family of protein kinases (RSK1-3). These activated RSKs then translocate to the nucleus where they phosphorylate CREB at Ser133. Ca^{2+} /CaM activation can lead to the activation of Ras by activating Ras-GRF (a GEF that bind Ca^{2+} /calmodulin, and activates Ras by catalysing the exchange of GDP for GTP). PYK2, a calcium-activated tyrosine kinase, can also activate Sos and results in the induction of the Ras pathway. PYK2 is a tyrosine kinase activated by Ca^{2+} , and is capable of translating Ca^{2+} stimulus into Ras activation. PYK2 functions by recruiting GEFs that activate Ras to the membrane (Shaywitz and Greenberg, 1999; Rosen and Greenberg, 1996; Lev et al., 1995).

In order to regulate the duration of Ca^{2+} -induced CREB phosphorylation, CREB phosphatases must be modulated. The rate at which CREB is dephosphorylated may reflect stimulus-induced activation of a phosphatase, or the action of constitutively active phosphatases as kinase activity decreases. Calcineurin, (PP-2B or CaN) is a Ser/Thr

phosphatase, which can be activated by $\text{Ca}^{2+}/\text{CaM}$, and was shown to dephosphorylate Ser133-phosphate *in vitro* (Enslen et al., 1994). PP-1 and PP-2A can also dephosphorylate CREB at Ser133. Certain patterns of membrane depolarization induce prolonged Ser133 phosphorylation, which suggests that a mechanism may exist to suppress CaN activity. Inactivation of CaN may be modulated by $\text{Ca}^{2+}/\text{CaM}$, so depending on neuronal stimuli that produce different patterns of Ca^{2+} influx which may result in either stimulation or inhibition of CaN activity (Shaywitz and Greenberg, 1999).

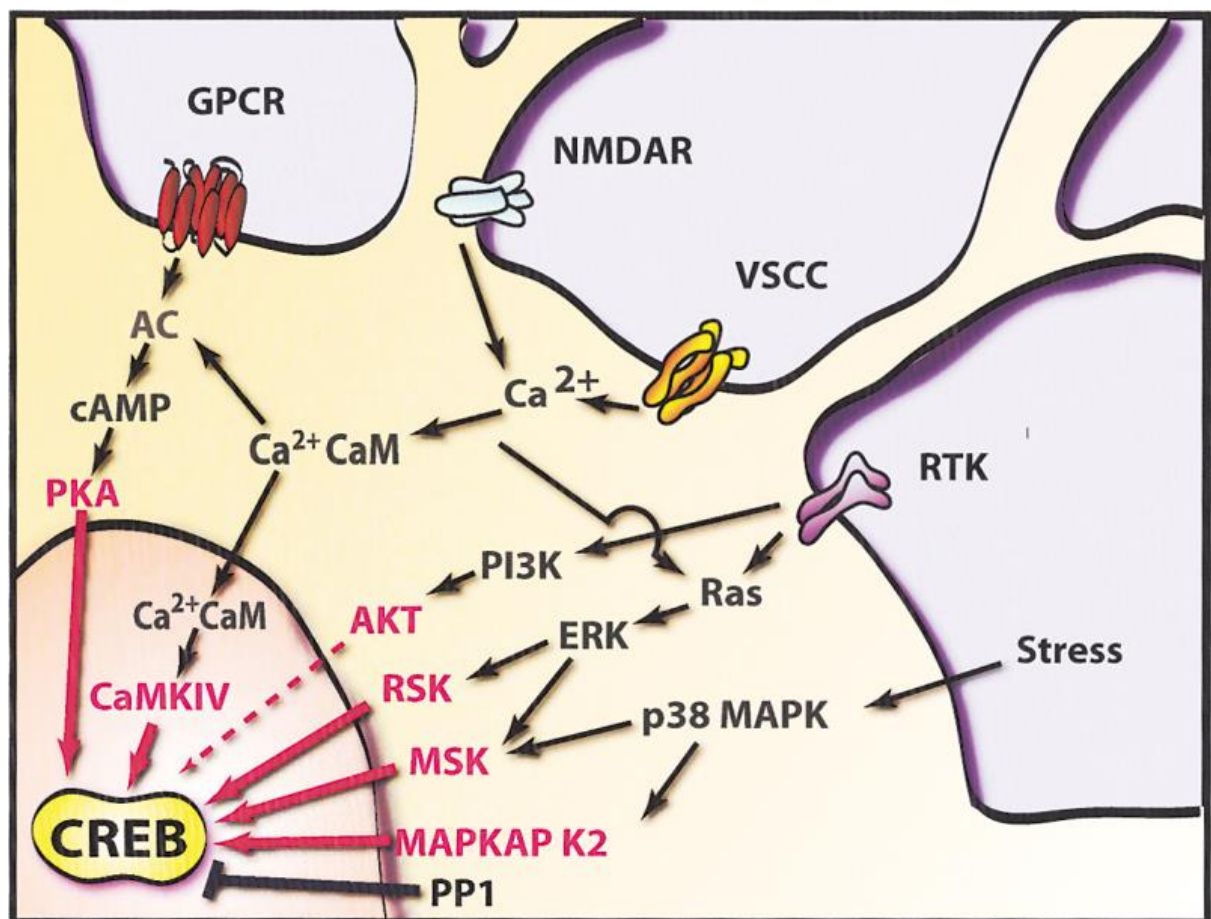


Figure 1.5 A diagrammatical overview of the signalling pathways that converge on CREB and its stimuli, in the form of stress, excitatory neurotransmitters, GPCR ligands and neuronal growth factors. The multiple signalling kinases involved in CREB

activation is also shown, as well as the intricate crosstalk between the pathways (Lonze and Ginty, 2002).

The path of Ca^{2+} entry plays a central role in determining which Ca^{2+} -dependent signalling pathways are activated. Activation of both Ligand gated VSCCs (L-VSCCs) and NMDA receptors enhance the intracellular Ca^{2+} levels, however, distinct features of each channel type and their subcellular localization will affect the signal transduction molecules that are activated (**Figure 1.5**) (Lonze and Ginty, 2002).

Ginty et al (1993) utilized hippocampal neurons to show that membrane depolarization-induced activation of L-VSCCs and glutamate-induced activation of NMDA receptors, both leading to Ser133 phosphorylation, but Bading and colleagues (1993) showed that activation of NMDA receptors fails to stimulate CREB-dependent transcription, whereas stimulation of L-VSCCs stimulate vigorous activation of CREB-dependent transcription. This effect is partly due to the fact that NMDA receptors cause a transient Ser133 phosphorylation, but the activation of L-VSCCs stimulate prolonged phosphorylation. NMDA receptors are generally located at post-synaptic dendritic sites many microns from the soma, while L-VSCCs are often localized to the proximal dendrites which are nearer to the cell body. When Ca^{2+} makes its entry into the neuron via these channels, it engages with different intracellular environments. These Ca^{2+} channels may affect the intracellular environment, and these channels themselves have the ability to alter the intracellular environment encountered by incoming Ca^{2+} ions, because the cytoplasmic domains of Ca^{2+} channels bind specific signal transduction molecules that could contribute to propagation of the Ca^{2+} signal. For L-SVCCs, data from experimentation infers that as Ca^{2+} enters the neuron, it does not diffuse very far from the site of entry and must activate a signalling pathway close to the membrane. Signalling molecules close to

the membrane could be the components of the Ras pathway (Ca^{2+} -sensitive adenylyl cyclase), or CaM, as Ca^{2+} influx via L-VSCCs stimulates translocation of CaM from the cytoplasm to the nucleus, where it has the ability to activate nuclear CaMKs. The Ca^{2+} that entered via L-VSCCs may also activate the Ras/MAPK pathway, which results in the induction of RSKs. The phosphorylation of Ser133 is initiated by Ca^{2+} that acts near its entry site and doesn't need an increase in nuclear Ca^{2+} , however nuclear Ca^{2+} maybe imperative for activating other events that are needed for CREB-dependent transcription. (Shaywitz and Greenberg, 1999).

1.3.2. ERK

1.3.2.1 The ERK pathway

The Ras/Raf/MEK/ERK pathway was found activated downstream of many membrane-bound receptors which may or may not display tyrosine kinase activity. The activation of this pathway was implicated in many cellular functions such as cell proliferation, cell-cycle arrest, terminal differentiation and apoptosis (Kolch, 2000). These pleiotropic effects have been ascribed to duration, intensity of its activation and cooperation with parallel intracellular signalling pathways (Peyssonnaud and Eychène, 2001).

As displayed in **figure 1.6** below, the ERK cascade is a signal transduction pathway that conveys signals from extracellular stimuli e.g. Growth factors, hormones and neurotransmitters. Following the interaction with membrane receptors, the signals of extracellular ligands are relayed via various mechanisms to their suitable sites of action (Segar and Krebs, 1995). The Raf/MEK/ERK cascade has been detected only in metazoans, and in this cascade Raf family functions as Mitogen activated protein kinase kinase kinase (MAPKKK). (Chen et al., 2001). They then phosphorylate and activate MEKs (MAP kinase kinases), which subsequently phosphorylate and activate ERK

(MAP kinases) and MAPK-activated protein kinases (MAPKAPKs). Following their activation, ERK translocate to the nucleus where they phosphorylate transcription factors, thereby modulating their activity. This nuclear translocation is often needed for growth factor gene regulation, cell cycle entry and cell differentiation (Peyssonnaud and Eychène, 2001; Brunet et al., 1999). The occurrence of three or more tiers in the MAPK cascade might be critical for the amplification and tight regulation of the transmitted signals, and each tier consists of many distinct protein kinases or isoforms (Seger and Krebs, 1995).

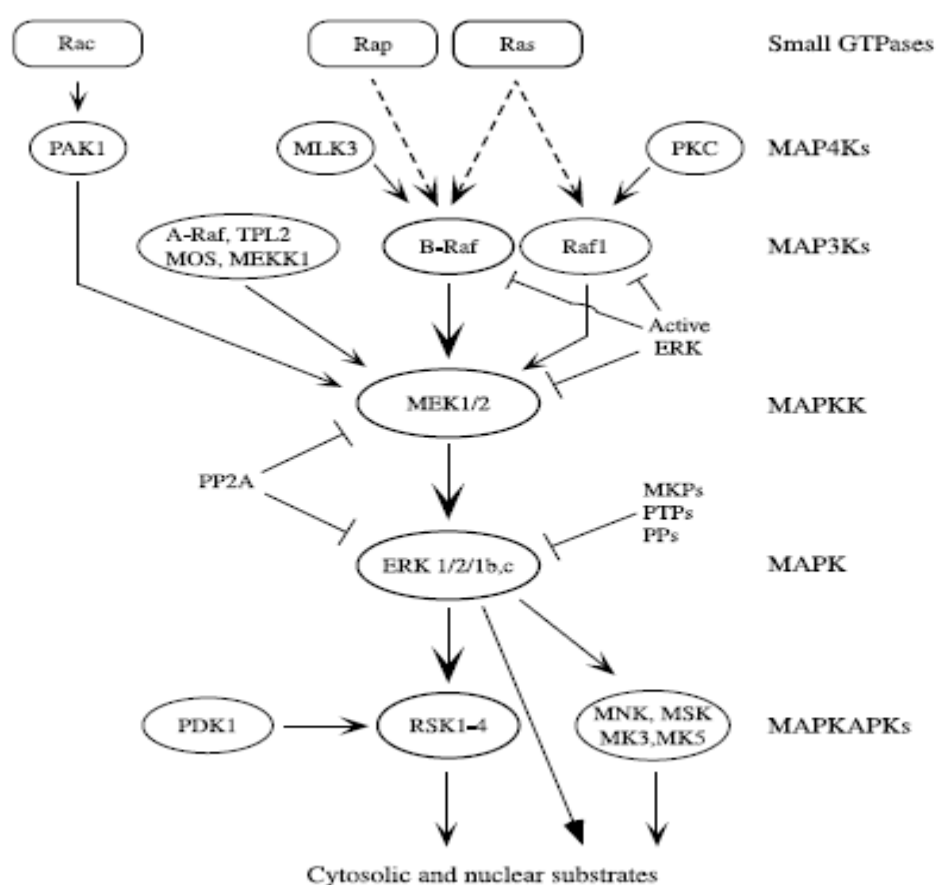


Figure 1.6 The ERK signalling cascade, schematically representing the activation and inactivation processes. Dashed lines designate activations, whilst dark arrows indicate main pathways upon growth factor activation. The phosphorylation by P21 Protein-

activated Kinase 1 (PAK1), found on Ser298, increases MEK-ERK interaction, which facilitates the kinetics of ERK activation. This can be suppressed by a feedback phosphorylation by ERK (on Thr292). ERK is negatively regulated by B-Raf and Raf1 by feedback phosphorylation. The constituents of the ERK cascade were originally elucidated as building blocks that participate in the transmission of mitogenic signals by both growth factors and hormones. The cascade can be activated by small GTP binding protein Ras, that uses Raf1 or B-Raf in MAPKKK tier, MEK 1/2 in the MAPKK tier and ERK 1/2 in the MAPK tier, as well as RSK 1/2 in the MAPKAPK tier of the cascade (Yoon and Seger, 2001).

1.3.2.2. Upstream to ERK: MAP kinase kinase kinase kinases

Upstream of the ERK cascade is Rap1, a GTP binding protein, which is a convoy to Ras and induces a prolonged activation of the ERK cascade. MAPKKKK have been implicated in the activation of the cascade, including Protein Kinase C (PKCs) and Tyrosine kinases (Yoon and Seger, 2001).

1.3.2.3. Upstream to ERK: MAP kinase kinase kinase (Raf-1 and B-Raf)

The intricate nature of the ERK cascade is evident in the MAPKKK tier, where Raf kinases may play a central role in signal transmission under certain conditions. There are three different *raf* genes which encode A-Raf, B-Raf and Raf-1. A-Raf is a poor MEK activator, whilst Raf-1 is ubiquitously expressed, and B-Raf displays highest levels of expression in neural tissues. B-Raf is the major MEK activator as it has a higher affinity for MEK-1 and MEK-2 and has a greater efficiency in phosphorylating MEKs (Peyssonnaud and Eychène, 2001).

B-Raf has been identified as the main signalling isoform under various conditions, and can be activated by Ras and Rap1 depending on the kinetics. The prolonged activation of

Rap1 in combination with the short term activation of Raf1 by Ras may induce a sustained ERK signalling in certain cell types (Busca et al., 2000). B-Raf also has a simpler activation which consists of the phosphorylation of the two main residues, while Raf-1 activation involves association with many proteins. This suggests that B-Raf and Raf1 undergo regulation by unique mechanisms and therefore can transmit signals from distinct extracellular stimuli. The activation of Raf1 involves Kinase suppressor of Ras (KSR), which serves as both a scaffold protein and a protein kinase (Yoon and Seger, 2006).

In megakaryotic and PC12 cells treated with Nerve Growth Factor (NGF), the initial peak of ERK activation is mediated by Ras/Raf-1 complexes, while the next phase of ERK activation results from the formation of Rap1/B-Raf complexes (Garcia et al., 2001). The variability in subcellular localization of Rap1, Ras and Raf proteins seen between different cell types can account for the differences observed between the different cell systems (Prior and Hancock, 2001).

There is a physical and uninterrupted interaction between Raf and Ras oncoprotein, which is a membrane-anchored GTPase detected in tumours. Raf binds to GTP-loaded Ras, following the recruitment of Raf to the plasma membrane. Raf is then subjected to phosphorylation events that further participate in conformational changes that result in its activation (Marais et al., 1997).

1.3.2.4. Upstream to ERK: MAP kinase kinase (MEK)

Two genes encode for MAPKs (ERK-1 and ERK-2), and two genes are encoded for MAPKKs (MEK-1 and MEK-2). MAPKKs phosphorylate and activate the kinases in the MAPKK levels, which are the MEKs, consisting of p45-MEK1, p46-MEK2 and an alternatively spliced form p43-MEK1b. The phosphorylation by MAPKKs occurs on

two serine residues in the activation loop of MEKs, namely Ser218 and Ser222, and this stimulates the activation of kinases. The activated MEKs are the only kinases able to specifically phosphorylate and activate ERK1 and ERK2, as well as less frequent, alternatively spliced form of these genes such as ERK1b, ERK1c, and possibly p41ERK2 (Boulton et al., 1991).

1.3.2.5 ERK: MAP kinase

ERK plays a crucial role in survival and neurogenesis, as well as a part in long-term alterations in synaptic transmissions in both the striatum and the hippocampus. The activation of p38 and ERK1/2 have been involved in the expression of POMC, which as stated earlier, is a precursor of ACTH, in corticotrophs (Van Kolen et al., 2009).

ERKs activation needs phosphorylation of Thr202, Glu203 and Tyr204 for ERK1 and Thr183, Glu184 and Tyr185 for ERK2, and these residues are found in Thr-Xaa-Tyr, which is MAPKs activation motif. ERKs also have the ability to phosphorylate RSK 3/4, mitogen-and stress activated kinase (MSK), MAPK-interacting kinases (MNKs) and MAPKAPK3 and MAPKAPK5 to a certain extent (Yoon and Seger, 2001).

The ERK molecule can interact with MEKs, which implies close interplay between the two molecules, which may be displayed in the activation process and in functions like cytosolic anchoring, as well as nuclear export of inactive ERKs. The phosphorylation of regulatory residues in the activation loop of ERKs by MEK stimulates a prominent change in conformation, dramatically enhancing the catalytic activity of ERKs, and also forcing the detachment of the two proteins. This dissociation allows the translocation of the two proteins separately to the nucleus, where ERKs persists for periods of time that vary between minutes and hours (Pouyssegur et al., 2002). MEKs on the other hand, are

rapidly exported to the cytosol, due to their nuclear export signal in their N-terminal region (Jaaro et al., 1997).

The activation of ERKs seem to be executed explicitly by the dual specificity MAPKKs, MEK1 and MEK2, that display a unique specificity to ERKs, phosphorylating them on their activatory Tyr and Thr residues. Inactive ERKs and MEKs tend to associate with each other directly, or with the aid of scaffold proteins (Morrison and Davis, 2003).

Different scaffold proteins induce interactions of the appropriate constituents of the cascade, and subsequently attract them to their proper activators and targets (Takekawa et al., 2005). In MEKs, the interaction with ERKs is facilitated by a basic and hydrophobic region in their N-terminal region (D-domain). The D-domain associates mostly with the cytosolic retention sequence (CRS) of ERK, or the common docking (CD) motif in residues 312-320 of ERK2. The hydrophobic residues found in D domain interact with hydrophobic and other residues found in different parts of the molecule. The D domain is located up to 20 residues apart from the phosphorylation site on the substrates as there is distance between CRS/CD domain and the catalytic site of ERKs (Yoon and Seger, 2006; Sharrocks et al., 2000).

The DEF (docking site for ERK) motif consists of two Phenylalanine residues, which are separated by one residue, and is often followed by a Proline residue. DEF binding site on ERKs consists of a hydrophobic pocket in the large lobe of ERKs, which is positioned next to their catalytic pockets. Only active ERK molecules can bind to the DEF motif. The phosphorylation site binding region, a CD motif and the DEF binding region contribute to the substrate specificity and affinity necessary for the proper ERKs action under differing conditions (Yoon and Seger, 2006).

ERKs activity is also regulated by phosphatases, and ERKs inactivation can be achieved by the removal of phosphates from either one of the regulatory residues, or both of them concurrently. Protein Ser/Thr phosphatase, protein Tyr phosphatase and dual specificity phosphatase were connected to the downregulation of ERKs. MAP Kinase phosphatase 3 (MKP3), is the most studied of the ERK phosphatases, it is a product of an inducible gene and is highly specific to ERKs. MKP3 acts by an uninterrupted interaction with ERKs, facilitated by a region of the phosphatase labelled kinase interaction motif (KIM), which has a D-domain similar to that of MEKs. This interaction is facilitated by many regions, such as the CRS/CR region, the groove formed by $\beta 7$ - $\beta 8$ sheets and the αD and αE helices, as well as a region that exists between them (Yoon and Seger, 2006).

1.3.2.6. Downstream of ERK

There have been 150 reported substrates of ERK. ERK phosphorylation may stimulate different molecular effects in the discrete substrates. In majority of the cases, the phosphorylation stimulates a change in conformation of the substrate, which then induces their activation or modulates their interaction with other molecules. Phosphorylation can destroy protein-protein interaction with other proteins and therefore downregulate signalling by the affected pathways (Chunderland and Seger, 2005).

ERKs exact their functions via a great number of downstream molecules that vary dependent on condition, cell types and cellular compartments. The phosphorylation of substrates is predominantly achieved when the ERKs are phosphorylated on their regulatory Thr and Tyr residues in the activation loop of ERKs. (Yoon and Seger, 2006).

The MAPKAPKs, like RSKs can also propagate the signalling of the ERK cascade, therefore the quantity of downstream substrates of ERK can reach many hundreds of proteins. These substrates can be categorically separated into transcriptional factors,

protein kinases and phosphatases, cytoskeletal and scaffold proteins, receptors and signalling molecules, apoptosis-related proteins and others. Half of these proteins are localized in the nucleus, whilst the other half is strewn across the cytosol, plasma membrane and cellular organelles. Spatial and temporal constraints exist with majority of the substrates and the order of the phosphorylation is determined by scaffold proteins and other mechanisms (Yoon and Seger, 2006).

1.4 ACTH and Signalling Pathways

1.4.1 CRH signalling pathways

POMC, the precursor of ACTH, is primarily synthesized in the intermediate and anterior lobe of the pituitary gland (Bertagna, 1994). CRH and AVP regulate ACTH release through different mechanisms. Both CRH and AVP exert their effects on the release of ACTH via GPCRs, CRHR1 and V1bR (subtype of vasopressin receptor), respectively. The molecular cross-talk concerning PKC activation through V1bR, and cAMP-PKA activation through CRHR1 is involved in CRH/AVP effects in mammalian corticotrophs (Bonfiglio et al., 2011).

CRH increases POMC transcription and ACTH secretion by binding to G-protein-coupled CRH receptors. CRH exerts its actions by the activation of two different GPCRs, CRHR1 and CRHR2, which display different localization throughout the brain. Due to these receptors only having 70% of their amino acid sequence in common, they have differing preference in ligands, as there is a difference in their N-terminal ligand-binding domain. CRH has a high affinity for CRHR1, whilst urocortin II and III have a better affinity for CRHR2. Urocortin I does not display a preference between the two receptors. Ligand-activated CRHR signal by *Gas* coupling, leads to the activation of adenylyl cyclase. cAMP is generated, which activates PKA. The activation of PKA phosphorylate

downstream of cytosolic and nuclear targets, such as CREB, resulting in the stimulation of gene transcription. When CREB is activated, the orphan nuclear receptor Nur77 is activated, which is involved in CRH-dependent POMC transcriptional regulation in corticotrophs (Zhang et al., 2012; Maira et al., 1999).

As outlined in **Figure 1.7** below, ERK 1/2 activation via CRH by both pathways in corticotrophs involves the activation of small G-protein Rap1, B-Raf kinase and ERK 1/2. In cells where cAMP induces ERK 1/2 activity, B-Raf, which is highly expressed in neurons and corticotrophs, is the decisive player of the kinase cascade Raf-MEK 1/2-ERK 1/2. This activated ERK 1/2 phosphorylate various proteins that are responsible for transcriptional regulation (Bonfiglio et al., 2011).

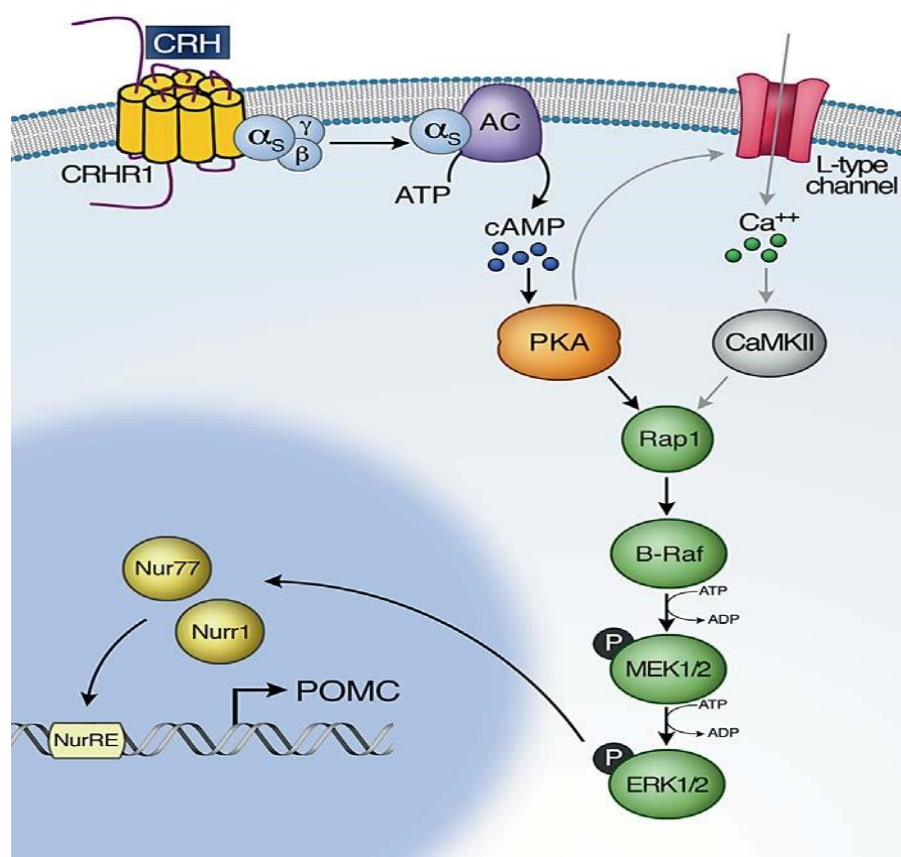


Figure 1.7. Ligand binding to CRHR1 triggers a MAPK signal transduction pathway, downstream to cAMP/PKA to regulate POMC transcription. PKA triggers two pathways,

one dependent on calcium entry via voltage-dependent L-type calcium channels at the plasma membrane, which concerns CaMK II, whilst the other pathway is calcium independent. ERK 1/2, that can be activated via both pathways, concerns the small G-protein Rap 1 and the B-Raf kinase. Activated ERK 1/2 (pERK 1/2) modulate induction and activity of orphan transcription factors Nur77 and Nurr1. This signal is passed through phosphorylation of the kinase cascade (Bonfiglio et al., 2011).

The Aim of this project was:

1. To compare levels of ACTH, CREB and ERK stimulated by the presence of D-amino acids in the pituitary.
2. To determine the effect that CRH combined with D-amino acids has on ACTH, CREB and ERK signalling in the pituitary.
3. To determine the effect that Dexamethasone (DEX) combined with D-alanine has on ACTH, CREB and ERK signalling in the pituitary.
4. To determine the effect that the NMDA receptor antagonist MK-801 has on ACTH, CREB and ERK signalling in the pituitary, in the presence of DEX and D-alanine.

Objectives:

1. Determine the optimum concentration to stimulate CREB and ERK in AtT20 cells treated with D-alanine or D-serine via western blot analysis.
2. Detect the effect ACTH has on AtT-20 cells treated with D-alanine or D-serine via ELISA.
3. Determine the optimum treatment schedule required to stimulate the activation of CREB and ERK in AtT20 cells treated with CRH and D-alanine or D-serine via western blot analysis.

4. Determine the effect CRH in the presence or absence of D-amino acids, have on ACTH produced by AtT-20 cells via ELISA.
5. Determine, using western blot and ELISA, the effect DEX has on CREB and ERK, and ACTH respectively, in AtT-20 cells, in the presence or absence of D-amino acids.
6. Determine using western blot and ELISA the effect MK-801 has on CREB and ERK, and ACTH respectively, in AtT-20 cells, in the presence or absence of DEX and D-amino acids

CHAPTER 2: METHODOLOGY

2.1 Introduction

The following questions had to be answered to assist with experimental planning:

1. What is the optimum concentration of D-amino acids required to illicit an effect on signalling protein levels and ACTH in AtT-20 cells?
2. What effect does CRH, in the presence or absence of D-amino acids, have on signalling proteins in AtT-20 cells?
3. What effect does CRH, in the presence or absence of D-amino acids, have on ACTH release in AtT-20 cells?
4. Is the effect of CRH and D-amino acids on signalling proteins in AtT-20 cells time dependent?
5. What effect does Dexamethasone (DEX), in the presence or absence of D-amino acids, have on signalling proteins in AtT-20 cells?
6. What effect does DEX, in the presence or absence of D-amino acids, have on ACTH release in AtT-20 cells?
7. Is the effect of DEX and D-amino acids on signalling proteins in AtT-20 cells time dependent?
8. What effect does the NMDA receptor antagonist, MK-801, have on signalling proteins in AtT-20 cells treated with DEX and D-amino acids.
9. What effect does MK-801, in the presence or absence of D-amino acids and DEX, have on ACTH release in AtT-20 cells?
10. Is the effect of MK-801 on signalling proteins in AtT-20 cells treated with DEX and D-amino acids time dependent?

2.2. Culturing AtT-20 cells

The AtT-20/D16v-F2 cells (**Figure 2.1**) were purchased from ATCC (CRL 1795). This tumour cell line is derived from the anterior pituitary of mice, is loosely adherent and is utilized in many studies involving the HPA axis, as it expresses ACTH.

The cells were defrosted following purchase, and grown in initial plating media (Appendix): Dulbecco's Modified Eagle's Media (DMEM) (high glucose, Sigma Aldrich, D5546, Missouri USA), containing 17% Fetal Bovine Serum (FBS) (Sigma Aldrich, F9665, Missouri USA) and 4mM L-glutamine (ThermoFisher, 25030081, Massachusetts, USA). The D16v strain of the AtT-20 cells are derived from the D1 strain of the AtT-20 cells and grow as a monolayer. These cells have relatively high rates of basal secretion, due to the frequent spontaneous membrane depolarization and neuron-like action potentials which have been documented in electrophysiological studies and supported by the qualitative spontaneous excursions in intracellular free calcium levels (Woods et al., 1992).

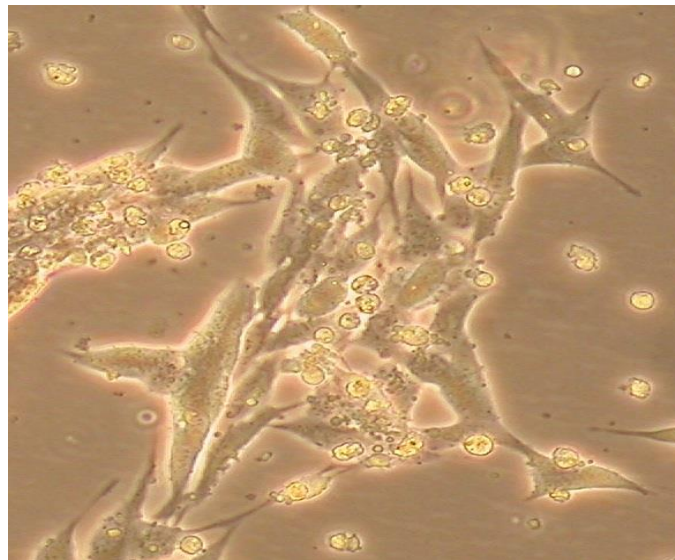


Figure 2.1. The AtT-20/D16v-F2 mouse pituitary cell line.

AtT-20 cells need to be seeded at a high density as cultures do not become fully confluent, as only about 70% of the cells attach after initial defrosting, and those that do

attach take at least 48 hours to do so. In order to achieve a high density of cells, the cells were initially grown in a T25 flask (Sarstedt, 83.3910.002, Germany) in 5mls of initial plating medium (Appendix), as these cells grow better in small volumes after defrosting. The following day, another 5 ml of initial plating medium was added, bringing up the volume to 10 ml. The 17% FBS DMEM initial plating media was used for the first week, and when the cells reached confluence, the concentration of FBS was dropped to 10%. This is the growth medium (Appendix), constituting 10% FBS and 4mM L-glutamine in DMEM.

Cells were maintained at 3×10^5 cells/ml growth media, and whilst cultures take a while to start up, once cells are confluent, they can be transferred to a T75 flask (Sarstedt, 83.3911.002, Germany), and eventually to a T175 flask (Sarstedt, 83.3912.002, Germany). The growth rate is much better once cells are in a T75 flask, and splitting of cells occurs more often (every 2-3 days). Cells trypsinize well with 0.05% EDTA Trypsin (Trypsin EDTA Solution 1X, Sigma Aldrich, 59417C, Missouri, USA) and usually take 2-3 minutes to detach.

2.3 Dose-dependent D-amino Acid study in AtT-20 cells

2.3.1 Treatment protocol

Previous studies in our lab involving the effects that D-amino acids have on the release of ACTH in AtT-20 cells, served as a measure for the concentration of D-amino acids utilized in our experiments. In these studies, D-alanine and D-serine were utilized at concentrations between 100 μ M – 200 μ M, and showed that the release of ACTH in AtT-20 cells were dose dependent (unpublished data). Therefore, in our study, we first sought to detect the optimum dosage of D-alanine (Sigma Aldrich, A7377, Missouri USA) and D-serine (Sigma Aldrich, S4250, Missouri, USA), to exert an effect on signalling

proteins in AtT-20 cells. We looked at the CREB and ERK 1/2 signalling proteins in particular, and utilized D-alanine and D-serine at concentrations of 100 μ M, 200 μ M and 500 μ M. AtT-20 cells were seeded at 2×10^5 cells/ well in a 6-well plate (Sarstedt, 83.3920, Germany) in 2 ml growth medium (Appendix, 10% FBS and 4mM L-glutamine in DMEM) per well. These cells were left to adhere for 48 hours, and then growth medium was replaced with treatment medium (Appendix, 0.2% FBS and 2mM L-glutamine in DMEM).

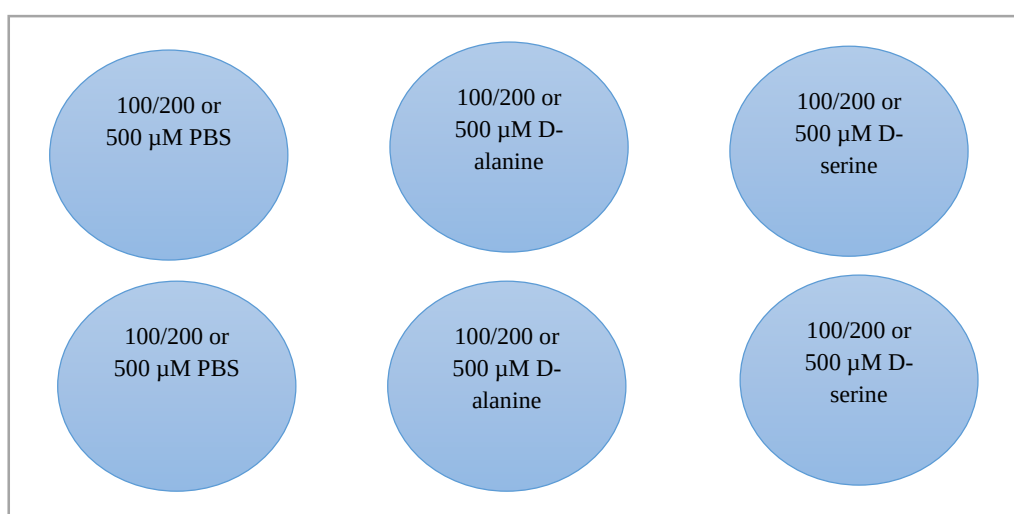


Figure 2.2. Diagrammatic representation of D-amino acid treatment of AtT-20 cells in 6-well plate.

Stock solutions of 1 mM D-alanine and D-serine were prepared (Appendix). Cells were then treated with 100 μ M, 200 μ M or 500 μ M D-alanine or D-serine, with PBS (Lonza, 17-516F, Switzerland) used as a control at equivalent quantities (See **Figure 2.2** above). This treatment lasted 24 hours, after which the cells were collected via cell scraping, and suspended in 2 ml of PBS. This 6-well plate treatment was conducted 6 times, therefore 12 replicates of each treatment were collected. The cell culture medium was also collected and stored at -80 degrees Celsius for later ACTH analysis using ELISA.

2.3.2. Cell Lysing

The cell suspension was spun at 1200 rpm for 5 minutes, the supernatant discarded and the cell pellet re-suspended in 1 ml PBS and protease inhibitor (cOmplete ULTRA Tablets, 05892791001, Roche, Switzerland) solution (Appendix). The cell suspension was then spun for 5 minutes at 1500 g at 4 degrees Celsius. Following centrifugation, the supernatant was discarded and the pellet was suspended in 100 µl of RIPA buffer (Sigma Aldrich, R0278, Missouri, USA) with protease inhibitor (Appendix). Sterile ball bearings were added to each tube, and the cell suspension was then centrifuged at the highest speed (14800 rpm) for 5 minutes at 4 degrees Celsius.

2.3.3 Bradford Assay

In order to determine the amount of protein of sample to add in gels for western blot, a Bradford assay (Quickstart Bradford 1X Dye reagent, Bio-Rad, 5000205, California, USA) was performed on the cell lysates collected (see **Figure 2.3.** below).

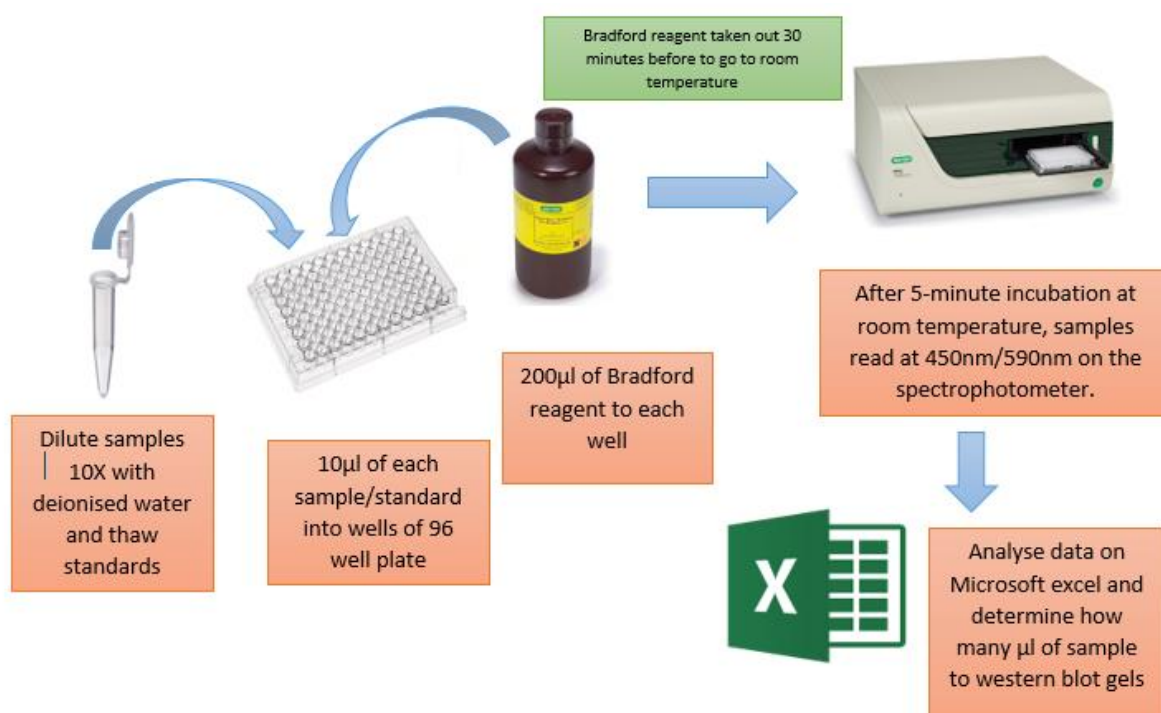


Figure 2.3. Diagrammatic representation of the Bradford assay for protein quantification of cell lysate

As per the description in **Figure 2.3**, duplicates of 10X diluted samples were prepared, by adding 2 μ l of cell lysate to 18 μ l of de-ionised water. Bovine Serum Albumin (Bio-Rad, 5000206, California, USA) (BSA) Standards were prepared in stock (Appendix), and aliquoted and stored at -20 degrees Celsius. The Bradford reagent was left at room temperature for 30 minutes. During this time, 10 μ l of standards/samples were pipetted into individual wells of the 96-well plate (Sarstedt, 82.1581, Germany) in duplicate. 200 μ l of the Bradford reagent was then added to each well containing standards/ samples, and the 96-well plate was incubated for 5 minutes at room temperature. The plate was then read on the microplate reader (Spectramax 190, Beckman Coulter, DS849B, California, USA) at 450/590nm. These readings were then analysed on Microsoft Excel, a standard curve drawn up and utilized to quantify the samples, and the quantity of cell lysate needed for 5 μ g of protein was calculated.

2.3.4. Western Blot Analysis for CREB-1

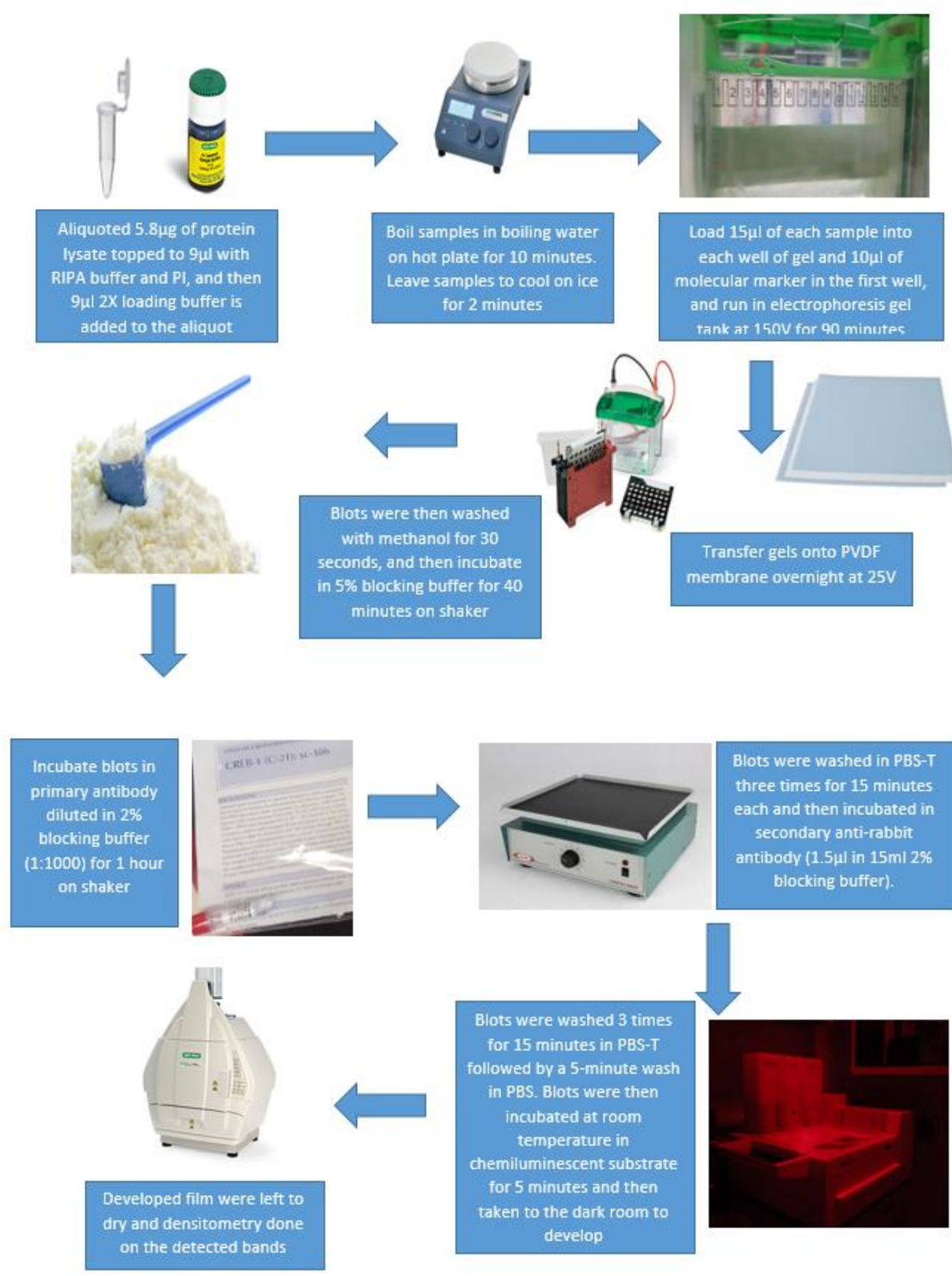


Figure. 2.4 The above diagram represents the steps involved in western blot procedure.

2.3.4.1. Sample preparation

Following the quantification of protein lysate, the quantity of lysate needed for 5 µg of protein was determined, and topped up to 9 µl with RIPA buffer and protease inhibitor solution (Appendix). 9 µl of 2X loading buffer (Fisher Scientific, BP673-1, Massachusetts, USA) was added to this, and then the samples were boiled for 10 minutes in boiling water on a hot plate. Samples were then left to cool for 2 minutes on ice.

2.3.4.2. Running the gel

As described in **Figure 2.4**, gels (4-20% Mini-PROTEAN TGX precast protein gels, Bio-Rad, 4561096, California, USA) were loaded with 15 µl of each sample (6 samples of each treatment), with the first well reserved for 10 µl of molecular weight marker (Precision plus protein standards Kaleidoscope, Bio-Rad, 1610375, California, USA). 1X Running Buffer (Appendix) was added to the gel tank (Mini PROTEAN Tetra Vertical Electrophoresis cell for mini precast gels, Bio-Rad, 1658004, California, USA). The gels were then run at 150 V for 90 minutes.

2.3.4.3. Transfer Gel

Transfer buffer (Appendix) was prepared and poured into transfer tanks. Gels were removed from the tank, and transferred onto PVDF membrane (Immobilon-P membrane, Merck Millipore, IPVH00010, Germany). A sandwich consisting of a single layer of foam pads, 2 layers of blotting paper, gel, PVDF membrane, 2 layers of blotting paper and a single layer of foam pads, were enclosed in a gel holder cassette and placed in a transfer tank (Criterion Blotter, Bio-Rad, California, USA), where it was run overnight at 25 V.

2.3.4.4. Incubation

Blots were removed from cassettes and placed in methanol (Fisher chemical, A457-4, United Kingdom) for 30 seconds. 5% blocking buffer (Appendix) was prepared, and the blots were then placed in this buffer on a shaker for 40 minutes. Primary antibody of CREB-1 (Santa Cruz, sc-186, California, USA) was prepared at a 1:1000 dilution in 2% blocking buffer (Appendix), and blots were placed in primary antibody solution for 1 hour. Following this incubation, blots were washed in PBS-T (Tween 20, P9416, Sigma Aldrich, Missouri, USA) (Appendix) 3 times for 15 minutes each, and then incubated in secondary goat anti-rabbit antibody (goat anti-rabbit antibody conjugated to HRP, Bio-Rad, 1662408, California, USA) at a 1: 10 000 dilutions in 2% blocking buffer for 40 minutes. Blots were then washed three times for 15 minutes each with PBS-T, and then for 5 minutes with PBS (Sigma Aldrich, P4417, Missouri, USA) (Appendix). Blots were then incubated at room temperature with ECL prime western blotting detection reagent (Appendix) (GE Healthcare, RPN2236, United Kingdom) for 5 minutes.

2.3.4.5. Developing

Blots were then developed in the dark room, by exposing it against a film (Autoradiography film Hyperfilm ECL, GE Healthcare, 10752067, United Kingdom) until relevant bands are detected. Films were then developed in developer (Ilfosol 3 film developer, Harman Technology, United Kingdom) solution for 60 seconds, washed in water, and then fixed in fixing (Ilford Hypam, Harman Technology, United Kingdom) solution for 60 seconds.

2.3.4.6. Stripping

Blots were then stripped by incubating it in stripping buffer (Appendix) at 45 degrees Celsius in a shaking incubator (Stuart, SI600, United Kingdom) for 30 minutes. Blots

were then washed 3 times for 10 minutes in PBS-T, and were ready to incubate with another antibody.

2.3.4.7. Densitometry

Films were left to dry, and then bands were measured by measuring its density using scanner/imaging system (AlphaImager 3400, Alpha Innotech, California, USA).

2.3.4.8. Statistical Analysis

The densities collected were collated on Microsoft Excel, where data was presented as ratios, where phosphorylated protein was represented as phosphorylated antibody/ total antibody, and total protein as total antibody/ beta-actin. This data was then analysed on IBM SPSS statistics software version 23, using One Way ANOVA statistics, followed by Tukey's Test. Graphs of the data were created using GraphPad Prism version 6, and values were expressed as means $\pm$ SE. $p < 0.05$ was used as indication of statistical significance.

2.3.5. Western Blot Analysis for PhosphoCREB

The stripped blot was probed for phosphorylated CREB using the same protocol outlined above, with the exception being the incubation step. Primary antibody of phospho-CREB (Cell signalling technology, 9198, Massachusetts, USA) was prepared at a 1:7000 dilution in 2% blocking buffer (Appendix). Blots were incubated with secondary goat anti-rabbit antibody at a 1: 10 000 dilution in 2% blocking buffer.

2.3.6. Western Blot Analysis for β -actin

The stripped blot was probed for the loading control antibody, β -actin, using the same protocol outlined above, with the exception being the incubation step. Primary antibody of beta actin (conjugated Beta actin (HRP) AC-15, Abcam, Ab49900, United Kingdom) was prepared at a 1:1000 000 dilution in 2% blocking buffer.

2.3.7. Western blot analysis for ERK 1/2

Samples were prepared similar to the above description in **Figure 2.4**, with exception of the sample buffer used. Samples were prepared using 5X loading buffer (Appendix), therefore 5.8µg of protein was quantified, and topped up to 14µl with RIPA buffer and Protease inhibitor. 3.5µl of 5X loading buffer was then added to the samples and then boiled, loaded, run and transferred onto PVDF membranes.

The resultant blots were probed for ERK 1/2 (Abcam, ab54230, Cambridge, United Kingdom) in 2% blocking buffer, and goat-anti-mouse secondary antibody (Bio-Rad, 1706516, California, USA) at a concentration of 1: 20 000 in 2% blocking buffer.

2.3.8. Western blot analysis for pERK 1/2

The blots were then stripped and probed for phosphorylated ERK using the pERK 1/2 antibody (Merck Millipore, 05-797R, Germany) at a concentration of 1:500 in 2% milk, and goat anti-rabbit secondary antibody (1: 10 000) in 2% milk. This blot was stripped and probed for β-actin as described above.

2.3.9. ELISA analysis for ACTH in AtT-20 culture media

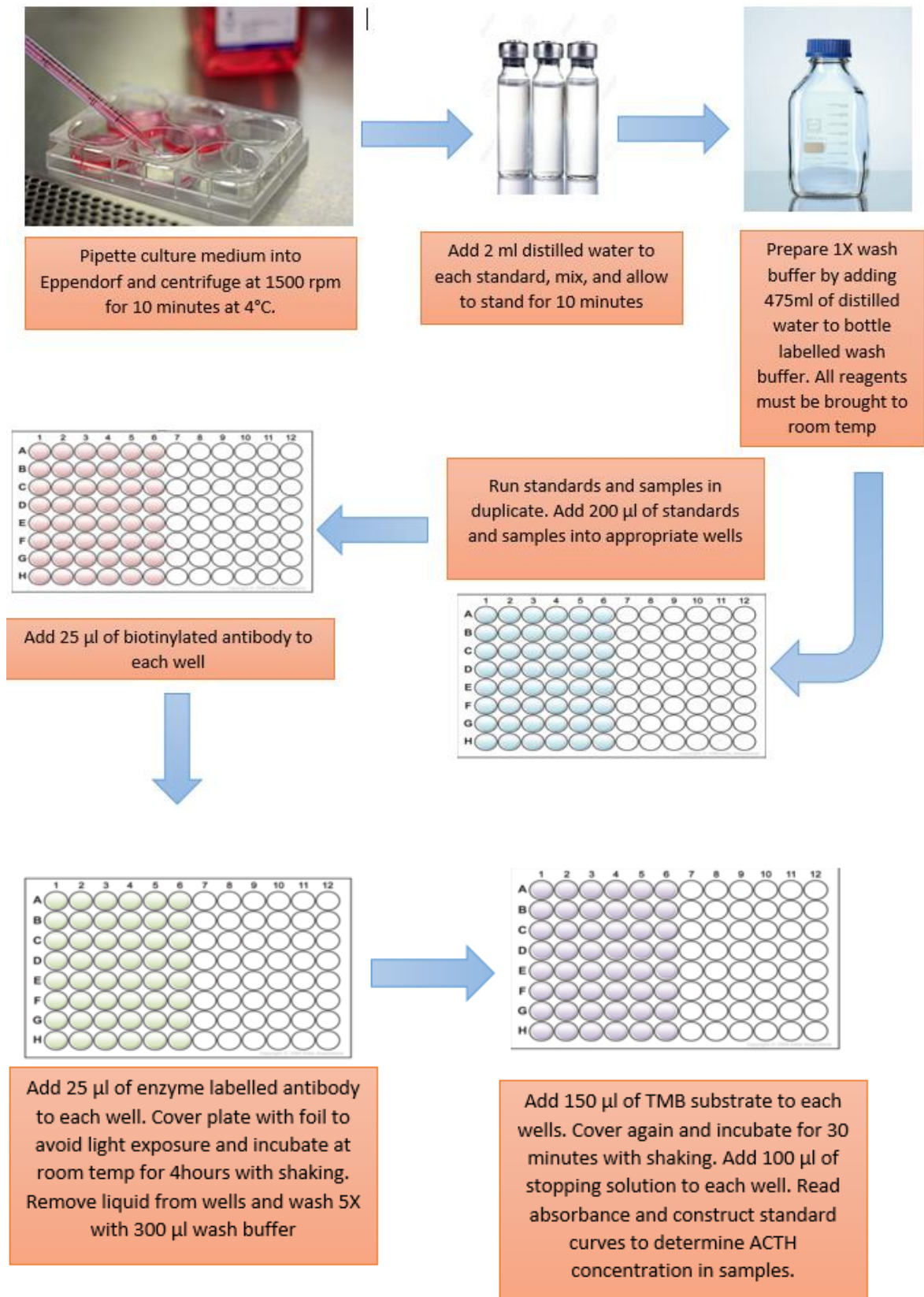


Figure 2.5 Diagram representing ACTH assay protocol

Collected cell culture media was analysed for ACTH (Adrenocorticotrophic hormone ELISA (Mouse/Rat) kit, EA100849, Insight Biotechnology, United Kingdom) following the protocol described in **Figure 2.5** above. In summary, cultured media was spun at 1500 rpm for 10 minutes at 4 degrees Celsius and resultant supernatant was collected. Standards were diluted in 2 ml distilled water and mixed well, and allowed to stand for 10 minutes, mixing well by inversion afterwards. Wash buffer (1X) is prepared by adding 475 ml of distilled water to the wash buffer concentrate provided. All reagents and controls were left to warm to room temperature. All standards and samples were run in duplicate. 200 µl of standards and samples were added to individual wells, then 25 µl of biotinylated antibody to appropriate wells, followed by 25 µl enzyme labelled antibody to each well. The plate was then covered and incubated for 4 hours at room temperature with shaking. Liquid was removed from wells and washed 5X with 300 µl wash buffer, and 150 µl of TMB substrate is then added. Plate was again covered and incubated at room temperature for 30 minutes with shaking. 100 µl of stopping solution was then added to wells, and then mixed gently. Absorbance was read at 450nm (Spectramax 190, Beckman Coulter, DS849B, California, USA) against 250 µl distilled water, and then once more at 405 nm against distilled water. A standard curved was created from the data, and quantity of ACTH in samples calculated.

2.4. AtT-20 cells treated with CRH, in the presence or absence, of D-amino Acids

2.4.1. Introduction

CRH stimulates the transcription factors AP1, CREB and Nur, these in turn stimulate ACTH biosynthesis and the promotion of POMC. Therefore, CRH is a potent stimulator of ACTH production in the pituitary gland (Vila et al., 2004). The binding of CRH to its

receptor in ACTH- producing anterior pituitary gland cells stimulates the cAMP-dependant pathway, it is likely that the subsequent phosphorylation of CREB by cAMP-dependant protein kinase A, acts as a transactivator of several cAMP-responsive genes (Waeber et al.,1998).

Therefore, our next set of experiments involved investigating the effect CRH has on AtT-20 cells, in the presence of D-amino acids. For these studies, based on the previous study, we used D-alanine and D-serine at 500 μ M, as it was the optimum concentration that initiated a significant change in the CREB signalling in AtT-20 cells. We used CRH at a concentration of 100 nM based on studies utilizing CRH in AtT-20 cells (Strowski et al., 2002; Aoki et al., 1996), and treatment lasted 24 hours which was also based on literature (Páez-Pereda et al., 2001). The second part of this study, which consisted of a time dependent study of CRH treatment, was based on findings by Kageyama et al. (2008), where they found that incubation with CRH for 5 minutes significantly increased ERK phosphorylation in a dose-dependent manner. They then examined AtT-20 cells to determine if treatment with CRH affected CREB phosphorylation. Their findings showed that incubation with 100nM of CRH increased CREB phosphorylation most effectively at 5 minutes after its addition.

2.4.2. Treatment Protocol

AtT-20 cells were seeded as described above and prepared for treatment. Cells were then treated with CRH (Sigma Aldrich, C3042, Missouri, USA) at 100 nM, in the absence or presence of D-alanine or D-serine at 500 μ M, and PBS was used as a control (**Figure 2.6**). This treatment lasted 24 hours and was conducted in triplicate for each plate, therefore 6 replicates of each treatment was collected.

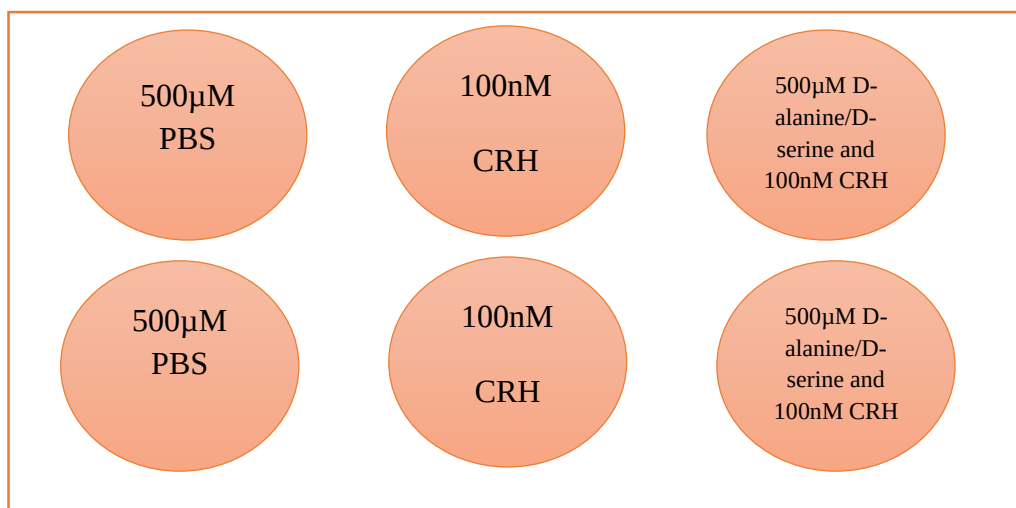


Figure 2.6. Diagrammatic representation of treatment of AtT20 cells with CRH in the absence or presence of D-amino acids in 6-well plate.

For the second part of this study, AtT-20 cells were seeded at 2×10^5 cells/well as previously described. Cells were then treated with 500 µM D-alanine or D-serine for 24 hours, followed by 5 or 15-minute treatment with CRH at 100nM. Duplicate wells were treated with just CRH for 5 and 15-minutes, to determine the effect that CRH has on AtT-20 cells in the absence of D-amino acids. PBS was used as a control at equivalent concentrations. This treatment was conducted in triplicate for each treated plate, therefore 6 replicates of each treatment was collected.

Cells were then lysed and protein quantified via Bradford assay as described in **Figure 2.4** above. Cultured medium, that was collected, was analysed for ACTH using the protocol explained in **Figure 2.5**, whilst cells were prepared for western blot analysis for signalling proteins CREB (Chapter 2.3.4 and 2.3.5) and ERK 1/2 (Chapter 2.3.7 and 2.3.8). β -actin was used as the loading control (Chapter 2.3.6).

2.5. Effect of dexamethasone (DEX) and D-amino acids on AtT-20 cells

2.5.1 Introduction

Glucocorticoids exert their physiological function by binding to receptors, therefore its effects are dependent on the number of cellular receptors available for binding.

Glucocorticoid hormones inhibit the secretion of ACTH by anterior pituitary corticotrophs. There are two phases in which glucocorticoids exert feedback inhibition, an early (0.5 to 2 hours) phase which is mediated by type II glucocorticoid receptors, and a slow phase (>6-8 hours), which involves the suppression of biosynthesis of POMC and may also involve the suppression of cAMP generated by CRH (Woods et al., 1992).

The concentration of DEX at 100nM was chosen for our study, as Rahnert et al (2016) showed that DEX at this concentration caused an increase in the phosphorylation of CREB, following its 48-hour treatment with muscle cells. A time dependent study of DEX treated to primary cultures of glomerular mesangial cells from Sprague-Dawley rats, showed that a 24-hour treatment of DEX resulted in the most prominent band for CREB-1, in comparison to 1 hour and 5 hour treatments (Eberhardt et al., 2002).

The successful phosphorylation of ERK was detected in A7r5 rat vascular smooth muscle cells after 10-minute treatment with 1 μ M DEX, and the successful phosphorylation of CREB detected in HT22 hippocampal murine cells after 15-minute treatment with 1 μ M DEX, lead to our investigating the time dependent effects of DEX on AtT-20 cells in the presence of D-amino acids (Zhang et al., 2013, Föcking et al., 2003).

2.5.2. Treatment Protocol

AtT-20 cells were seeded as described above. Cells were then treated with 500 μ M D-alanine or D-serine and DEX (Sigma Aldrich, D4902, Missouri USA) at 100nM (Appendix) for 24 hours (**Figure 2.7**). PBS was used as a control and cells were treated with DEX alone (100 nM). This treatment lasted 24 hours.

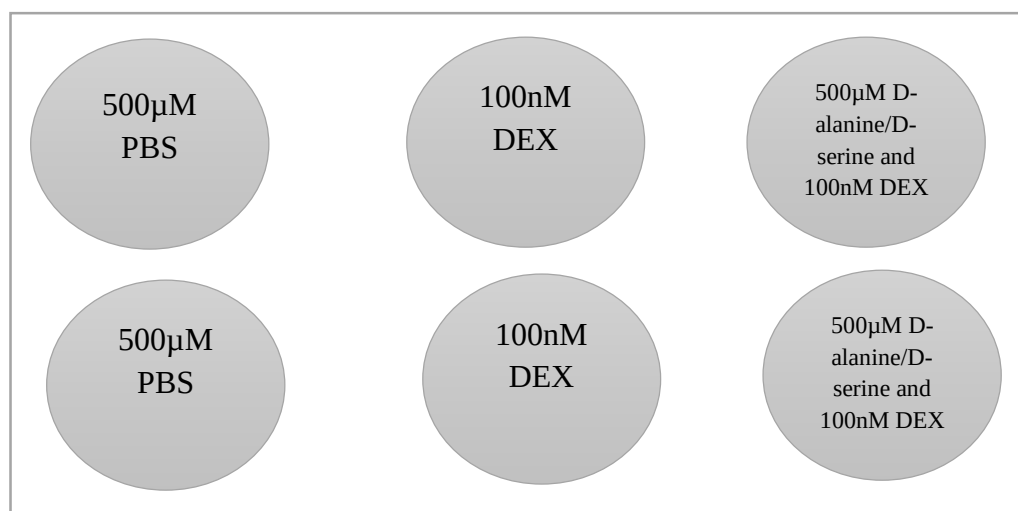


Figure 2.7. Diagrammatic representation of 24-hour treatment of DEX and D-amino acids on AtT20 cells in 6-well plate.

AtT-20 cells were seeded again and treated as described in **Figure 2.8**.

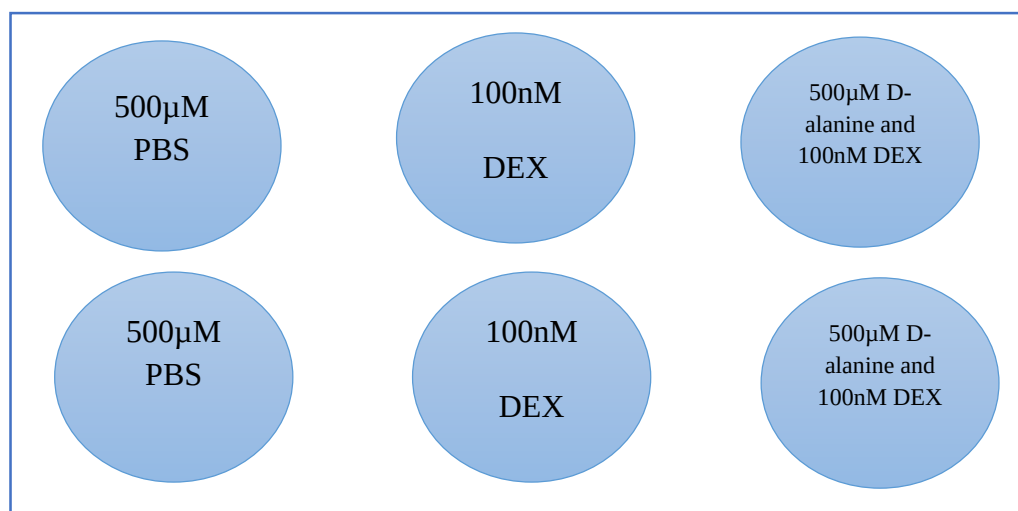


Figure 2.8. Diagrammatic representation of time dependent DEX treatment of AtT20 cells in 6-well plate.

The second part of our study constituted cells treated with 500 μ M D-alanine for 24 hours, followed by 5 or 15-minute treatment with DEX at 100nM, just DEX for 5 or 15 minute at 100 nM and PBS used as a control at equivalent concentrations (**Figure 2.8**). Following this treatment, cells were collected via cell scraping, and suspended in 2mls of PBS. This treatment was conducted in triplicate for each sample, therefore 6 replicates of each treatment was collected. Cells were then lysed and lysate protein quantified via Bradford assay as described in **Figure 2.3** above, and then probed for ERK 1/2 and CREB protein via western blot analysis as described in Chapter 2.3 above. ACTH was detected in culture medium as per description in **Figure 2.5**.

2.6. Effect of MK-801 on AtT-20 cells treated with DEX and d-amino acids

2.6.1. Introduction

Based on data obtained in the DEX and D-amino acid study, we sought to determine the receptor mediated pathway that D-alanine utilizes to exert its effects. This is as D-alanine and D-serine appear to carry out their effects via two separate signalling pathways (CREB and ERK respectively) in the presence of DEX. It is our deduction that this is due to the binding of two different receptors. As D-serine has a high affinity to NMDA receptors, it is possible that it is D-alanine that is binding to another receptor i.e. the GlyR. To confirm this, we used the MK-801 NMDA receptor antagonist, which should have an inhibitory effect on D-serine induced ERK phosphorylation, whilst having no effect on D-alanine induced CREB stimulation.

Therefore, we seeded 200 000 cells per well, left these cells to attach over 48 hours in 10% FBS 4mMol L-glutamine DMEM, replaced this media with treatment media

consisting of 0.2% FBS, and treated the cells for 24 hours with D-serine or D-alanine (500 μ M) and DEX (100nM), followed by a 1-hour treatment of MK-801 (10 μ M).

This treatment garnered no effect, therefore we decided to treat the cells for a longer period of 8 hours, based on literature showing the phosphorylation of CREB after 8-hour treatment with MK-801 in hippocampal neurons (Hardingham et al., 2002).

A study by Ahn et al (2006) showed that MK-801 can have a varying effect on the phosphorylation of ERK in the frontal cortex dependent on its dosage concentration, where there was a decrease in phosphorylation in ERK when the dosage concentration was 0.5-1 mg/kg or 4-8 mg/kg, but there was an increase in the phosphorylation of ERK when treated with 2mg/kg.

Based on the literature, we have therefore conducted an experiment where AtT-20 cells were treated with DEX and D-amino acids and MK-801 for 24 hours, to determine whether the effect MK-801 has on ERK and CREB signalling, in the presence DEX and D-amino acids, is time dependant.

2.6.2 Treatment Protocol

AtT-20 cells were seeded as per protocol previously described and treated as per **Figure 2.9** below. Cells were then treated with 500 μ M D-alanine or D-serine and DEX at 100 nM (Appendix) for 24 hours, followed by an 8-hour treatment with MK-801 (Sigma Aldrich, M107, Missouri, USA) (Appendix). To ascertain the effects DEX has on AtT-20 cells in the presence of D-amino acids, but in the absence of MK-801, cells were treated with 100nM DEX and 500 μ M D-alanine or D-serine. PBS was used as a control at equivalent concentrations.

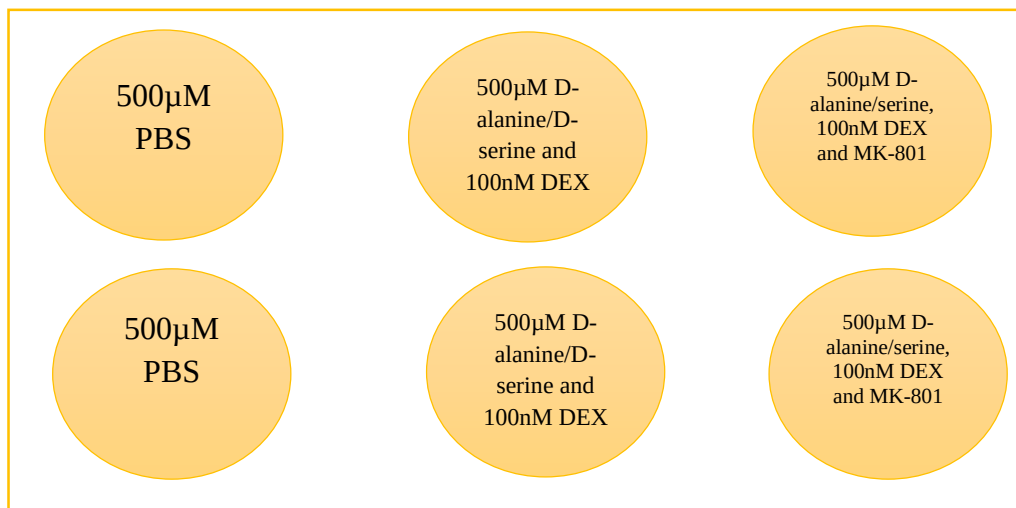


Figure 2.9. Diagrammatic representation of treatment of DEX and D-amino acids, and MK-801, on AtT20 cells in 6-well plate.

The second part of our study consisted of cells treated with MK-801 at 10 µM (Appendix) for 24 hours, in the presence or absence of 500 µM D-alanine or D-serine and DEX at 100nM (Appendix). PBS was used as a control and this treatment was conducted in triplicate for each plate, therefore 6 replicates of each treatment was collected (**Figure 2.9**). Cells were analysed for CREB (phosphorylated and total) and ERK 1/2 (phosphorylated and total) as described above, whilst collected media was analysed for ACTH (**Figure 2.6**)

**CHAPTER 3: THE EFFECT OF CRH ON ACTH RELEASE AND
INTRACELLULAR SIGNALLING IN THE PRESENCE OR ABSENCE OF D-
ALANINE**

3.1 Introduction

The co-localization of D-alanine with ACTH in pituitary cells suggests that the D-amino acid may have a regulatory role in the secretion of the stress hormone. This influence may be exerted either at basal level of ACTH secretion and/or when the hormone is actively released by CRH. Studies have shown that the CREB and ERK signalling pathways are integral to the modulation of ACTH secretion, and their activation increases ACTH release. In this chapter, cultured AtT-20 cells were used to determine the influence of D-alanine on ACTH secretion and the levels of CREB and ERK signalling proteins. The effect of D-serine was also explored for comparison.

3.2 Methods

3.2.1 Effect of D-amino acids on ACTH release, and CREB and ERK levels

The AtT-20 cells were cultured as described in Chapter 2.2. The effect of a 24-hour incubation with D-alanine or D-serine (100, 200, 500 μ M) on ACTH release into the culture media, and the levels of CREB and ERK proteins in cells, were measured following protocols detailed in Chapter 2.3.4 and 2.3.7, respectively. pCREB is represented as a ratio of pCREB/tCREB, whilst CREB-1 is represented as CREB/ β -actin (tCREB). pERK 1/2 is represented as pERK/tERK, whilst ERK 1/2 is represented as ERK 1/2/ β -actin (tERK 1/2). Twelve replicates of each condition were run. A diagrammatic representation of this study is shown below (**Fig 3.1**).

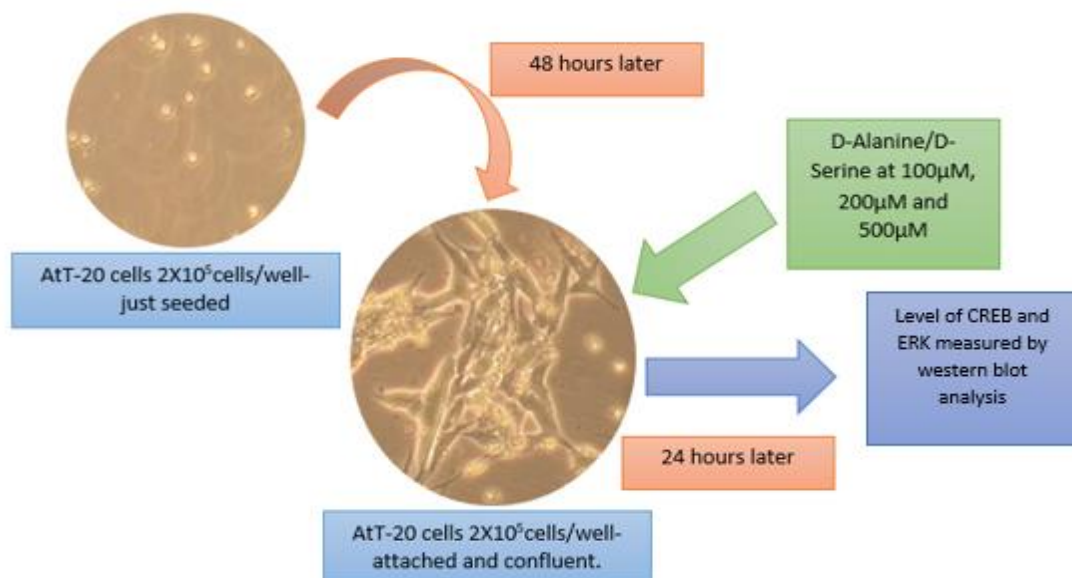


Figure. 3.1. Diagrammatic representation of dose-dependent D-amino acids study.

3.2.2 Effect of CRH and D-amino acid on ACTH release, and CREB and ERK levels

The influence of D-amino acids on CRH-mediated ACTH secretion, was tested at the highest concentration (500 μ M) of D-alanine or D-serine. The experimental procedure is outlined below (**Fig 3.2**). To test the rapid effects of CRH on signalling proteins, cells were incubated with the peptide for 5 and 15 minutes, following a 24 hour incubation of D-amino acids. All cell culture, assay and western blot techniques are described in Chapter 2. Results are mean of 6 replicates $\pm$ SEM.

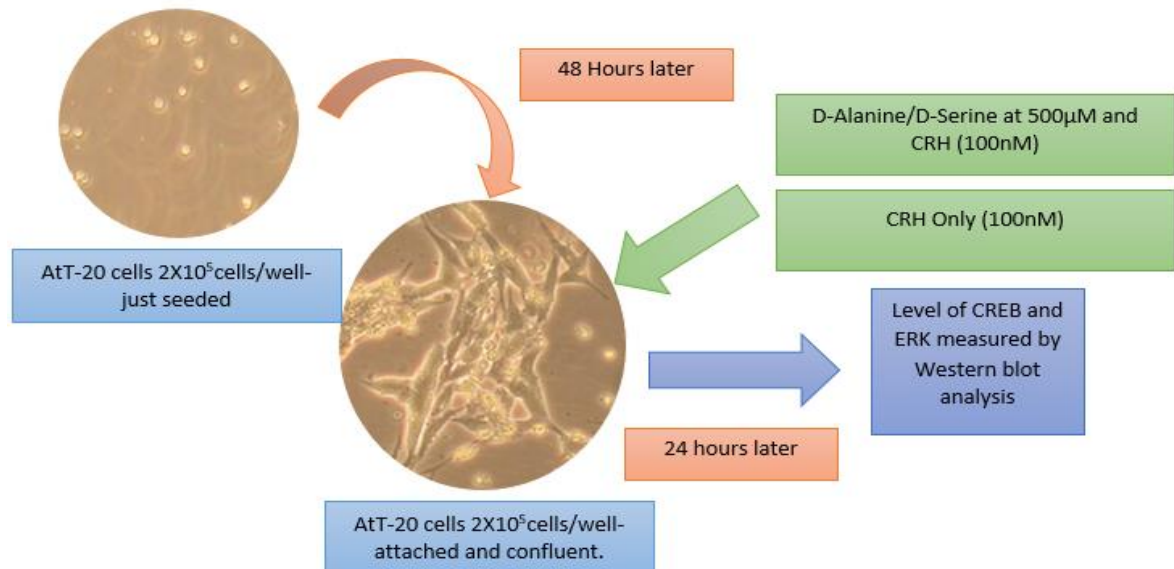


Figure 3.2. Overview of treatment protocol of AtT-20 cells treated with D-amino acids and CRH for 24 hours

3.3 Results

3.3.1 Effect of D-amino acids on ACTH release, and CREB and ERK levels

An increased secretion of ACTH from AtT-20 cells into the culture media, was observed following a 24-hour incubation of AtT-20 cells with D-alanine (+12%, $p=0.038$) or D-serine (+13%, $p=0.043$) at the highest concentration only (**Fig 3.3**). Under these conditions, the intracellular levels of tCREB were significantly increased by D-alanine (+35%, $p=0.011$) and D-serine (+29%, $p=0.028$) at 500 μ M (**Fig 3.4**). There were no significant differences between groups in pCREB/tCREB levels at this concentration (PBS= 1.000 ± 0.096 ; D-alanine = 1.247 ± 0.058 ; D-serine = 1.019 ± 0.086). No significant differences in ACTH levels, pCREB/tCREB or tCREB levels were observed between groups at lower concentrations of these D-amino acids (**Table 3.1 and Table 3.2**). The levels of ERK1/2 signalling proteins, were not affected by neither D-alanine, nor D-serine, at 500 μ M (**Fig 3.5**), or at a lower concentration (data not shown).

Table 3.1: Level of ACTH detected in culture media of AtT-20 cells treated with 100 μ M or 200 μ M D-amino acids for 24 hours. Data is mean of 6 replicates $\pm$ SEM.

Treatment	ACTH level
D-alanine (100 μ M)	0.98 $\pm$ 0.033
D-serine (100 μ M)	1.10 $\pm$ 0.048
PBS (100 μ M)	1.00 $\pm$ 0.012
D-alanine (200 μ M)	1.09 $\pm$ 0.053
D-serine (200 μ M)	1.06 $\pm$ 0.057
PBS (200 μ M)	1.00 $\pm$ 0.011

Table 3.2: Level of pCREB/tCREB and tCREB expressed in AtT-20 cells treated with 100 μ M or 200 μ M D-amino acids for 24 hours. Data is mean of 6 replicates $\pm$ SEM.

	pCREB/tCREB	tCREB
D-alanine (100 μ M)	0.952 $\pm$ 0.024	1.052 $\pm$ 0.051
D-serine (100 μ M)	0.978 $\pm$ 0.057	0.970 $\pm$ 0.043
PBS (100 μ M)	1.000 $\pm$ 0.053	1.000 $\pm$ 0.061
D-alanine (200 μ M)	1.000 $\pm$ 0.036	1.025 $\pm$ 0.090
D-serine (200 μ M)	0.973 $\pm$ 0.051	1.002 $\pm$ 0.131
PBS (200 μ M)	1.000 $\pm$ 0.047	1.000 $\pm$ 0.054

3.3.2 Effect of CRH and D-amino acid on ACTH release, and CREB and ERK levels

The incubation of AtT-20 cells with CRH alone resulted in a significant increase in ACTH levels (+17%, $p=0.035$) in culture media, compared to PBS control (**Fig 3.6**). The levels of intracellular CREB was not affected by CRH, or CRH in the presence of D-alanine (**Fig 3.7**).

There was no significant difference in either the pERK/tERK levels, or the tERK 1/2 levels in cells incubated with D-alanine and CRH for 24 hours (**Fig 3.8**). However, there was an increased trend in pERK/tERK in cells incubated with CRH only, when compared to the control (+68%, $p = 0.074$). In corresponding experiments with D-serine, CRH did not elicit ACTH release, and also reduced ERK signalling (see Appendix). Since this is

contrary to current (Fig 3.6) and published findings, it was concluded that this set of experiments failed.

There were no significant differences ($p>0.05$) detected in CREB or ERK signalling in AtT-20 cells treated with short incubation of CRH in the presence or absence of D-alanine (Table 3.3 and Table 3.4).

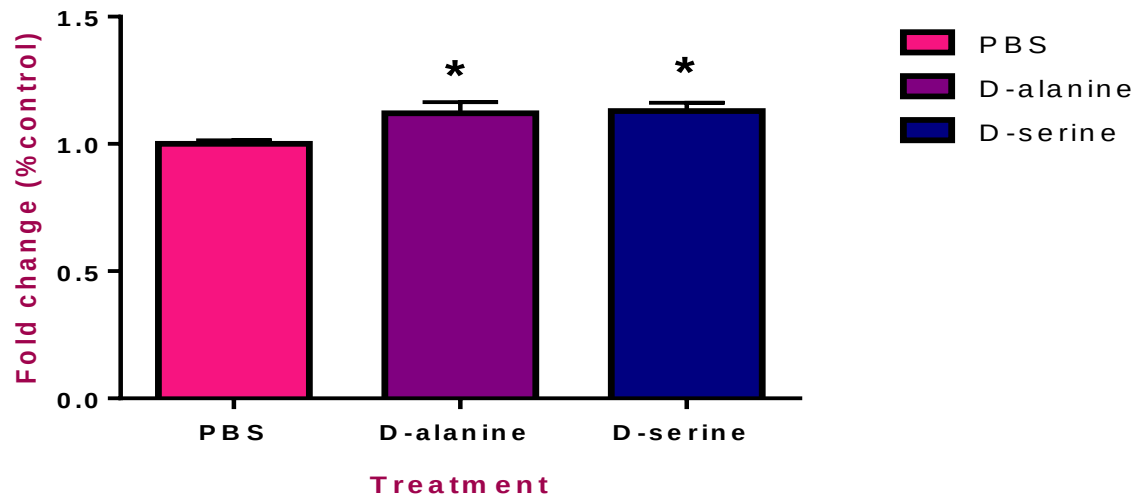


Figure 3.3. Level of ACTH detected in culture media of AtT-20 cells treated with 500 μ M D-alanine or D-serine, where PBS was used as control. Data are mean of 12 replicates $\pm$ SEM, and are normalized to PBS (i.e. PBS = 1) * $p<0.05$

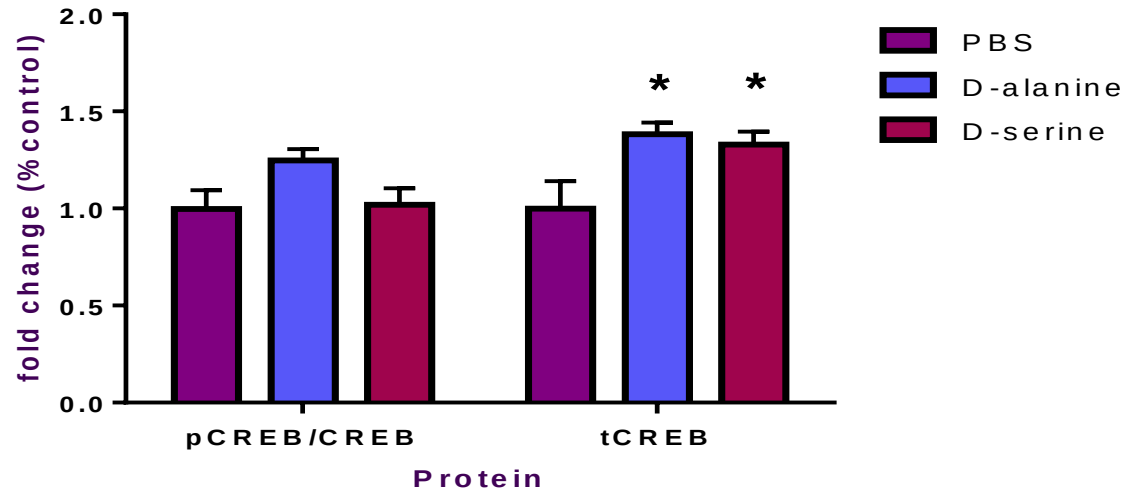


Figure 3.4. Western blot analysis of pCREB/tCREB and tCREB levels of AtT-20 cells treated with D-alanine or D-serine at 500 μ M. PBS was used as a control. Cells were incubated for 24 hours. Results are expressed as the mean $\pm$ SEM of 12 replicates in each group, following normalisation to the controls (i.e. PBS= 1). * p <0.05.

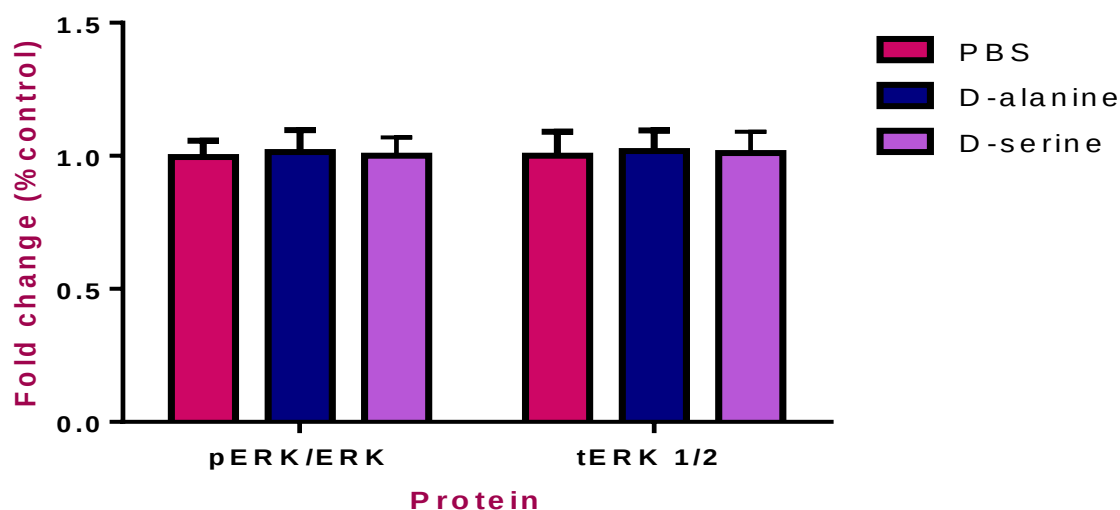


Figure 3.5. Effect of D-alanine or D-serine at 500 μ M on pERK/tERK and tERK 1/2 in AtT-20 cells. PBS was used as a control in the absence of either D-amino acid. Cells were treated for a duration of 24 hours. Results are the mean $\pm$ SEM of 12 replicates of each treatment group, with all data normalized to PBS, which was normalized to 1.

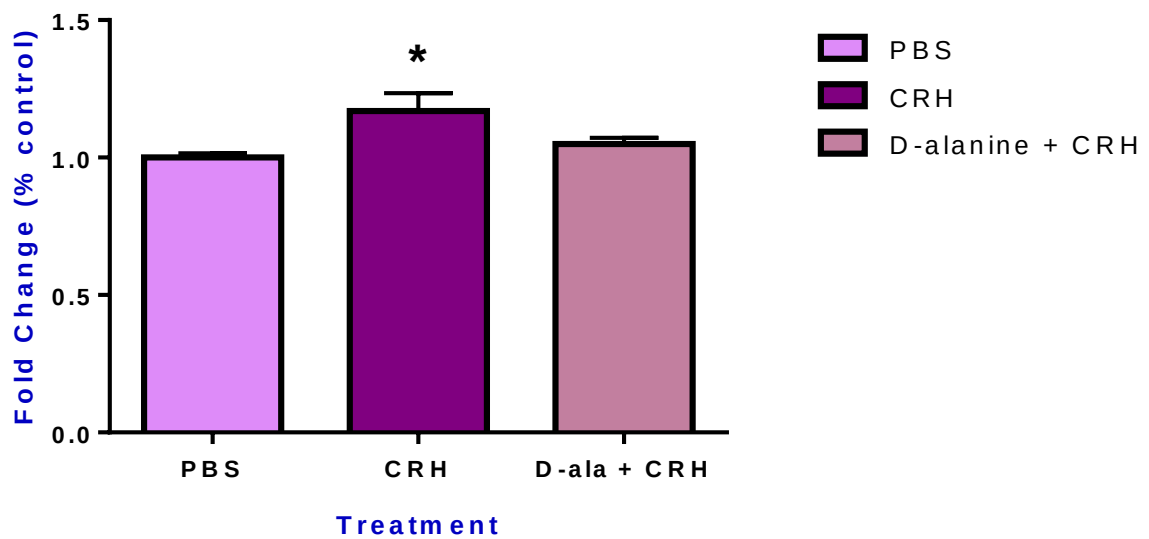


Figure 3.6. ELISA analysis of ACTH present in cultured media of AtT-20 cells treated with CRH (100 nM) in the presence or absence of D-alanine (500 μ M) for 24 hours. PBS was used as a control; data are normalized to PBS, which is normalized to 1, and are the mean of 6 replicates $\pm$ SEM. * $p < 0.05$ compared to control.

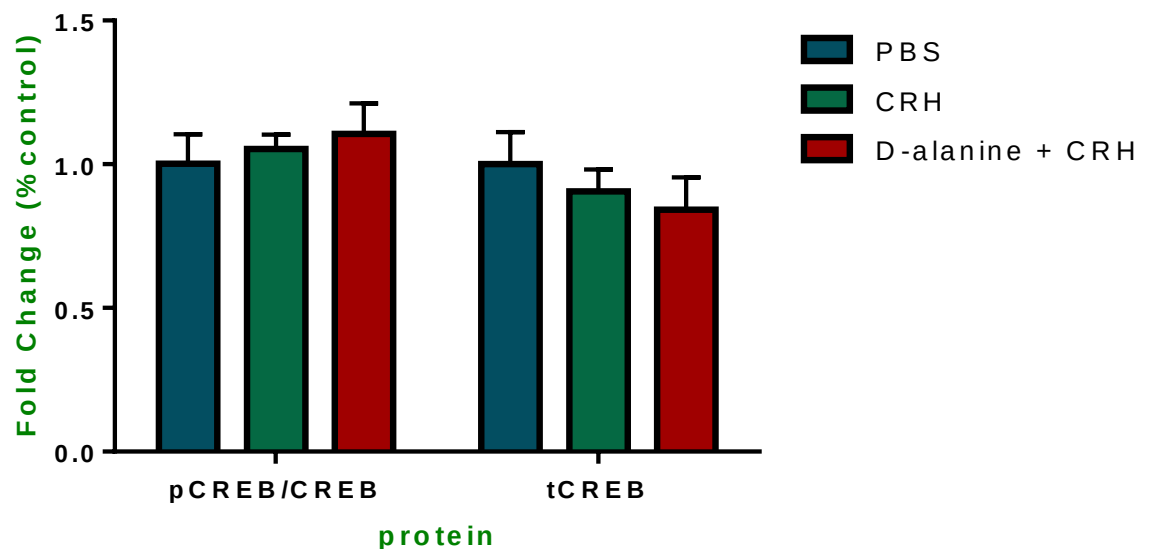


Figure 3.7. Western blot analysis of CREB protein expression in AtT-20 cells treated with D-alanine (500 μ M) and CRH (100 nM) for 24 hours, as well as CRH (100 nM) in

the absence of D-alanine for 24 hours. PBS was used as a control. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, with all data normalized to PBS, which was normalized to 1.

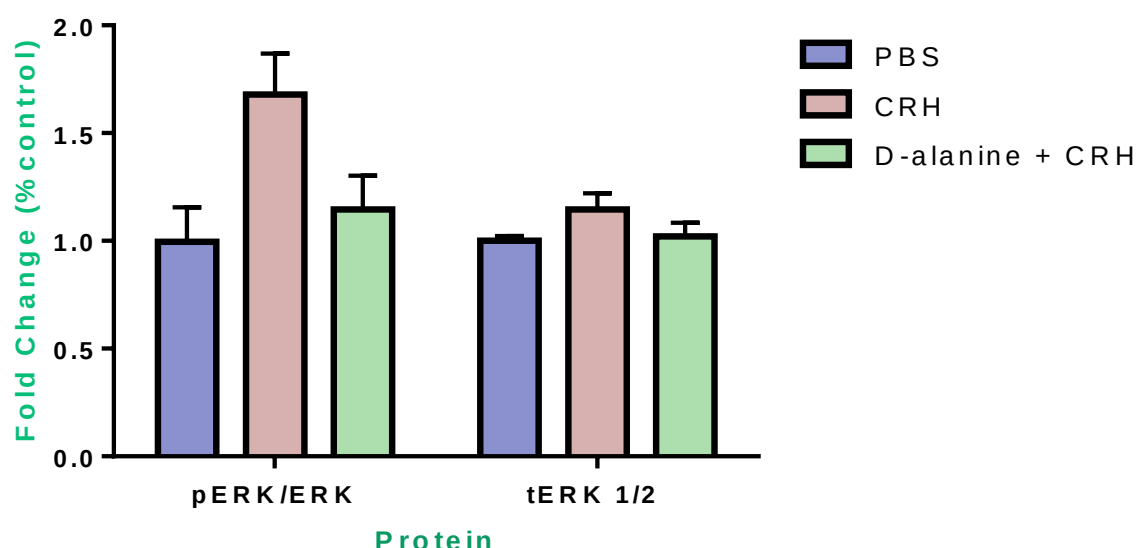


Figure 3.8 The effect of D-alanine (500 μ M) and CRH (100 nM) on ERK signalling in AtT-20 cells, where PBS is used as a control in the absence of D-alanine and CRH, and CRH (100 nM) in the absence of D-alanine. The duration of the treatment lasted 24 hours. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, following normalisation to the controls (i.e. PBS= 1).

Table 3.3: Western Blot analysis of CREB signalling in AtT-20 cells treated with D-alanine (500 μ M for 24 hours) followed by 5 or 15 minutes of CRH (100 nM). Data are mean of 6 replicates

	pCREB (mean $\pm$ SEM)	tCREB (mean $\pm$ SEM)
PBS	1.000 $\pm$ 0.023	1.000 $\pm$ 0.038
CRH only (100nM for 5min)	1.008 $\pm$ 0.044	0.982 $\pm$ 0.070
D-alanine (500 μ M for 24h) and CRH (100nM for 5min)	1.010 $\pm$ 0.037	1.022 $\pm$ 0.096

PBS	1.000 ± 0.045	1.000 ± 0.066
CRH only (100nM for 15min)	1.084 ± 0.019	1.048 ± 0.044
D-alanine (500µM for 24h) and CRH (100nM for 15min)	1.044 ± 0.034	1.063 ± 0.066

Table 3.4: Western Blot analysis of ERK 1/2 signalling in AtT-20 cells treated with D-alanine (500 µM for 24 hours) followed by 5 or 15 minutes CRH (100 nM). Data are mean of 6 replicates

	pERK (mean ± SEM)	tERK (mean ± SEM)
PBS	1.000 ± 0.087	1.000 ± 0.040
CRH only (100nM for 5min)	0.932 ± 0.068	1.025 ± 0.057
D-alanine (500µM for 24h) and CRH (100nM for 5min)	0.920 ± 0.066	1.000 ± 0.072
PBS	1.000 ± 0.126	1.000 ± 0.017
CRH only (100nM for 15min)	1.004 ± 0.068	0.935 ± 0.054
D-alanine (500µM for 24h) and CRH (100nM for 15min)	1.095 ± 0.097	0.897 ± 0.038

3.4. Discussion

3.4.1 D-alanine and D-serine increase ACTH release.

The increase in ACTH concentrations in the culture media of cells incubated with D-alanine and D-serine (**Fig 3.3**), suggests the activation of the NMDAR. Both D-amino acids are co-agonists of this receptor, and previous work in AtT-20 cells has demonstrated that the stimulation of NMDARs recues the alcohol-induced reduction of the POMC gene, which encodes ACTH (Zhou and Lapingo, 2014). However, D-alanine

is also an agonist of GlyRs, and the stimulation of these receptors, in spite of their inhibitory actions, can also mediate hormone release e.g. insulin from the pancreas (Yan-Do et al., 2016). Nevertheless, given that D-alanine increased the secretion of ACTH in a similar way to D-serine, which is the endogenous co-agonist of NMDAR and does not bind to other receptors, it is likely that these receptors alone were mediating ACTH release. This can be confirmed by testing if an NMDAR antagonist blocks the effect of both D-amino acids, and/or whether a GlyR antagonist attenuates the actions of D-alanine.

3.4.2 D-amino acids affect CREB

The significant increase in tCREB levels in AtT-20 cells following D-alanine or D-serine treatment (**Fig 3.4**) suggested that the binding of the D-amino acids to their receptors, was sufficient to initiate a downstream signalling cascade. The assumption is that this higher level of tCREB should be activated by phosphorylation, whereby it then achieves its effects in the cell via transcription factor activation. We did not detect an alteration in the levels of pCREB/tCREB between different treatments. One possible explanation for this is that the treatment exposure was too long a duration, as studies show that D-serine phosphorylated CREB in Müller cells after a 30 min incubation (Lamas et al., 2007). In AtT-20 cells, CREB phosphorylation by CRH occurred at 5 and 15 minutes (Kageyama et al., 2006). In the current experiment, therefore, CREB phosphorylation may have occurred before 24 hours, and might have been de-phosphorylated by phosphatases shortly after, resulting in a net increase of tCREB levels.

The same argument may also explain the lack of effect of the D-amino acids on ERK1/2 signalling (**Fig 3.5**) i.e. that changes in this signalling pathway were induced before 24 hours. One study incubated AtT-20 cells with the peptide encoded by the cocaine and

amphetamine regulated transcript (CART), at 5 min increments from 0-20 minutes, and showed that ERK phosphorylation occurred in a time dependent manner, with the 5-minute treatment garnering the greatest level of phosphorylated ERK (Lakatos et al., 2005). However, it is also possible that the NMDAR in AtT-20 cells are only linked to CREB signalling. The activation of CREB and ERK signalling by NMDARs depends on temporal and spatial dynamics between the receptor and signalling proteins (see Chapter 1), and in the current study, conditions may only favour NMDAR coupling to CREB. It is reasonable to conclude therefore, that NMDAR stimulation in AtT-20 cells leads to ACTH release through the CREB signalling pathway, with the caveat that active CREB (pCREB/tCREB) has not been demonstrated.

3.4.3. D-alanine reduces CRH-mediated ACTH release

The increase in ACTH concentration in the culture media of cells incubated with CRH alone, was expected, and consistent with previous work (Hofland et al., 2005). The inhibition of CRH-mediated ACTH release by D-alanine, however, was surprising given that preceding experiments showed a stimulatory action of D-alanine. Although speculative, it may be possible that the activation of CRH receptors in AtT-20 cells, led to the increase of GlyRs therein which D-alanine preferentially activated over NMDARs. Indeed, pancreatic endocrine cells that also contain D-alanine, express GlyRs (Morikawa et al., 2007; Weaver, 1998). The possibility that the AtT-20 cell-line and pituitary cells contain GlyRs that are modulated by CRH, can be studied by measuring the abundance of encoding mRNAs with QPCR, and encoded proteins with western blots and immunocytochemistry. Repeating corresponding experiments with D-serine, in cells that release ACTH in response to CRH, will demonstrate whether this D-amino acid augments hormone secretion, which is predicted since D-serine only binds to NMDARs.

3.4.2 D-alanine did not affect CRH-mediated cell signalling

The current data suggest that the CRH-induced ACTH secretion did not involve CREB signalling (**Fig 3.7**). A temporal study of CRH actions on AtT-20 cells, showed that it elicited the most significant elevation of pCREB/tCREB after a 5-minute incubation, but 30 and 60-minute treatments had no significant effect (Kageyama et al., 2006; Van Kolen et al, 2010). Therefore, a possible explanation for the lack of significant difference in both tCREB and pCREB/tCREB levels in the current study, might be that the duration of CRH incubation was too long. However, subsequent studies to address this, did not support that supposition as CREB signalling remained unaltered when AtT-20 cells were exposed to CRH for 5 min and 15 min (**Tables 3.3 and 3.4**). It is possible, therefore, that CRH receptors in the AtT-20 cells under the current conditions, were not coupled to CREB. The finding with ERK may support this.

When AtT-20 cells were treated with CRH alone, there was an increased trend (+68%, $p=0.074$) in pERK/tERK, compared to controls. Biologically, this change may have been sufficient to trigger the observed release of ACTH (**Fig 3.6**), but statistically, the experiment may have been underpowered. Nevertheless, it is interesting that in the presence of D-alanine, the effect of CRH was not observed. A replicate study with a greater sample size would need to be run to confirm that CRH increases ERK signalling and that D-alanine attenuates this effect. Before such experiments are carried out it is reasonable to propose, with caution, that under the current cell culture conditions, CRH receptors in AtT-20 cells may be linked to ERK signalling, whereas NMDARs are linked to CREB. This can be further explored using pharmacological agents directed against these receptors, or applying genetic manipulation methods.

Finally, unaltered CREB and ERK signalling at 5 min and 15 min incubations with CRH (**Table 3.3 and Table 3.4**) conflict with published data (Van Kolen et al., 2010, Kageyama et al., 2007). A possible explanation for this is that the cells were passaged too many times, causing an alteration in the physiology of the AtT-20 cells. These experiments were conducted when the cells were at their 10th passage, and microscopic analysis revealed that the cells tended to detach easily, and contained many vacuoles, at later passages. Future experiments will not be performed, therefore on AtT-20 cells beyond their 8th passage number.

In summary, the current data have provided a preliminary proof-of-concept that D-alanine in pituitary cells might modulate ACTH secretion. This D-amino acid increased the secretion of ACTH from AtT-20 cells, and since D-serine had the same effect, it is likely that their action was via the NMDAR. The release of ACTH by both D-amino acids appeared to be mediated by CREB signalling. The CRH-mediated ACTH secretion from AtT-20 cells, was attenuated by D-alanine, which suggests that when CRH receptors were activated, the D-amino acid preferentially bound to non-NMDAR, such as inhibitory GlyRs. This supposition is supported by observing that the elevation of CREB seen in cells incubated with D-alanine alone, was not apparent in the presence of CRH. This part of the study, therefore suggested that D-alanine may have an inhibitory effect on ACTH secretion when the HPA axis is activated, and hypothalamic CRH is secreted. Further work is needed to confirm this, as well as corroborate the differential coupling of CRH and NMDAR to CREB and ERK pathways.

**CHAPTER 4: THE EFFECT OF D-ALANINE AND DEXAMETHASONE ON ACTH
RELEASE AND INTRACELLULAR SIGNALLING IN THE PRESENCE OR
ABSENCE OF MK-801**

4.1. Introduction

Dexamethasone (DEX) is a synthetic glucocorticoid, which has a high affinity for glucocorticoid receptors (GR). Glucocorticoids (such as cortisol in humans and corticosterone in rats) are constantly present in the circulation, but increase in concentration during times of stress. Therefore, to model the natural environment of the anterior pituitary, AtT-20 cells were incubated with DEX, and the effect of D-amino acids on ACTH release and CREB and ERK signalling was measured. In other experiments, the NMDAR antagonist, MK-801, was used to determine whether the D-amino acids exerted their effects via this receptor.

4.2. Methods

4.2.1. Effect of DEX and D-amino acids on ACTH release, and CREB and ERK levels

The AtT-20 cells were cultured as described in Chapter 2.2. AtT-20 cells were treated with D-alanine or D-serine (500 μ M) and DEX (100 nM) for 24 hours, and the level of ACTH released, as well the effect on CREB and ERK signalling, were measured following protocols detailed in Chapter 2.3.5 and 2.3.7, respectively. To test the fast effects of DEX on signalling proteins, cells were incubated with the DEX for 5 and 15 minutes, following a 24-hour incubation with D-alanine. All cell culture, assay and western blot techniques are described in Chapter 2. pCREB is represented as a ratio of pCREB/tCREB, whilst CREB-1 is represented as CREB/ β -actin (tCREB). pERK 1/2 is represented as pERK/tERK, whilst ERK 1/2 is represented as ERK 1/2/ β -actin (tERK).

1/2). 6 replicates of each condition were run. A diagrammatic representation of this study is shown below (**Fig 4.1**).

4.2.2. Effect of DEX and D-amino acids on ACTH release, and CREB and ERK levels, in the presence, or absence, of MK-801

The effect of MK-801 (10 μ M) on ACTH secretion and CREB and ERK signalling, was tested in the presence of D-alanine or D-serine (500 μ M), and a constant concentration of 100 nM of DEX. Diagrammatic representation of experimental procedure can be found below (**Fig 4.2**) The initial treatment duration of MK-801 was 1 hour, but this garnered no effect (data not shown), so we increased the time of duration to 8 hours and 24 hours, whilst keeping the DEX and D-amino acid treatments constant at 24 hours. All cell culture, assay and western blot techniques are described in Chapter 2. Results are mean of 6 replicates $\pm$ SEM.

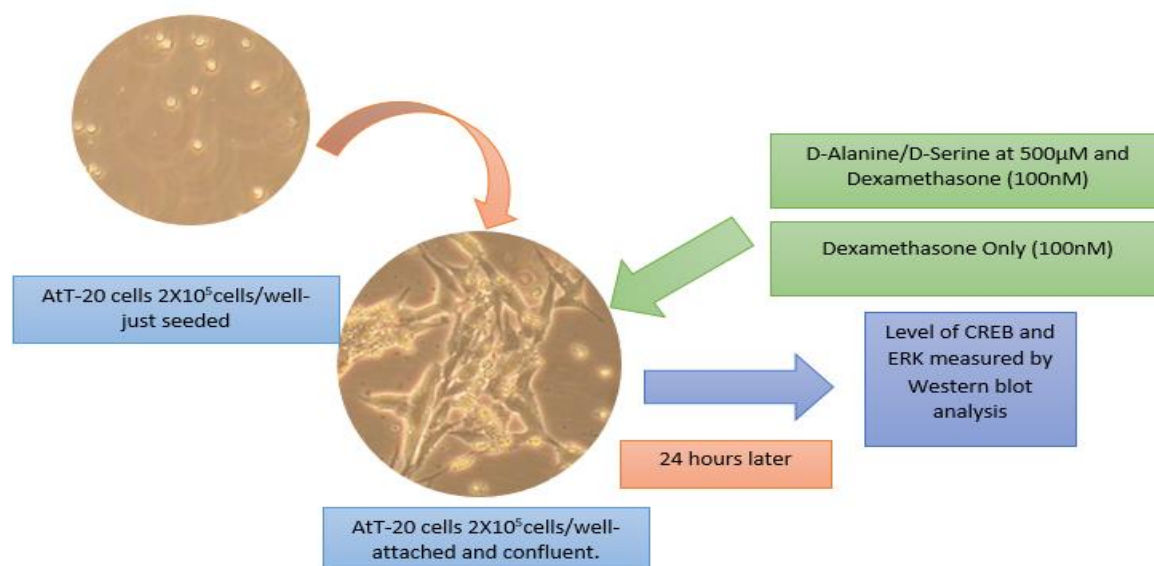


Figure 4.1 Overview of treatment protocol of AtT-20 cells treated with D-amino acids and DEX for 24 hours

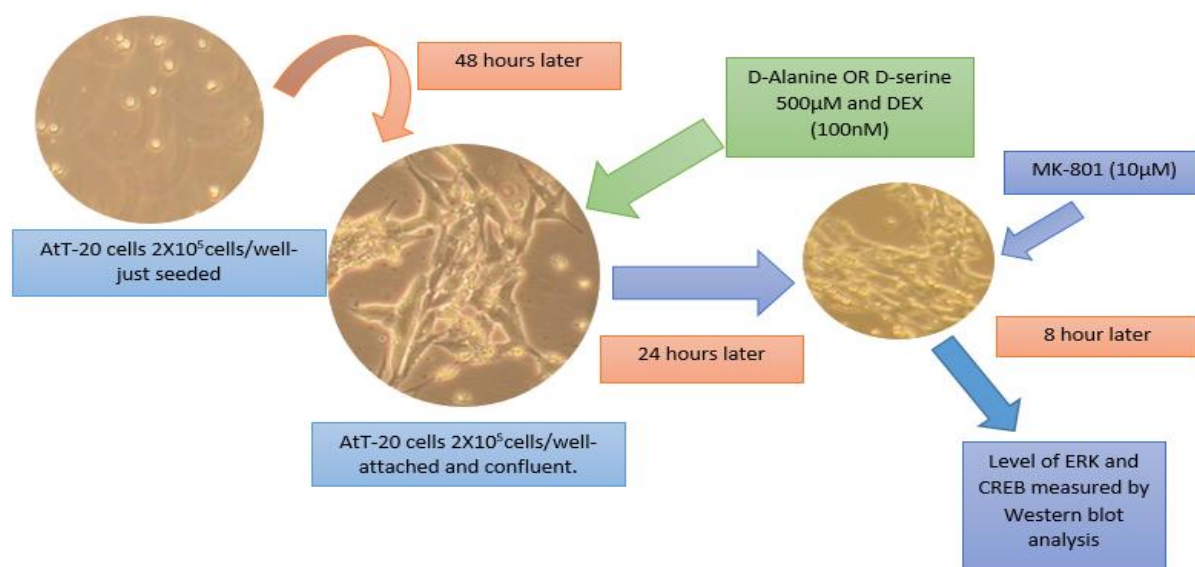


Figure 4.2. The above diagram is the overview for the treatment protocol of MK-801 treatment to AtT-20 cells, in the presence of DEX and D-amino acids.

4.3. Results

4.3.1. Effect of DEX and D-amino acids on ACTH release, and CREB and ERK levels

The incubation of AtT-20 cells with DEX (100 nM) and D-alanine (500 μ M) for 24 hours, significantly decreased the levels of ACTH released into the culture medium, when compared to PBS control (-44%, $p=0.03$, **Fig 4.3**). The combination of DEX and D-alanine did not significantly alter the levels of pCREB/tCREB (**Fig 4.4**), but significantly reduced tCREB levels compared to controls (-30%, $p=0.022$). Cells exposed to the same conditions displayed no significant difference in ERK signalling (**Table 4.1**).

There was a significant increase in ACTH released in culture medium of cells treated with DEX and D-serine for 24 hours, when compared to PBS, and cells treated with only DEX (+16%, $p=0.038$ and +38%, $p=0.008$ respectively, **Fig 4.5**).

No significant effects on CREB signalling were observed in AtT-20 cells incubated with DEX and D-serine (**Table 4.2**). However, there was a significant elevation of

pERK/tERK levels in cells incubated with D-serine and DEX compared to the PBS group (+38%, $p=0.039$, **Fig 4.6**).

The incubation of cells with DEX for 5 and 15 min following a 24-hour incubation with D-alanine, did not alter pCREB/tCREB or tCREB levels (**Fig. 4.7 and 4.8**). A decreased trend in the levels of tCREB in cells treated with DEX alone for 5 minutes compared to PBS (-21%, $p=0.063$), was observed. There was no significant difference between treatments in tCREB in the 15-minute treatment of DEX (**Fig 4.8**), but there was a trend of greater levels of tCREB present in cells treated with D-alanine and DEX vs. PBS (+39%, $p=0.068$).

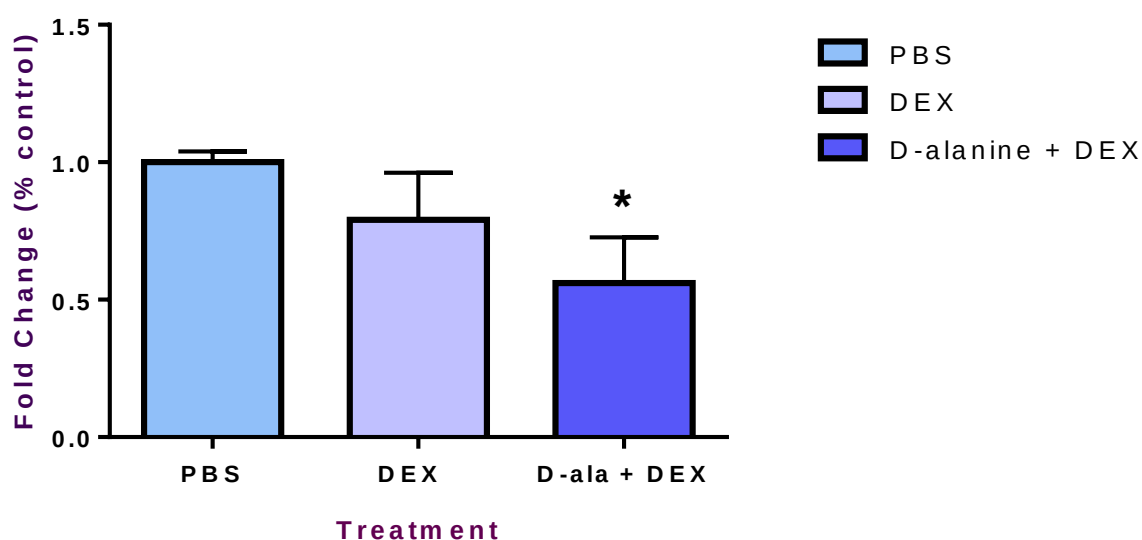


Figure 4.3. ACTH levels in AtT-20 cells exposed to 500 μ M D-alanine and DEX (100 nM) for 24 hours. PBS was a control, and cells were also treated with DEX in the absence of D-alanine. Data is mean of 6 replicates $\pm$ SEM, where data are normalized to PBS (PBS =1) * $p<0.05$

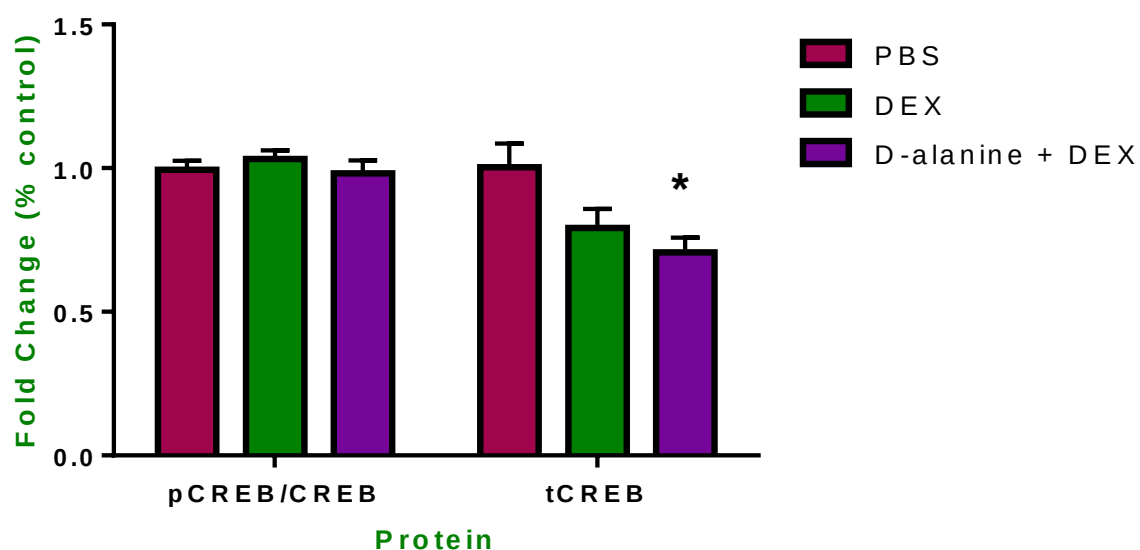


Figure 4.4. Western blot analysis on CREB signalling in AtT-20 cells treated with D-alanine (500 μ M) and DEX (100 nM) for 24 hours. Control was cells treated with PBS in the absence of DEX and D-alanine. DEX represents AtT-20 cells treated with DEX (100 nM for 24 hours) in the absence of D-alanine. Data are mean of 6 replicates $\pm$ SEM. (* $p < 0.05$)

Table 4.1. Effect of D-alanine and DEX on ERK 1/2 signalling in AtT-20 cells. Data is represented as mean of 6 replicated $\pm$ SEM.

	pERK/tERK	tERK 1/2
PBS	1.000 $\pm$ 0.064	1.000 $\pm$ 0.022
DEX (100nM) for 24 hours	1.207 $\pm$ 0.188	0.978 $\pm$ 0.042
DEX (100nM) and D-alanine (500 μ M) for 24 hours	1.232 $\pm$ 0.193	0.998 $\pm$ 0.041

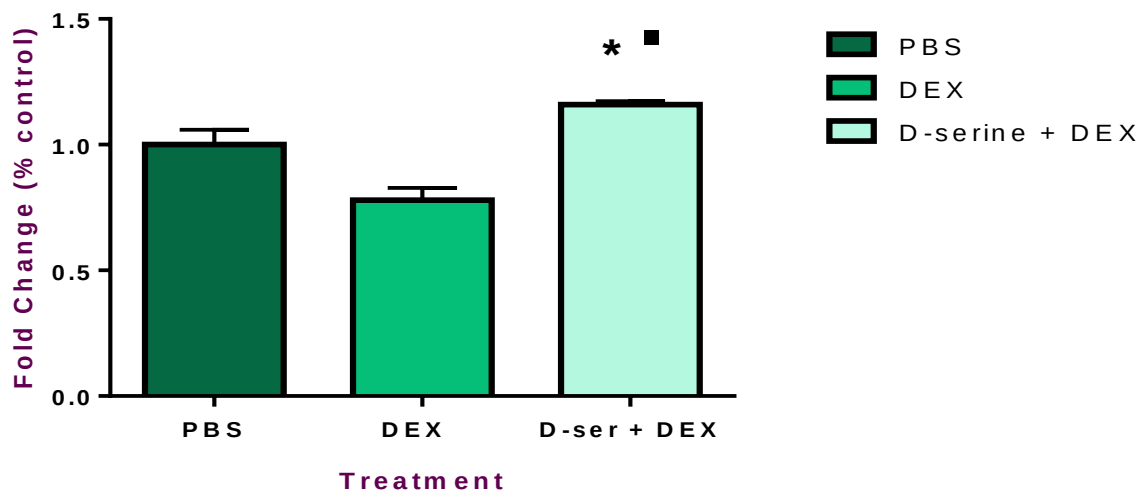


Figure 4.5. ELISA analysis of ACTH released in the cultured media of AtT-20 cells treated with DEX (100 nM) in the absence, or presence, of D-serine (500 μ M). Treatment lasts 24 hours, and PBS is control. All data are normalised to PBS (PBS = 1). Data are mean of 6 replicates $\pm$ SEM. * $p < 0.05$, ■ $p < 0.01$

Table 4.2. Western blot analysis of pCREB/tCREB and tCREB in AtT-20 cells treated with D-serine and DEX for 24 hours. Data are the mean of 6 replicates $\pm$ SEM

	pCREB/tCREB	tCREB
PBS	1.000 $\pm$ 0.102	1.000 $\pm$ 0.102
DEX (100nM) for 24 hours	0.930 $\pm$ 0.054	0.978 $\pm$ 0.087
DEX (100nM) and D-serine (500 μ M) for 24hours	0.822 $\pm$ 0.088	1.168 $\pm$ 0.098

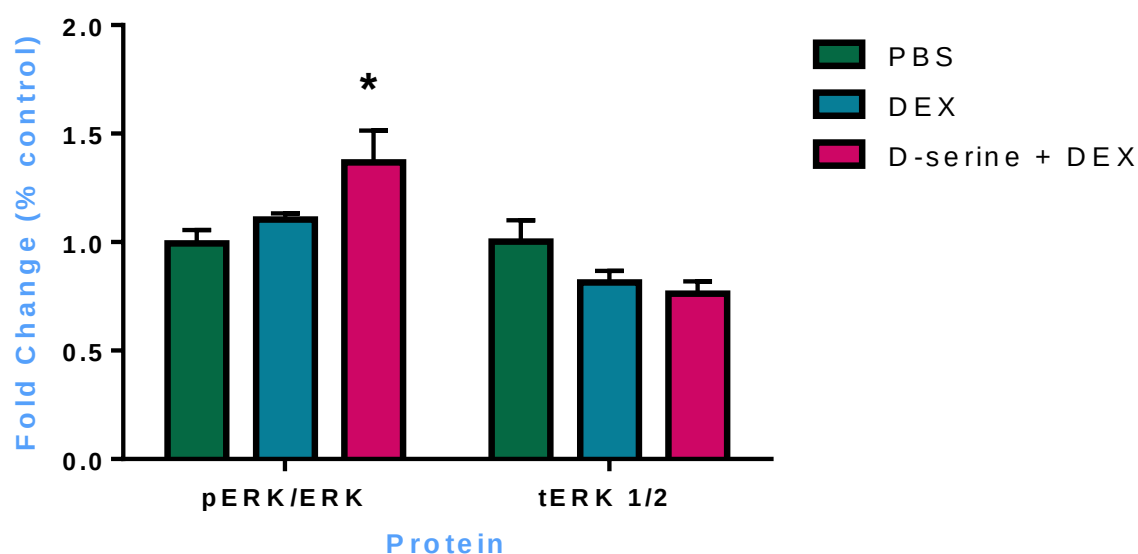


Figure 4.6. The effect of D-serine (500 μ M) and DEX (100 nM) on pERK/tERK and tERK 1/2 for 24 hours in AtT-20. Cells were also treated with DEX in the absence of D-serine (100 nM for 24 hours), and PBS was utilized as a control. The data is represented as mean of 6 replicates $\pm$ SEM. (* $p < 0.05$)

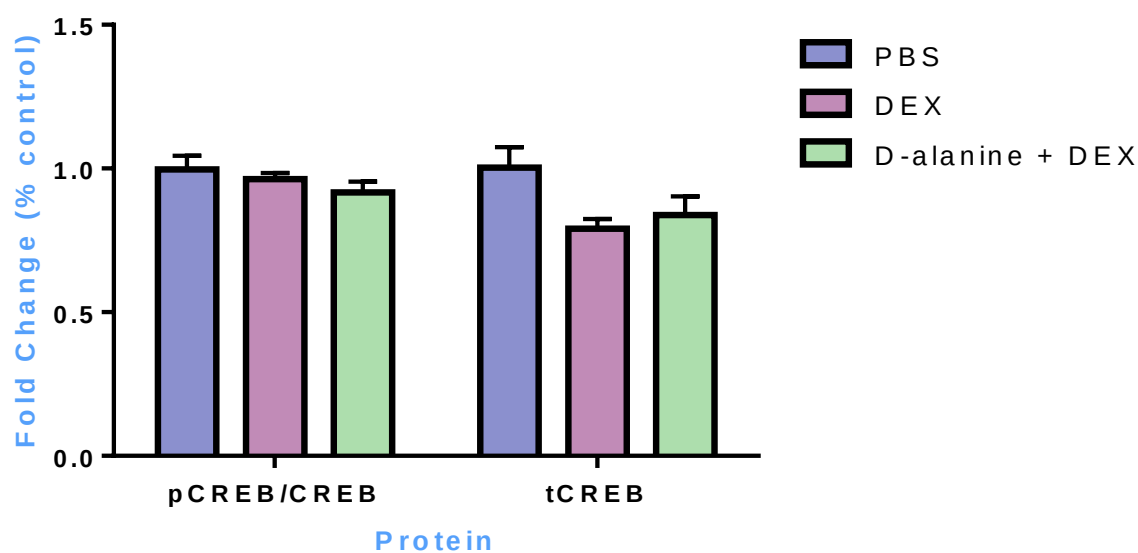


Figure 4.7. The effect of D-alanine (500 μ M) for 24 hours, followed by 5 minutes of DEX (100 nM) on CREB signalling in AtT-20 cells. DEX was treated to cells for 5

minutes, and PBS was used as control in the absence of D-alanine and DEX. Data are mean of 6 replicates $\pm$ SEM, where all data are normalized to PBS, which is normalized to 1.

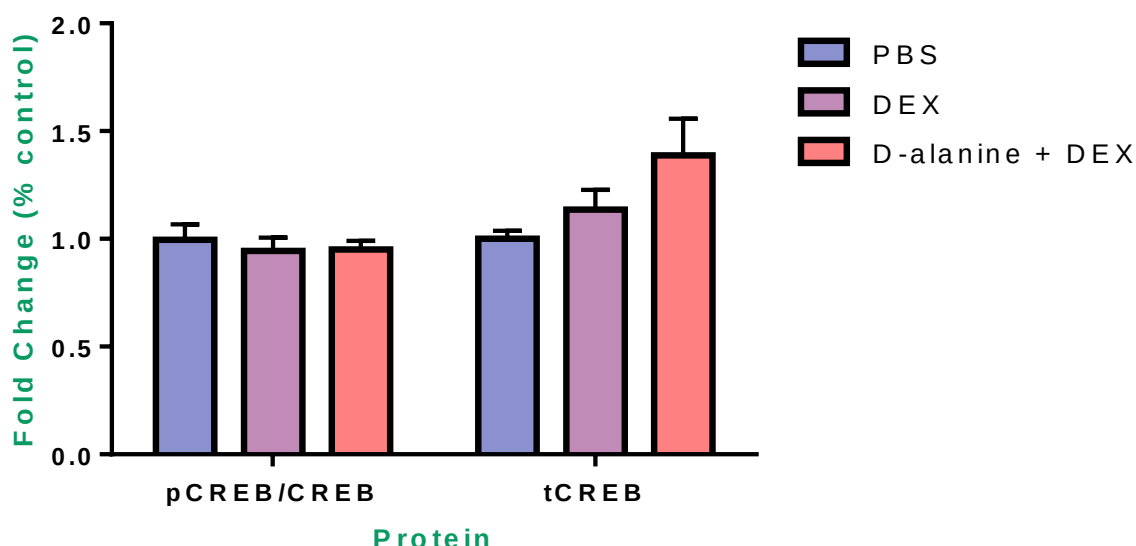


Figure 4.8. Western blot analysis of pCREB/tCREB and tCREB in AtT-20 cells treated with D-alanine (500 μ M) for 24 hours, followed by 15 minutes of DEX (100nM). DEX alone was also added to cells for 15 minutes, and PBS was used as a control in the absence of D-alanine and DEX. Data are mean of 6 replicates $\pm$ SEM, where all data is normalized to PBS (PBS =1).

4.3.2. Effect of DEX and D-amino acids on ACTH release, and CREB and ERK levels, in the presence, or absence, of MK-801

Figure 4.9 depicts the significantly lesser levels of ACTH detected in culture medium of AtT-20 cells following treatment with DEX and D-alanine (-42%, $p=0.015$), and DEX and D-alanine (24 hours) followed by 8 hours of MK-801 (-47%, $p=0.021$) compared to PBS control.

The incubation of cells with DEX and D-alanine in the presence or absence of MK-801, did not alter the levels of pCREB/tCREB (**Fig. 4.10**), but did reduce the levels of tCREB (DEX+D-alanine: -15%, $p=0.025$; DEX + D-alanine + MK801: -16%, $p = 0.009$). The DEX + D-alanine effects were consistent with previous experiments (see **Fig.4.4**). Neither DEX + D-alanine, nor DEX + D-alanine +MK801 affected ERK signalling (**Table 4.3**).

The presence of D-serine and DEX, as well as D-serine and DEX followed by MK-801, significantly increased the level of ACTH present in cultured medium (+15%, $p=0.035$ and +19%, $p=0.020$ respectively) when compared to controls (**Fig. 4.11**)

A greater level of pERK/tERK was observed in cells incubated with DEX and D-serine compared to controls (+200%, $p=0.035$, **Fig. 4.12**), which replicated previous experiments (see **Fig. 4.6**). The levels of pERK/tERK were further increased in cells incubated with DEX, D-serine and MK-801 (+365%, $p=0.019$). There was a lack of significant effect on the CREB signalling in cells treated to D-serine and DEX followed by MK-801 (**Table 4.4**).

The effect on ACTH that D-serine, DEX and MK-801 treated to AtT-20 cells for 24 hours was consistent with our previous finding (**Fig 4.11**), where there were significantly higher levels in cells incubated with D-serine, DEX and MK-801 (+26%, $p=0.010$) and D-serine and DEX (+18%, $p=0.022$) than control (**Fig 4.13**).

When cells were incubated with D-serine, DEX and MK-801 for 24 hours (**Fig 4.14**), significantly greater levels of pERK/tERK were observed (DEX+D-serine: +56%, $p=0.023$; DEX+D-serine+MK801: +98%, $p=0.000$), which was consistent with previous experiments examining 8 hr incubations with MK801 (see **Fig 4.12**). However, there was also significant decrease in tERK 1/2 in cells treated with MK-801, DEX and D-serine

compared to PBS (-12%, $p=0.021$). These treatments did not have a significant effect on CREB signalling (Table 4.5).

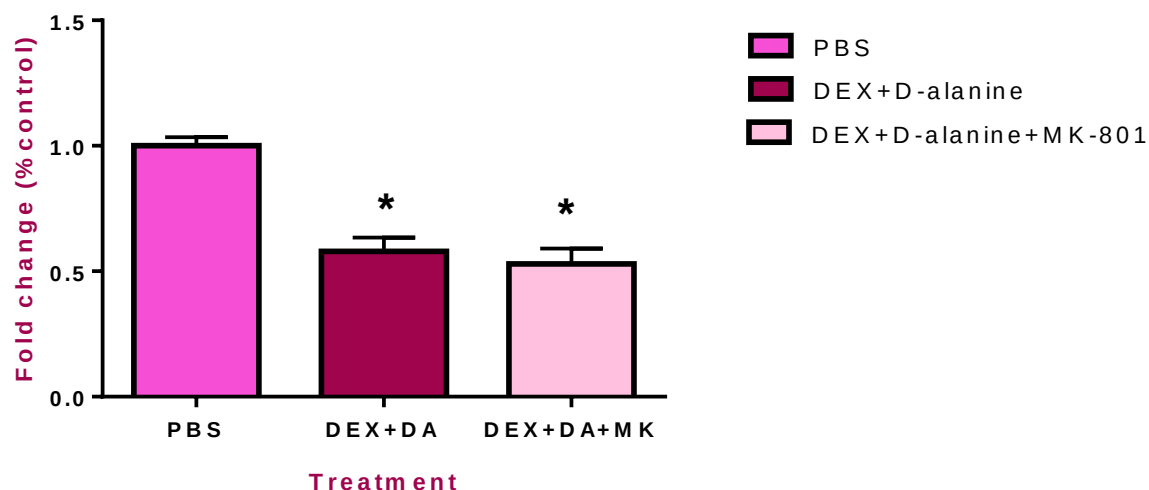


Figure 4.9 Level of ACTH expressed in AtT-20 cells treated with DEX (100 nM) and D-alanine (500 μ M), in the presence and absence of MK-801 (10 μ M). PBS was used as a control, and data are the mean of 6 replicates $\pm$ SEM, normalized to PBS (PBS =1)

* $p<0.05$

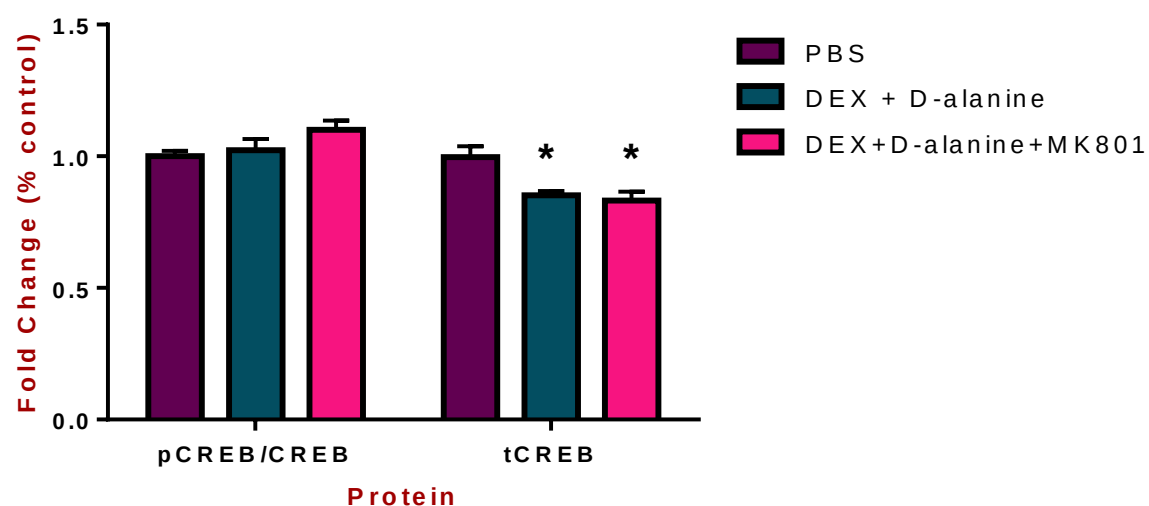


Figure 4.10. Western blot analysis of CREB protein expression in AtT-20 cells treated with D-alanine (500 μ M) and DEX (100 nM) for 24 hours, followed by MK-801 (10 μ M)

for 8 hours, as well as DEX (100 nM) and D-alanine (500 μ M) in the absence of MK-801, for 24 hours. PBS was used as the control, where PBS was delivered to AtT-20 cells in the absence of D-alanine, DEX or MK-801, at the same quantity as the quantity of D-alanine treatment delivered. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, with all data normalized to PBS, which was normalized to 1 (*p <0.05).

Table 4.3. The effect of ERK 1/2 protein expression in AtT-20 cells treated with D-alanine and DEX for 24 hours, followed by MK-801 for 8 hours. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, with all data normalized to PBS (PBS =1).

	pERK/tERK (mean $\pm$ SEM)	tERK 1/2 (mean $\pm$ SEM)
PBS	1.000 $\pm$ 0.061	1.000 $\pm$ 0.054
DEX (100nM) and D-alanine (500 μ M) for 24hours	1.165 $\pm$ 0.133	0.888 $\pm$ 0.031
DEX (100nM) and D-alanine (500 μ M) for 24hours and MK- 801(10 μ M) for 8hours	1.103 $\pm$ 0.083	1.022 $\pm$ 0.054

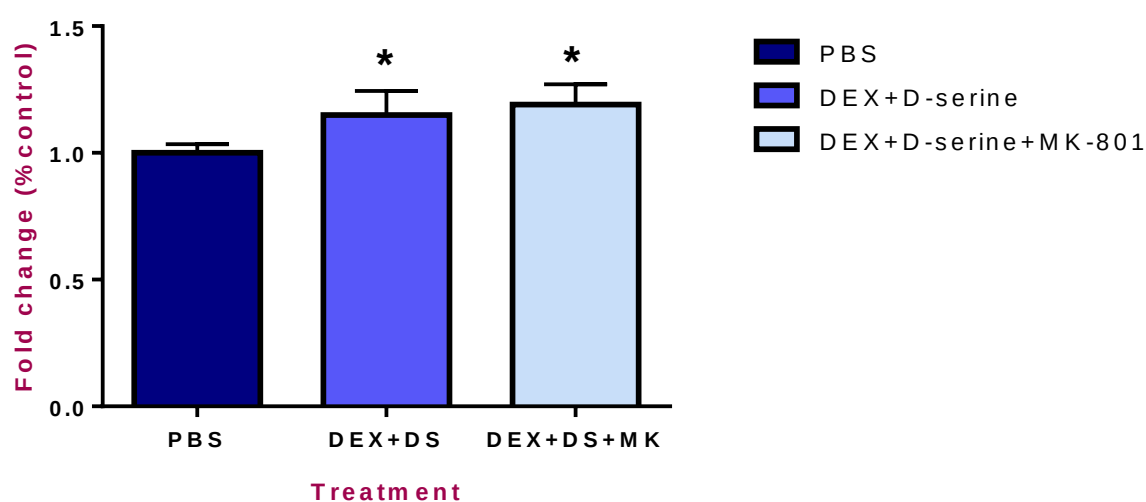


Figure 4.11 ELISA analysis of ACTH levels in culture media following treatment with DEX and D-serine (100 nM and 500 μ M respectively, for 24 hours) in the absence or presence of MK-801 (10 μ M for 8 hours post DEX and D-serine treatment). PBS was used as a control, and all data was normalised to it (PBS =1). Data are the mean of 6 replicates $\pm$ SEM.

Table 4.4. Western blot analysis of CREB protein expression in AtT-20 cells treated with D-serine and DEX for 24 hours, followed by MK-801 for 8 hours. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, with all data normalized to PBS, which was normalized to 1

	pCREB/tCREB (mean $\pm$ SEM)	tCREB (mean $\pm$ SEM)
PBS	1.000 $\pm$ 0.040	1.000 $\pm$ 0.090
DEX (100nM) and D-serine (500 μ M) for 24hours	0.970 $\pm$ 0.024	1.090 $\pm$ 0.068
DEX (100nM) and D-serine (500 μ M) for 24hours and MK-801(10 μ M) for 8hours	0.973 $\pm$ 0.0662	0.968 $\pm$ 0.088

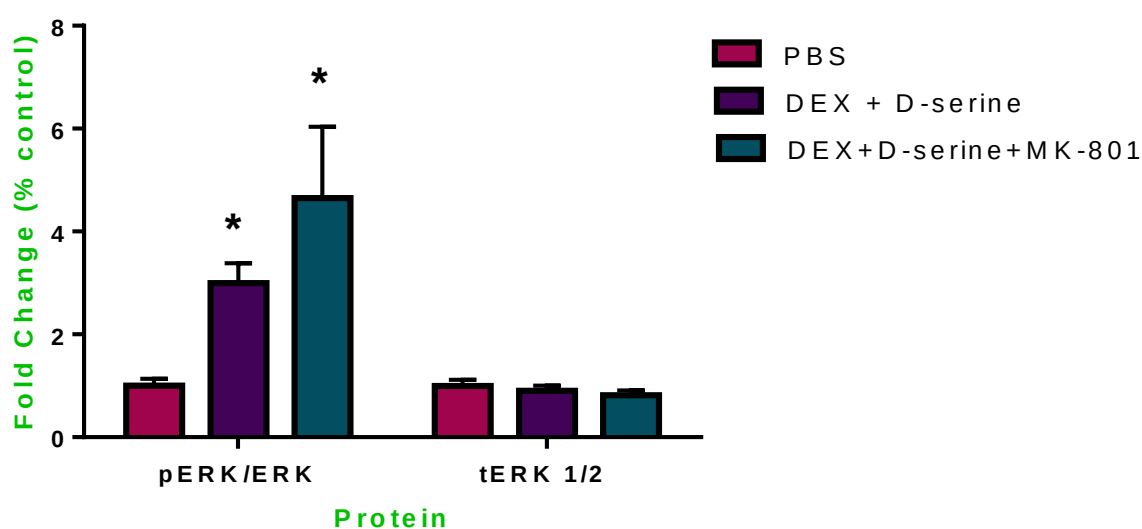


Figure 4.12. The effect of ERK 1/2 protein expression in AtT-20 cells treated with D-serine (500 μ M) and DEX (100nM) for 24 hours, followed by MK-801 (10 μ M) for 8 hours, as well as DEX (100nM) and D-serine (500 μ M) in the absence of MK-801, for 24 hours. PBS was used as the control. Results are the mean $\pm$ SEM of 6 replicates of each treatment group, with all data normalized to PBS, which was normalized to 1 (*p<0.05).

Table 4.5: Western Blot analysis of CREB expression in AtT-20 cells treated with DEX, D-amino acids and MK-801 for 24 hours (mean of 6 replicates).

	pCREB (mean $\pm$ SEM)	tCREB (mean $\pm$ SEM)
PBS	1.000 $\pm$ 0.125	1.000 $\pm$ 0.079
DEX (100nM) and D-alanine (500 μ M) for 24hours	1.275 $\pm$ 0.205	0.763 $\pm$ 0.100
DEX (100nM), D-alanine (500 μ M) and MK-801 (10 μ M) for 24hours	1.265 $\pm$ 0.100	0.813 $\pm$ 0.056
PBS	1.000 $\pm$ 0.039	1.000 $\pm$ 0.071
DEX (100nM) and D-serine (500 μ M) for 24hours	0.988 $\pm$ 0.020	0.982 $\pm$ 0.037
DEX (100nM), D-serine (500 μ M) and MK-801 (10 μ M) for 24hours	0.953 $\pm$ 0.021	1.027 $\pm$ 0.069

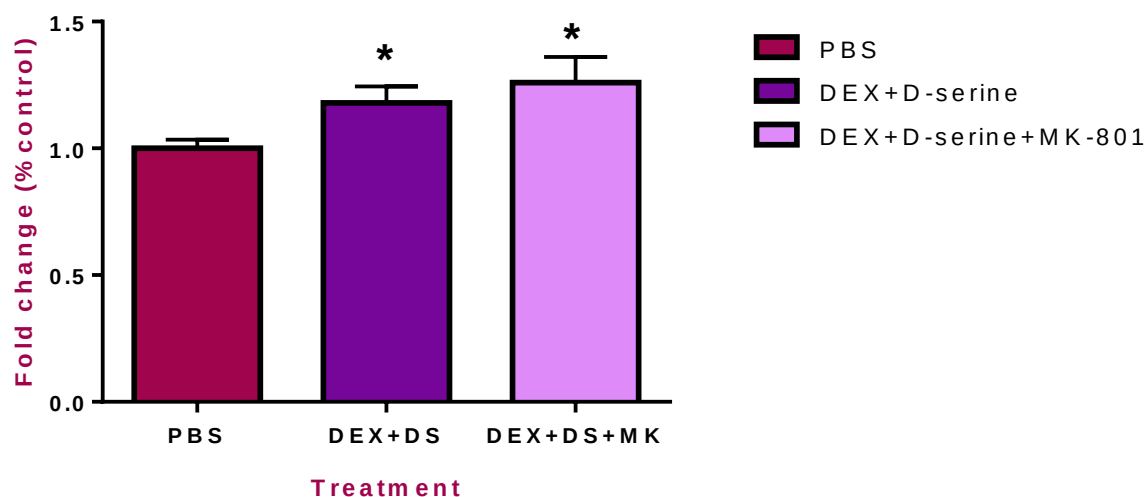


Figure 4.13 Level of ACTH in culture media following treatment with DEX and D-serine and MK-801 (100 nM, 500 μ M and 10 μ M respectively for 24 hours) in the absence or presence of MK-801 (10 μ M for 24 hours). PBS was used as a control, and all data was normalised to it (PBS =1). Data are the mean of 6 replicates $\pm$ SEM.

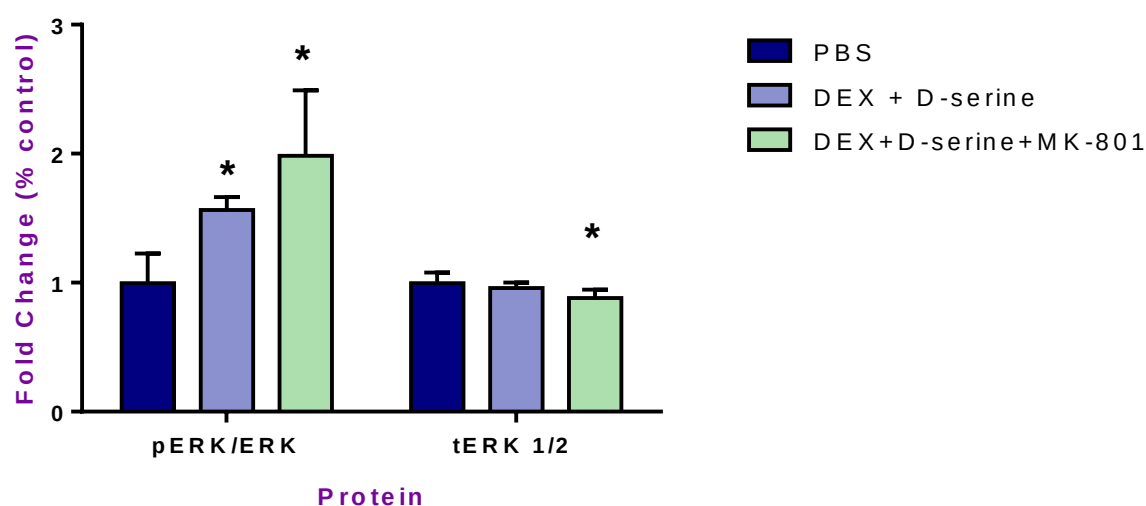


Figure 4.14. Western blot analysis of ERK protein expression in AtT-20 cells treated with D-serine, DEX and MK-801 (500 μ M, 100nM and 10 μ M respectively) for 24 hours.

PBS was used as the control. Cells also received DEX and D-serine only (500 μ M and 100nM respectively). Results are the mean $\pm$ SEM of 12 replicates of each treatment group, with all data normalized to PBS (PBS =1, * p <0.05).

4.4. Discussion

4.4.1. D-alanine and DEX decrease ACTH release

Previous studies have demonstrated the suppression of ACTH release from anterior pituitary corticotropes, by glucocorticoids (Shipston, 1995). In the current study, the concentration of DEX used only resulted in a non-significant reduction in ACTH release from AtT-20 cells. However, in the presence of D-alanine a significant reduction of hormone release was observed. This finding is contradictory to the results obtained in Chapter 3, where D-alanine increased ACTH secretion i.e. it was predicted that D-alanine might counter-balance the effect of DEX and maintain ACTH release at control levels. In the previous chapter, the stimulatory effect of D-alanine on ACTH secretion was attributed to NMDAR activation, since the potent endogenous co-agonist, D-serine, had a similar effect. However, although conflicting, the literature suggest that NMDAR expression is modulated by glucocorticoid receptor activation. A study by Kamphuis et al (2003) showed that DEX had an effect on the assembly and targeting of NMDARs in the hippocampus, and reduced the concentration of GluN2B subunits in rats. In contrast, another study demonstrated that the activation of hippocampal GRs increased NMDAR, GluN2A and GluN2B subunits, via the serum-and glucocorticoid-inducible kinase-1 (SGK1) (Tai et al, 2009). Nevertheless, altered levels of NMDAR do not explain the current findings. Thus, if NMDARs were reduced in AtT-20 cells following DEX, the D-alanine might not sufficiently increase ACTH release, which will therefore remain unchanged. Alternatively, if NMDARs were increased by DEX, the D-alanine might be expected to elevate ACTH release sufficiently to oppose GR activation, resulting in

ACTH secretion that is either the same or greater than control levels. In either case, therefore, NMDAR stimulation by D-alanine does not explain why this D-amino acid reduces ACTH secretion in the presence of DEX.

One possibility, though speculative, is that SGK1 activation by DEX, which has been demonstrated in pituitary cells (Keller-Wood et al, 2006)), not only increases NMDAR expression (as in the hippocampus), but also inhibitory GlyRs. If this was the case in the current study, then D-alanine may have preferentially bound to these receptors rather than NMDARs, and thereby suppress ACTH release. There are currently no reports on the effect of GRs/SGK1 activation on the levels of GlyRs or NMDARs in pituitary cells. Therefore, a direct measure of the expression of these genes in AtT-20 cells, from the current experiments will support or refute the notion that glucocorticoid-mediated increase of GlyRs may have mediated the effect of D-alanine on ACTH release. The possibility of DEX elevated NMDARs, is suggested in corresponding D-serine experiments (see below).

4.4.2. D-alanine and DEX suppress tCREB levels in AtT-20 cells

The effect of D-alanine in combination with DEX on tCREB, was similar to that seen with ACTH release, and was again unexpected given that the D-amino acid increased tCREB in AtT20 cells (see Chapter 3). The same argument as used to explain the ACTH results, could also be applied to interpret the changes in CREB signalling. That is, in the presence of DEX, GlyR are expressed which bind D-alanine and inhibits the levels of tCREB. Although there is no direct evidence for the interaction between GlyRs and CREB, when activated these receptors can hyperpolarise membranes by increasing influx of intracellular chloride ions, which reduces NMDARs activity and calcium entry into cells (Hoffman et al., 1989). The decrease in intracellular calcium would then lead to a

reduction in CREB signalling (Hardingham et al., 2001). The possibility that DEX initiates the expression of a receptor through which D-alanine acts, is also suggested by the experiments testing the effects of a short-term exposure to the glucocorticoid (**Fig 4.7**). In this case, even in the presence of D-alanine, the incubation of cells to DEX for 5 min and 15 min, did not significantly reduce tCREB as observed with a 24-hour incubation. It is therefore, reasonable to propose that a longer incubation time with DEX is required to establish the expression and function of the gene (GlyRs?) that mediates the actions of D-alanine. Gene expression studies of GlyR subunits in the presence or absence of DEX at 5 min., 15 min and 24-hour incubation, will test this theory.

4.4.3. D-serine and DEX increase ACTH release

Contrary to a decrease in ACTH in the presence of D-alanine and DEX, D-serine and DEX resulted in an increase in ACTH secretion. In the absence of D-serine, there was a decrease in ACTH levels which, whilst not significant, was consistent with the suppressive effect of DEX on ACTH release (see above). Therefore, the presence of D-serine resulted in the rise of ACTH, which was similar to the effect it had on AtT-20 cells in the absence of DEX (**Fig 3.3**). Moreover, it would seem that the D-serine mediated stimulation was stronger than the inhibitory action of DEX, to the point where an elevation in ACTH release was the net effect. This finding supports the previous conclusion (see Chapter 3) that D-serine is acting through the NMDAR, as there are no other receptors/genes known to bind D-serine with such high affinity. This is also suggested at the level of cell signalling.

4.4.4. D-serine and DEX increase pERK/tERK levels

The elevation of pERK/tERK by D-serine in the presence of DEX, is likely to have mediated the increase in ACTH (**Fig 4.6**). Indeed, inhibition of ERK signalling in AtT-20

cells, has been shown to reduce ACTH secretion (Van Kolen et al., 2010). However, the preceding experiments (Chapter 3) suggested that in the absence of DEX, CREB signalling- which is also linked to ACTH secretion (Zhang et al., 2012)- may have mediated the elevation of ACTH by D-serine. It is possible, therefore, that the presence or absence of DEX (level of GR activation), determines which signalling pathway NMDARs are coupled to in pituitary cells. Thus, in the current study, GR activation may have promoted NMDAR coupling to ERK signalling proteins, perhaps as a homeostatic response to the inhibitory actions of GRs. The involvement of ERK signalling in D-serine mediated ACTH release can be tested by repeating the current experiments in the presence of the ERK inhibitor, PD98059 (Zheng et al, 2016). In this instance, the combination of D-serine, DEX and PD98059, will not affect ACTH release or ERK signalling.

4.4.5. MK801 did not block the effects of D-alanine or D-serine on ACTH secretion and cell signalling

The lack of effect of MK-801 on the D-alanine + DEX mediated reduction of ACTH secretion and tCREB, is consistent with the notion that this D-amino acid was not acting through NMDARs in the presence of DEX (see above). However, the finding that MK-801 also failed to block the stimulatory effect of D-serine, on ACTH release (**Fig 4.11 and 4.13**) and ERK signalling (**Fig 4.12 and Fig 4.14**) was unexpected. Seo et al (2007) showed that treatment of MK-801 caused an increase in the phosphorylation of the MEK-ERK-p90RSK pathway in the rat frontal cortex. This, together with the finding by Ahn and colleagues (2006), showing that MK-801 caused a time dependent increase in ERK phosphorylation, may suggest that the overall effect of an increase in pERK/tERK by MK-801, may have overcome a potential decrease in pERK/tERK levels due to reduced NMDAR activity, as MK-801 may be a more potent simulator of ERK phosphorylation

than D-serine. To ascertain whether MK-801 has such a potent effect on the phosphorylation of ERK, experiments should be conducted where cells will be treated with MK-801 independent of D-serine or DEX.

In summary, the present data suggests that D-alanine and D-serine exert their effects on AtT-20 cells, via two separate pathways. Thus, D-alanine decreased ACTH release and tCREB levels in the presence of DEX, whereas D-serine increased ACTH secretion pERK/tERK levels in the presence of DEX. However, although experiments using the NMDAR blocker, MK-801, supported the notion that D-alanine may have mediated its effects through non-NMDARs, they did not confirm that NMDARs mediated the actions of D-serine. Further studies are required to test if DEX increases GlyR expression in AtT20 cells, which would explain the inhibitory effects of D-alanine on ACTH secretion and tCREB. Confirmation that GlyRs are involved can also be achieved by using strychnine, a potent antagonist of this receptor, and taurine, another potent agonist. Finally, AtT-20 cells should be treated with MK-801 alone, to determine if the level of pERK/tERK in these cells are sufficient to overcome the potential inhibition of ERK signalling that results from the blockade of NMDARs by this antagonist.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

The D-amino acid, D-alanine, has been localised to ACTH-containing cells of the rat pituitary, and therefore may have an endocrine role in the modulation of the mammalian HPA axis. The current study, therefore, used an anterior pituitary cell-line (AtT-20) to determine the influence of D-alanine on ACTH secretion and associated cell signalling proteins. This D-amino acid is a co-agonist of the NMDAR and a full agonist of the GlyR, and so its actions might be mediated via one or both of these receptors. To confirm the involvement of NMDARs, the actions of D-alanine were compared with those of D-serine, the natural endogenous co-agonist of these receptors only.

Both D-alanine, and D-serine, appear to be stimulants of ACTH release, and this stimulation seems to be mediated by CREB signalling, inferred by the fact that both these D-amino acids significantly increased tCREB levels. This also suggests that CREB signalling was modulated at the level of the gene, since alterations in tCREB occur when the levels of encoding mRNA are changed. The lack of effect on the pCREB/CREB levels in these cells was unexpected, as the phosphorylation, thus activation, of CREB is required to increase ACTH release i.e. studies have shown that this ratio is increased (more pCREB) with ACTH secretion. However, the current study may also suggest elevated pCREB, given that the pCREB/tCREB ratio was maintained; that is, if pCREB remained unaltered when tCREB increased after D-amino acid incubations, then the ratio would have decreased. Further studies are therefore required to test if the increase in tCREB reflects an active CREB signalling pathway.

In spite of this unknown, it is likely that the NMDAR mediated the elevation of CREB signalling since D-serine, which binds only to these receptors, had the same effect as D-alanine. In this instance, D-serine acted as a positive control to confirm the involvement

of NMDARs. This D-amino acid is mainly localised to the mammalian forebrain, such as the hippocampus and cortex, but there are small amounts found in the hypothalamus, and trace levels in the pituitary. Therefore, the influence of D-serine on ACTH release in mammals, is likely to mainly arise from the circulation via the diet, and/or through D-serine-NMDAR signalling from the hypothalamus.

To better represent the environment of the anterior pituitary, cells were incubated with either CRH or DEX, (**Fig 5.1**) with the latter representing circulating corticosterone. The addition of CRH increased ACTH secretion from AtT-20 cells, but not in the presence of D-alanine. This could be due to inhibitory effects of the GlyR which D-alanine also binds to. Although the expression of GlyRs in the pituitary have not been reported, the pancreas which also contains high levels of D-alanine, expresses high levels of GlyRs. The data suggest, therefore that when the HPA axis is activated, and CRH is released into the pituitary, the subsequent increase in ACTH secretion can be inhibited by D-alanine, which may have been co-released with this hormone, as it is present in the same cells. In this instance, D-alanine has an autocrine role. The suppressive effect on ACTH secretion by D-alanine may be a homeostatic response to stress in mammals, which is similar to the glucocorticoid negative feedback system, except it is within the pituitary. The current study could not confirm, however, the signalling pathways mediating the effects of D-alanine.

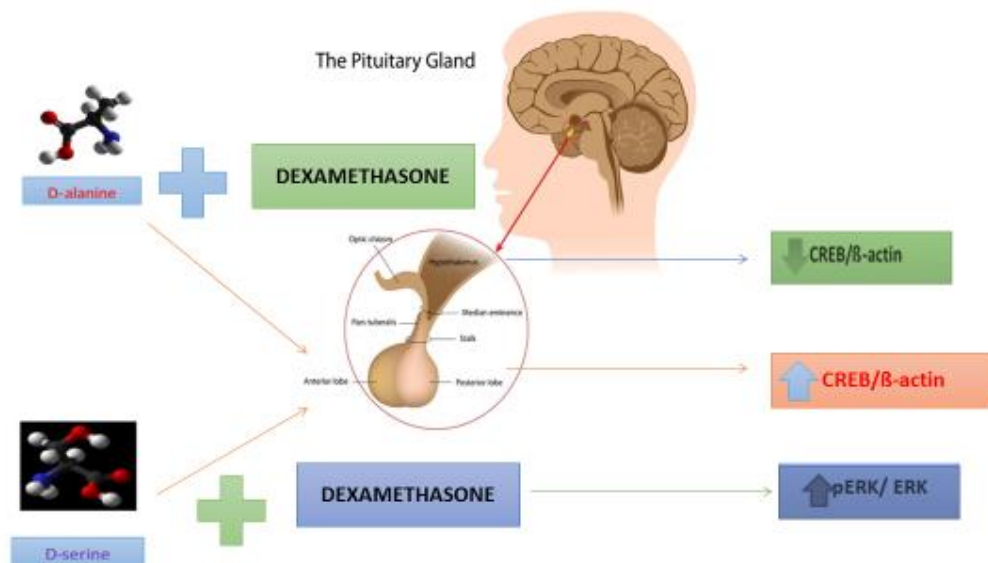


Figure 5.1. Diagrammatic summary of results obtained when AtT-20 cells were treated with D-amino acids in the presence of DEX.

The study did show however, that D-alanine and D-serine acted on two different signalling pathways (CREB and ERK respectively) in the presence of DEX. The suppressive effects on CREB signalling by D-alanine continues to support the notion that inhibitory GlyRs may be involved, and this is reinforced by the fact that D-serine increases ERK signalling, which it can only achieve through NMDARs. The corresponding effects on ACTH release supports the interpretation that D-alanine reduces hormone secretion by decreasing CREB signalling, whereas D-serine elevates ACTH release by augmenting ERK pathways. The experiments with DEX also endorse the potential role of D-alanine in glucocorticoid negative feedback, since the D-amino acid only had an inhibitory action when GRs were activated. Future studies should, therefore, examine the expression of GlyRs in AtT20 cells in the presence and absence of DEX, and perform experiments in *in vivo* to confirm that D-alanine has the same effect on

ACTH secretion and subsequently, corticosterone release, in healthy and stressed animals.

In order to confirm that the NMDAR mediated the effects of D-serine, but not D-alanine, experiments were conducted using the potent receptor antagonist MK-801 (**Fig 5.2**).

Although these experiments did show that the action of D-alanine on ACTH and CREB signalling were unaffected by NMDAR blockade, they did not confirm the role of these receptors in the effects of D-serine. Indeed, inspection of the data suggests that MK-801 may have augmented the actions of D-serine. The persistent increase in pERK/tERK levels may be explained by MK-801 being a potent stimulant of phosphorylation of ERK, and to determine if this is true, the effects of MK-801 alone on ERK signalling should be analysed.

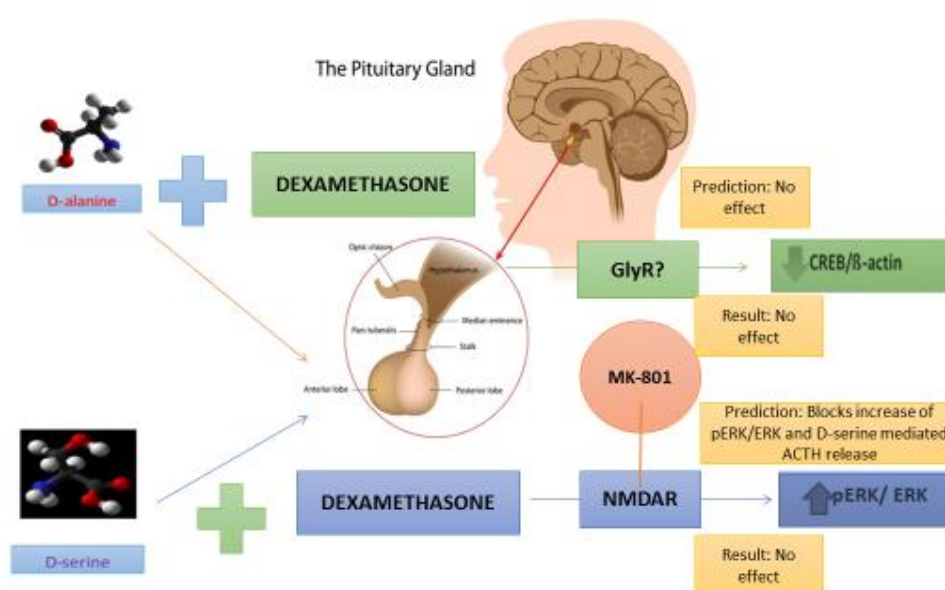


Figure 5.2. The effect of MK-801 on D-serine and D-alanine on intracellular signalling, in the presence of DEX in AtT-20 cells

Overall, the study confirms that D-alanine does have an influence on ACTH secretion, and appears to be a negative modulator, probably through its inhibition of CREB signalling. Its actions are not likely to be mediated by NMDARs since the potent co-agonist of this receptor, D-serine, elevates ACTH release via ERK signalling. However, these conclusions are based on the measurement of protein levels, and so the study lacks functional information. For instance, knowing whether the levels of CREB is a proxy indicator of a functional pathway is a limitation of this study. Furthermore, without animal data, it is difficult to know whether the current findings can be translated to the *in vivo* system. The MK801 experiments were also limited because the effect of this compound alone on the parameters measured, was not determined. Future studies, therefore, should investigate the influence of D-alanine on CREB and ERK activity, use GlyR antagonists and agonists, or even genetic knockdown, to confirm the role of this receptor in the D-alanine effect, measure receptor gene expression and finally, and perhaps more importantly, test the influence of D-alanine on ACTH secretion and HPA axis activity *in vivo*.

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APPENDIX

1. Initial plating medium: 17% FBS (FBS, Sigma Aldrich, F9665, Missouri USA) and 4mM L-glutamine (ThermoFisher, 25030081, Massachusetts, USA) in DMEM (high glucose, Sigma Aldrich, D5546, Missouri USA)
2. Growth medium: 10% FBS and 4mM L-glutamine in DMEM
3. Treatment medium: 0.02% FBS and 4mM L-glutamine in DMEM
4. Preparing D-alanine stock solution:

Molecular weight of D-Alanine: 89.09318g/mol

For 1M need 89.09 g in 1L, therefore for 1M in 1ml need 0.089g.

Dissolve 0.089 g in 1ml PBS for 1M stock

Dissolve 100µl of above solution in 1ml for 0.1M stock solution

For 100µM D-alanine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.1\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 2\mu\text{l}$$

Therefore, for 100µM in 2ml media, add 2µl 1mM stock solution

For 200µM D-alanine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.2\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 4\mu\text{l}$$

Therefore, for 100µM in 2ml media, add 4µl 1mM stock solution

For 500 μ M D-alanine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.5\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 10\mu\text{l}$$

Therefore, for 100 μ M in 2ml media, add 10 μ l 1mM stock solution

5. Preparing D-serine stock solution:

Molecular weight of D-serine: 105.09258 g/mol

For 1M need 105.09258 g in 1L, therefore for 1M in 1ml need 0.105g.

Dissolve 0.105 g in 1ml PBS for 1M stock

Dissolve 100 μ l of above solution in 1ml for 0.1M stock solution

For 100 μ M D-serine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.1\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 2\mu\text{l}$$

Therefore, for 100 μ M in 2ml media, add 2 μ l 1mM stock solution

For 200 μ M D-serine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.2\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 4\mu\text{l}$$

Therefore, for 100 μ M in 2ml media, add 4 μ l 1mM stock solution

For 500 μ M D-serine:

$$C_1V_1 = C_2V_2$$

$$(100\text{mM}) V_1 = (0.5\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.1)(2000)/(100)$$

$$= 10\mu\text{l}$$

Therefore, for 100 μ M in 2ml media, add 10 μ l 1mM stock solution

6. PBS and Protease inhibitor (PI) solution: Add 1 tablet of PI to 10ml of PBS
7. RIPA and PI: Add 1 tablet of PI to 10ml of PBS
8. BSA standard was prepared as per Bio-Rad Bradford Assay Protocol:

Microplate Standard Assay

Tube #	Standard Volume (μ l)	Source of Standard	Diluent Volume (μ l)	Final [Protein] (μ g/ml)
1	20	2 mg/ml stock	0	2,000
2	30	2 mg/ml stock	10	1,500
3	20	2 mg/ml stock	20	1,000
4	20	Tube 2	20	750
5	20	Tube 3	20	500
6	20	Tube 5	20	250
7	20	Tube 6	20	125
8 (blank)	-	-	20	0

Figure 1. Microplate Standard Assay from Bradford Assay Protocol from Bio-Rad

9. Preparing 1 X Running Buffer:

15.1 g of Tris, 72g of Glycine, 5g SDS mixed into 1 litre of MilliQ water

(Millipore) makes a 5X running buffer. Dilute to 1X by mixing 200ml of 5X running buffer with 800ml MilliQ water (Millipore).

10. Preparing Transfer Buffer:

14.4 g of glycine, 3.03 g Tris base, 800ml distilled water and 200ml of methanol.

11. 5% Blocking buffer: 5g of dry non-fat milk in 100ml of PBS-T

12. 2% Blocking buffer: 2g of dry non-fat milk in 100ml PBS-T

13. PBS-T: 10 tablets of PBS mixed in 1 litre of distilled water. 2mls of Tween mixed into this solution.

14. PBS: 10 tablets of PBS mixed into 1 litre of distilled water

15. ECL substrate: 2mls of Substrate A mixed with 2mls of Substrate B

16. Stripping Buffer: 6ml of 1M Tris PH 6.8, 94mls of distilled water and 2g of SDS.

17. Stock solution of CRH: Molecular weight is 4757.45. Therefore, for 1M mix 4.757g in 1ml of PBS.

Dissolve 10 μ l of above solution in 1ml for 0.01M stock solution

Dissolve 10 μ l of above solution in 1ml for 0.0001M stock solution

For 100nM CRH:

$$C_1V_1 = C_2V_2$$

$$(0.1\text{mM}) V_1 = (0.0001\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.0001)(2000)/(10)$$

$$= 2\mu\text{l}$$

Therefore, for 100nM in 2ml media, add 2 μ l 0.1mM stock solution

18. 5X loading buffer: 3mls 20% SDS, 3.75mls 1M Tris buffer at pH 6.8, 9mg bromphenol blue, 1.16mg DTT and mix well. Add 4.5mls glycerol and mix well. Add distilled water to 9ml mark and mix well. Store at 4 degrees Celcius.

19. Stock solution of dexamethasone: Molecular weight is 392.46. Therefore, for 1M mix 0.392g in 1ml of PBS.

Dissolve 10µl of above solution in 1ml for 0.01M stock solution

Dissolve 10µl of above solution in 1ml for 0.0001M stock solution

For 100nM DEX:

$$C_1V_1 = C_2V_2$$

$$(0.1\text{mM}) V_1 = (0.0001\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.0001)(2000)/(10)$$

$$= 2\mu\text{l}$$

Therefore, for 100nM in 2ml media, add 2µl 0.1mM stock solution

20. Stock solution of MK-801: Molecular weight is 337.37. Therefore, for 1M mix 0.337g in 1ml of PBS.

Dissolve 10µl of above solution in 1ml for 0.01M stock solution

For 10 µM MK-801:

$$C_1V_1 = C_2V_2$$

$$(10\text{mM}) V_1 = (0.01\text{mM})(2000\mu\text{l})$$

$$V_1 = (0.01)(2000)/(10)$$

$$= 2\mu\text{l}$$

Therefore, in 2ml media, add 2µl 10mM stock solution

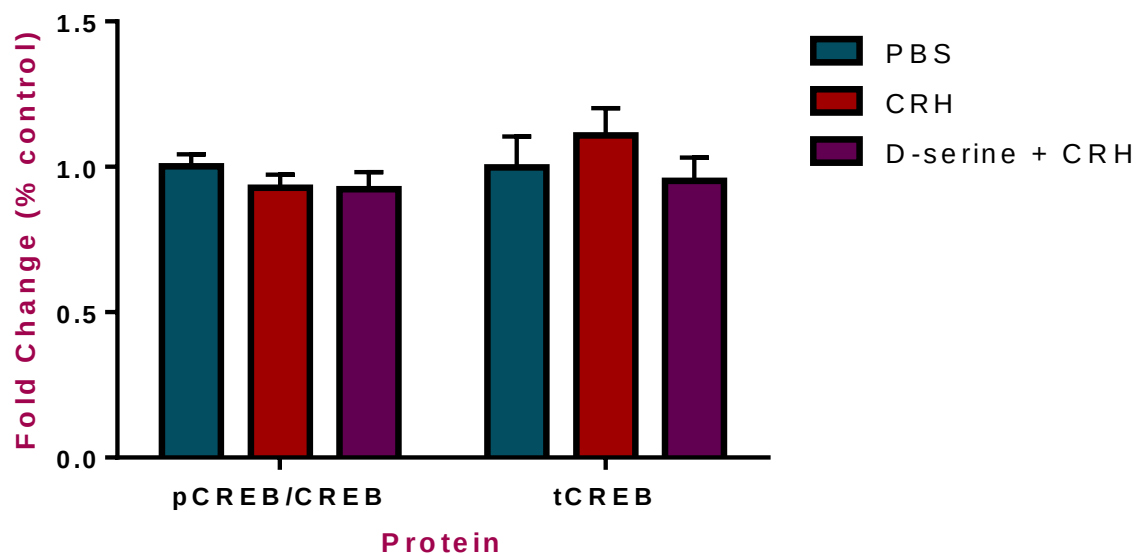


Figure 2. Western blot analysis of pCREB/CREB and tCREB levels in AtT-20 cells treated with D-serine and CRH (500 μ M and 100nM respectively) for 24hours. PBS was used as a control, and cells were also treated with CRH (100nM) alone for 24hours. Results are the mean $\pm$ SEM of 6 replicates of each group. Data was normalised against PBS, which was normalised to 1.

Western Blot Images

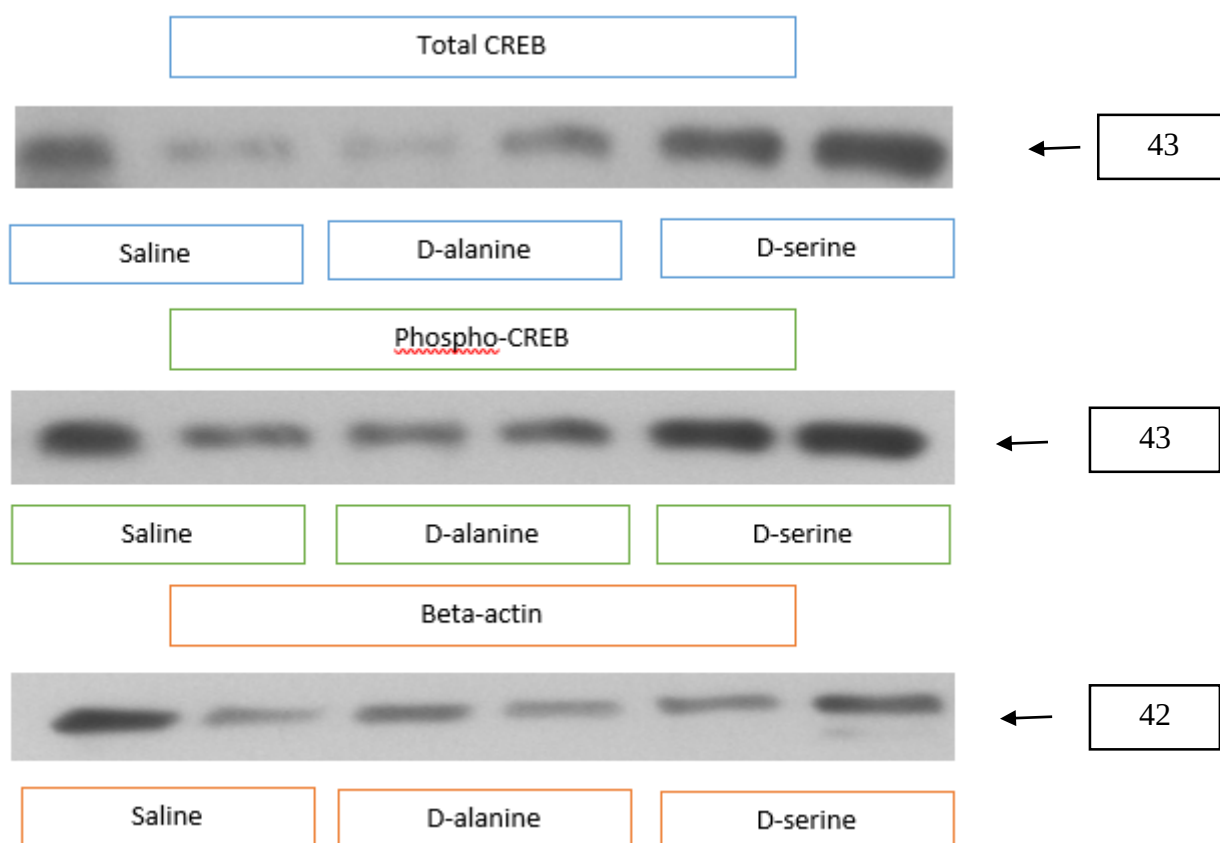


Figure 3. Western blot image of AtT-20 cells (2×10^5 cells per well) treated with D-alanine or D-serine (500 μ M) for 24 hours. PBS was used as a control.

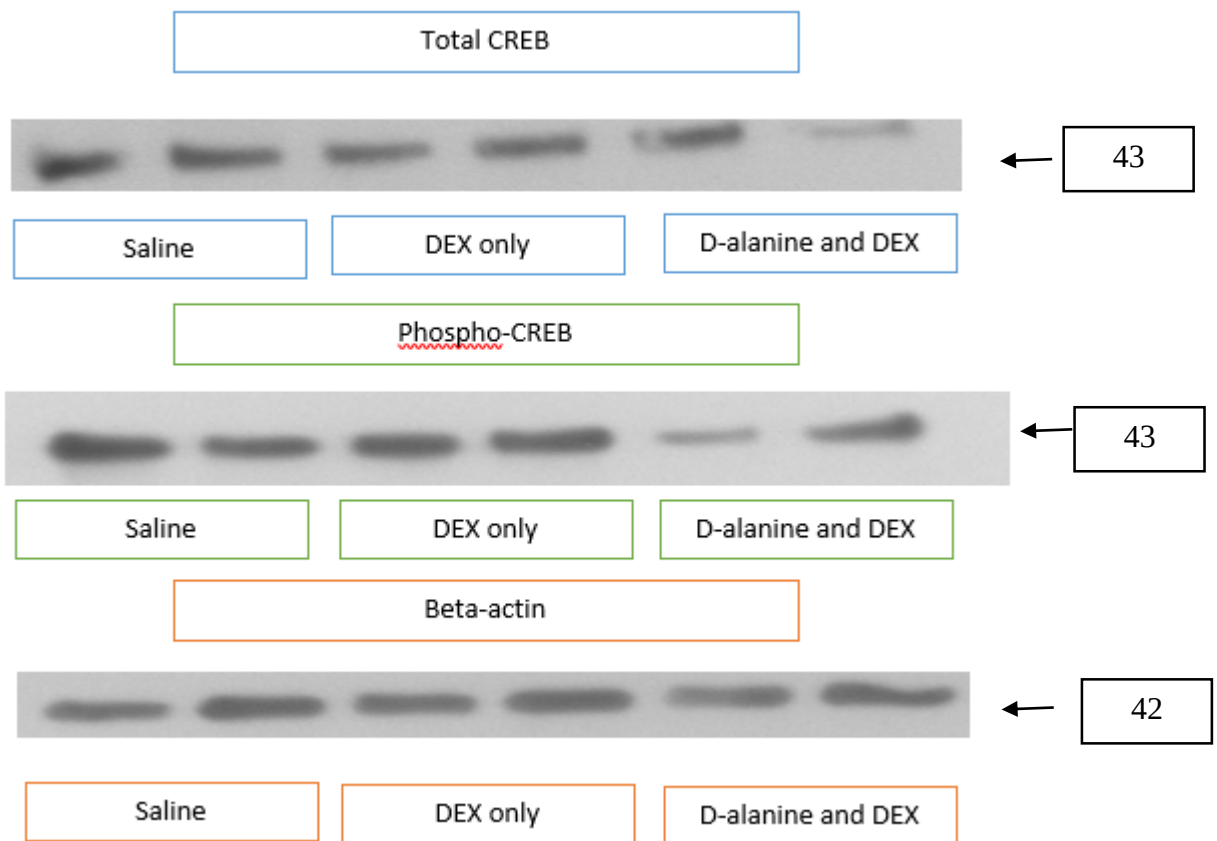


Figure 4. Western blot image of AtT-20 cells (2×10^5 cells per well) treated with Dexamethasone (DEX, 100nM) in the presence and absence of D-alanine (500 μ M) for 24 hours. PBS was used as a control.

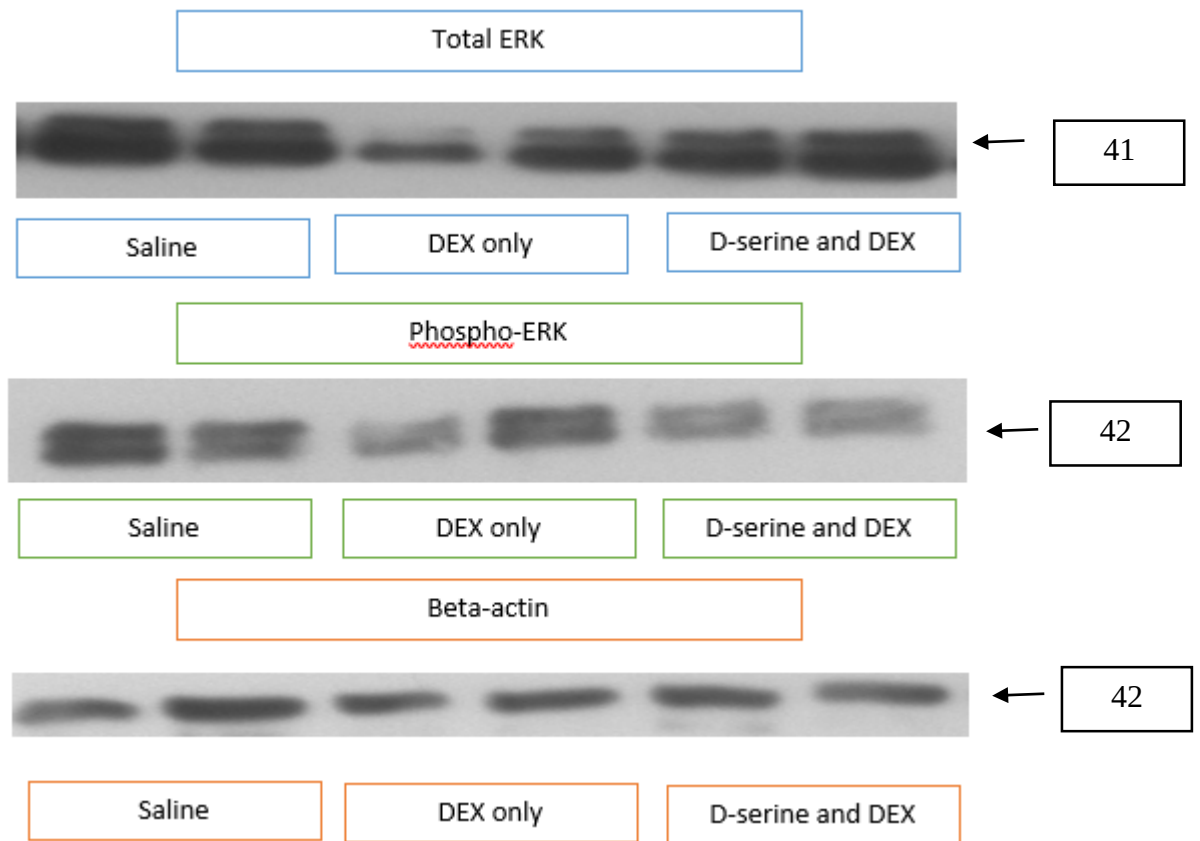


Figure 5. Western blot image of AtT-20 cells (2×10^5 cells per well) treated with DEX (100nM) in the presence and absence of D-serine (500 μ M) for 24 hours. PBS was used as a control.



Figure 6. Western blot image of AtT-20 cells (2×10^5 cells per well) treated with MK-801 ($10 \mu\text{M}$) in the presence and absence of D-serine ($500 \mu\text{M}$) and DEX (100nM) for 24 hours. PBS was used as a control.

